Analysis of Coagulation Cascade and Endothelial Cell Activation During Inhibition of Vascular Endothelial Growth Factor/Vascular Endothelial Growth Factor Receptor Pathway in Cancer Patients


Objective—The angiogenesis inhibitor SU5416 is a potent inhibitor of vascular endothelial growth factor (VEGF) receptor-1 and -2. VEGF may be involved in hemostasis by altering the hemostatic properties of endothelial cells. We analyzed the effects of SU5416 on the coagulation cascade and the vessel wall in patients with advanced cancer.

Methods and Results—Markers for thrombin generation, activation of the protein C pathway, fibrinolysis, and endothelial cell activation were measured in patients with renal cell carcinoma, soft tissue sarcoma, or melanoma on days 0, 14, and 28 of treatment with SU5416. Three of 17 sampled patients developed a thromboembolic event in the fifth week of treatment. Markers for thrombin generation and fibrinolysis did not show significant changes. We observed a significant increase in endogenous thrombin potential and of parameters reflecting endothelial cell activation (von Willebrand antigen, soluble tissue factor, and soluble E-selectin) in all patients (P≤0.001). In patients experiencing a thromboembolic event, endogenous thrombin potential, soluble tissue factor, and soluble E-selectin increased to a significantly greater extent (P=0.029, P=0.021, and P=0.007, respectively).

Conclusions—VEGF is not only a permeability, proliferation, and migration factor, but it is also a maintenance and protection factor for endothelial cells. (Arterioscler Thromb Vasc Biol. 2002;22:1500-1505.)

Key Words: vascular endothelial growth factor ■ endothelial cell function ■ hemostasis ■ SU5416

During adult life, angiogenesis is a physiological process that occurs during the menstrual cycle and wound healing, but it also has a pathophysiological role in cancer. Members of the vascular endothelial growth factor (VEGF) family play a critical role in angiogenesis.1 In addition to increasing vascular permeability, VEGF stimulates endothelial cell (EC) proliferation, migration, and tube formation.2 The effects of VEGF are mediated through several receptors located on ECs, in particular VEGF receptor (VEGFR)-1 (Flt-1 murine homologue) and VEGFR-2 (KDR human homologue, Flk-1 murine homologue). Disruption of the VEGF/VEGFR pathway may represent a potentially attractive target for anticancer therapy. One promising approach involves blockade of the intracellular tyrosine kinase domain of VEGFR-2. The compound SU5416 (Z-3-[(2,4-dimethylpyrrol-5-yl)methylidene]-2-indolinone) inhibits VEGFR-1 and -2 autophosphorylation, which follows the interaction of VEGF with its receptor and prevents the subsequent activation of the intracellular signaling pathways.3,4 SU5416 also inhibits c-kit (stem cell factor receptor).5

The role and importance of VEGF in the physiology of adult life is not completely clear. In addition to its role in angiogenesis, VEGF may affect hemostasis, inasmuch as it increases the expression of tissue factor (TF) and thrombomodulin (TM) in ECs.5–10 Via the formation of the TF–factor VIIa complex, TF triggers the extrinsic coagulation cascade. If the expression of TF is affected by treatment with SU5416, a decrease in the activation level of the coagulation cascade can be expected. TM is expressed on ECs as a membrane-bound protein that, when complexed with thrombin, increases the activation of protein C and thrombin-activatable fibrinolysis inhibitor (TAFI) by ≥1000-fold.11,12 Activated protein C (APC) is an inhibitor of the coagulation cascade, inasmuch as it neutralizes activated factor V and activated factor VIII. TAFI is involved in the inhibition of clot lysis. Fibrinolysis may be further affected by direct perturbation of ECs, which synthesize, store, and bind fibrinolytic proteins. Because VEGF stimulates the expression levels of tissue plasminogen activator (tPA), urokinase plasminogen...
activator, plasminogen activator inhibitor-1 (PAI-1), and the receptor for the urokinase plasminogen activator, fibrinolysis may be modified by VEGF.\textsuperscript{13,15} EC perturbation may also be reflected by the release of von Willebrand factor (vWF) and E-selectin, which is expressed exclusively by ECs.

It has been shown that activation of VEGFR-2 influences the hemostatic properties of ECs.\textsuperscript{16} Because cancer patients often exhibit a procoagulant state, resulting in a high incidence of thromboembolic events, we analyzed the effect of the inhibition of the VEGF/VEGFR-1 and -2 pathway on parameters of coagulation, fibrinolysis, and EC activation.

Methods

Patients and Treatment Schedule

The Department of Medical Oncology at the Vrije Universiteit medical center participated in 3 multicenter phase II trials investigating SU5416 as single-agent therapy. Enrollment was limited to patients with histologically proven, advanced, and progressive soft tissue sarcoma, melanoma, or renal cell carcinoma, for whom no standard treatment was available. SU5416 was administered in a dose of 145 mg/m\textsuperscript{2} IV twice weekly. To prevent allergic reactions to Cremophor, the solvent of SU5416, dexamethasone was given orally 12 and 6 hours before every infusion. Dexamethasone was administered in 5-mg doses before the first 2 infusions, in 2-mg doses before the third and fourth infusions, and, provided that no allergic reactions occurred, in 1-mg doses thereafter. In addition, 30 minutes before each infusion, clemastine (2 mg) and cimetidine (300 mg) were given intravenously. Signed informed consent was obtained before study entry.

Blood samples were collected in sodium citrate (9 vol blood/1 vol citrate, final concentration 0.32%) before the start of treatment and on days 14 and 28. The samples were centrifuged at 4000 rpm at 4°C for 10 minutes, and plasma was stored at −80°C in 1-mL aliquots in microtubes until further processing.

Assays

Plasma levels of thrombin-antithrombin complexes and prothrombin activation fragment 1+2, indicating thrombin generation, were determined by specific ELISAs (Behringwerke). In addition, the endogenous thrombin potential (ETP), an in vitro test reflecting the potential of plasma to form thrombin, was measured as previously described.\textsuperscript{13} APC resistance was measured with normalized APC sensitivity ratios.\textsuperscript{17} TAFI antigen was measured by using a previously described method.\textsuperscript{18} For soluble TM (s-TM), an ELISA was used (Diagnostica Stago). The fibrinolytic parameters, tPA antigen and PAI-1 antigen, were measured by ELISA (Innotest, Innogenetics).

For the measurement of vWF antigen, we used an in-house ELISA according to methods reported elsewhere.\textsuperscript{19} Soluble TF (s-TF) and soluble E-selectin (s-E-selectin) were determined by ELISA (American Diagnostics and R&D Systems, respectively).

Plasma concentrations of VEGF and basic fibroblast growth factor were assayed by quantitative sandwich enzyme immunoassays (catalogue Nos. DVE00 and HSFB75, respectively; R&D Systems, Inc).

Statistical Analysis

Data are reported as mean±SD. The paired-samples t test and corresponding 95% CIs were used to assess significance of differences for days 0, 14, and 28 and to estimate the magnitude of these differences, with an indication of the precision of these estimates. Because in the small groups a normal distribution could not safely be assumed, the data were also analyzed by a nonparametric method as a final check. The Wilcoxon signed rank test was used to again assess the significance of differences for days 0, 14, and 28. The Wilcoxon–Mann-Whitney 2-sample test was used to contrast patients with and without a thromboembolic event. Differences were considered significant at a level of P<0.05.

Results

Response rates and toxicities for all patients enrolled in these 3 multicenter phase II trials of SU5416 will be reported elsewhere. Between August 1999 and May 2000, 21 patients in our hospital were entered into these trials: 9 patients had advanced soft tissue sarcoma, 8 patients had advanced melanoma, and 4 patients had advanced renal cell carcinoma. Of a total of 80 patients entered in all centers, 5 developed a thromboembolic event during treatment. This incidence of 6.3% is not unusual in a patient population with advanced malignancies.\textsuperscript{20}

During SU5416 treatment in our center, thromboembolic or vascular events occurred in 3 patients (Table 1). The first patient (filled inverse triangle in the Figure) was a 58-year-old woman with melanoma who had widespread subcutaneous and cutaneous metastases located mainly on her right leg. After 5 weeks of treatment, she developed deep venous thrombosis of her left leg, which was confirmed by ultrasonography. Her only risk factors for thromboembolism were a history of smoking and the presence of cancer. The second patient (open inverse triangle in the Figure) was a 62-year-old man with lung metastases from a malignant fibrous histiocytoma and a large lesion on his left shoulder adjacent to a previously irradiated field. After 5 weeks of treatment, he developed thrombosis in the left subclavian vein, which was confirmed by ultrasonography. He had no other risk factors other than cancer and radiotherapy. The third patient (filled squares in the Figure) was a 55-year-old man with pulmonary metastases from renal cell cancer. After 5 weeks of treatment, he developed atypical chest pain with ECG changes and, before arriving at the hospital, died of an anteroseptal myocardial infarction. Autopsy was not performed. The patient’s only cardiovascular risk factor was obesity.

Eighteen patients experienced no thromboembolic or vascular events during SU5416 treatment, and 14 of these patients were used as a control group (Table 1). Of the remaining 4 patients, 2 were excluded because they were already receiving oral anticoagulant treatment for a previous deep venous thrombosis at the time of study entry. No samples were available for the other 2 patients, who were withdrawn from the study shortly after the start of treatment because of rapidly progressive disease. Only 1 serious adverse event probably related to SU5416 occurred. A patient with metastatic renal cell cancer developed diarrhea, resulting in dehydration with hypotension and renal insufficiency and a modest decrease in platelet count (lowest level 121×10\textsuperscript{3}/L). Hemolysis was not observed, and no fragmented erythrocytes were seen in the peripheral blood smears. After treatment with intravenous fluids for several days, renal function and platelet count returned to normal, and the patient recovered completely.

Growth Factors

In all patients, plasma VEGF levels were clearly elevated before the start of the treatment, probably as a result of malignancy. VEGF levels did not change considerably during treatment, and no differences were observed between the 2 groups before or during treatment. Basic fibroblast growth factor levels were within the normal range at baseline and...
remained so during treatment for the group as a whole and for the groups with and without an event (Table 2).

Platelets
Approximately 40% of the 17 patients had an elevated platelet count, as is often seen in patients with malignancy. The number of platelets did not change during treatment with SU5416, except for the patient with renal cell carcinoma who developed diarrhea. There were no differences in platelet counts between the 2 groups before or during treatment (Table 2).

Thrombin Generation
Plasma concentrations of thrombin-antithrombin complexes and prothrombin activation fragment 1 antigen 2 in 17 patients were not influenced by treatment with SU5416 (Tables 2 and 3). No differences were seen between the patients with and those without thromboembolic or vascular events.

A significant increase in ETP levels was observed in all patients (Table 3). Patients with a thromboembolic event had an increase to a significantly greater extent in ETP between days 0 and 28 compared with patients without an event ($P=0.029$, Figure 1A).

Protein C Pathway
s-TM levels in 14 patients (11 without and 3 with a thromboembolic event) were normal at baseline and did not change during treatment (Table 2). Levels of APC resistance in 14 patients did not change significantly during treatment, and no differences were observed between the 2 groups (Table 2).

Fibrinolysis
The fibrinolysis parameters tPA, PAI-1, and TAFI in 14 patients did not change during treatment with SU5416 either in the group as a whole or in the 2 comparison groups (Table 2). tPA levels were within normal limits, except in 2 patients in the control group. PAI-1 levels were elevated in 29% of the patients, ranging from 75 to 160 U/L, which is not exceptional in patients with malignancy. TAFI levels showed some variation, which was not significant, and did not change during treatment.

EC Parameters
In all patients, a significant increase in s-TF levels was observed during treatment with SU5416 (Table 3). Moreover, mean basal levels of s-TF were significantly higher in patients who developed a thromboembolic or vascular event ($n=3$) compared with control subjects ($n=14$, $P=0.035$; Figure 1B). During treatment with SU5416, s-TF increased to a significantly greater extent in patients who developed an event ($P=0.021$ on day 14 as well as day 28).

Also, all patients showed a significant increase in plasma vWF levels during treatment with SU5416 (Table 3). Mean vWF levels increased by $\approx 40\%$ in both groups. Mean basal levels of vWF were not statistically significant higher in the patients experiencing a thromboembolic event.

s-E-selectin levels increased significantly in all patients (Table 3, Figure 1C). This increase was significantly greater on day 14 and day 28 in patients who developed an event ($P=0.012$ and $P=0.007$, respectively). In addition, mean basal levels of s-E-selectin were significantly higher in patients who developed an event ($P=0.003$).

Discussion
In the present study, we investigated the effects of SU5416 on EC function and coagulation. We could document neither activation of the coagulation cascade nor changes in fibrinolysis during treatment with SU5416, an angiogenesis inhibitor
that targets the VEGF/VEGFR-1 and -2 pathway. However, we did observe a significant increase in the levels of s-E-selectin, which reflect activation of ECs, and the levels of vWF and s-TF, which reflect activation of endothelial and circulating cells. It is remarkable that s-TM, which is also known as an EC parameter, did not change at all. One possible, but speculative, explanation could be that the elevation of circulating EC parameters depends on the degree of damage to the ECs and that only in the case of substantial EC necrosis/apoptosis will the level of s-TM be elevated. Interestingly, levels of s-E-selectin and s-TF were significantly higher at baseline and increased to a significantly greater extent in patients experiencing a thromboembolic event compared with control patients. This finding probably indicates a higher state of EC activation and vulnerability in the patients with thromboembolisms. Increases in the concentration of these parameters (particularly s-TF) heighten the thrombin-forming potential of the plasma, which probably accounted for the significant increase in ETP in all patients and, moreover, the significantly greater increase in the patients experiencing a thromboembolic event. We hypothesize that VEGF has a role not only in increasing vascular permeability (and/or mitogenesis) and angiogenesis but also in maintaining the integrity of the endothelium. In case of local small damage to the endothelium, locally produced VEGF probably contributes in “repairing” ECs. Therefore, VEGF not only is a permeability, proliferation, and migration factor but also is a maintenance and protection factor for ECs during adult life.21–24

The endothelium has secretory, synthetic, metabolic, and immunological functions. In the event of perturbations such as injury or inflammation, ECs become activated and form a prothrombotic surface.25,26 One important event that occurs on EC activation is the release of vWF. When cultured ECs are stimulated with VEGF, vWF is rapidly released, and the expression of vWF mRNA increases.27,28 However, we observed an elevation of vWF levels during treatment with SU5416, suggesting increased synthesis and release of vWF, indicating low-grade activation of ECs. This finding contrasts with what we had expected and remains to be elucidated. Elevated plasma levels of vWF are found in a wide variety of diseases involving vascular pathology and are associated with an increased risk of thrombosis and cardiovascular mortality.26,29

The observed increase in s-E-selectin during treatment with SU5416 confirms the idea of an activated vascular endothelium. This finding was also unexpected inasmuch as VEGF, acting via VEGFR-2, induces immediate expression of E-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 at the EC surface and increases mRNA levels of these adhesion molecules in a time-dependent manner.30,31 Why ECs release E-selectin during treatment with SU5416 remains unclear.

In addition, we observed an increase in the plasma concentration of s-TF, which, like s-E-selectin, was especially prominent in the patients experiencing an event. This increase could also indicate a change of the ECs to a prothrombotic state. However, TF is not exclusively expressed on activated ECs; it is also expressed on activated mononuclear cells. The level of s-TF in plasma may reflect activation of these other cells as well. Increased levels of circulating s-TF have been associated with increased thrombogenicity in several disease states.32

It is known that high levels of VEGF, as may be seen during injury or inflammation, result in the activation of
and NO production in ECs.16,21 A potential mediator in maintaining the ECs in a nonactivated basal level of VEGF signaling and maybe become activated. During treatment with SU5416, these ECs are deprived of a quiescent ECs.33 In contrast, a low basal level of VEGF may be needed to keep nontumoral ECs in a nonactivated state.32 If eNOS expression and subsequent NO production are significantly depressed by SU5416, quiescent ECs may be vulnerable and activated. Significantly, if eNOS expression and subsequent NO production are significantly depressed by SU5416, quiescent ECs may be vulnerable and activated. The present study demonstrates several parameters that reflect increased vascular activation (vWF, s-E-selectin, and s-TF) and increased potential for coagulation (s-TF and ETP). Although SU5416 also inhibits (besides VEGFR-1 and -2) c-kit and perhaps other receptors, which could have attributed to the observed effects, the findings suggest that VEGFR-1 and -2 blockade affects EC function and hemostasis. It strengthens the idea that VEGF is also a maintenance and protection factor for ECs and that targeting the VEGF/VEGFR-1 and -2 pathway with SU5416 may increase the risk of a thromboembolic event. This hypothesis is supported by the fact that in a phase I trial investigating cisplatin-gemcitabine in combination plus SU5416 in 19 treated patients, 8 patients developed 9 thromboembolic events.39 For patients with malignancies who have other risk factors for thromboembolism, the use of anticoagulant treatment is indicated according to the individual risk profile and should be especially considered during SU5416 therapy.

Acknowledgment

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TABLE 2. Platelet Counts, Growth Factor Levels, and Coagulation, Fibrinolytic, and Cell Activation Parameters Before and During SU5416 Treatment

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Day 0, Mean±SD</th>
<th>Day 14, Mean±SD</th>
<th>Day 28, Mean±SD</th>
<th>Normal Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelets, x10^9/L</td>
<td>336±120</td>
<td>410±127</td>
<td>385±142</td>
<td>150–450</td>
</tr>
<tr>
<td>VEGF, pg/mL</td>
<td>92±120</td>
<td>70±42</td>
<td>65±49</td>
<td>&lt;2</td>
</tr>
<tr>
<td>BFGF, pg/mL</td>
<td>21±17</td>
<td>21±13</td>
<td>24±16</td>
<td>2–28</td>
</tr>
<tr>
<td>TAT complexes, μg/L</td>
<td>9.2±7.0</td>
<td>17.5±25.4</td>
<td>8.1±5.5</td>
<td>0–4.6</td>
</tr>
<tr>
<td>F 1+2, nmol/L</td>
<td>1.25±0.32</td>
<td>1.88±2.2</td>
<td>1.33±0.35</td>
<td>0.3–1.6</td>
</tr>
<tr>
<td>ETP, % of normal</td>
<td>110±13</td>
<td>122±16</td>
<td>138±41</td>
<td>58–139</td>
</tr>
<tr>
<td>APC:st,* ratio</td>
<td>0.92±1.0</td>
<td>1.06±1.59</td>
<td>0.51±0.49</td>
<td>men: 0.65–1.28; women: 0.87–2.15</td>
</tr>
<tr>
<td>t-PA,* μg/L</td>
<td>8.1±4.9</td>
<td>7.4±3.6</td>
<td>7.8±4.5</td>
<td>1.5–15</td>
</tr>
<tr>
<td>PAI-1,* μg/L</td>
<td>58±37</td>
<td>54±21</td>
<td>52±26</td>
<td>10–70</td>
</tr>
<tr>
<td>TAFI,* % of normal</td>
<td>117±28</td>
<td>130±32</td>
<td>110±30</td>
<td>80–120</td>
</tr>
<tr>
<td>s-TM,* ng/mL</td>
<td>18.9±9</td>
<td>20±12</td>
<td>20±11</td>
<td>5–50</td>
</tr>
<tr>
<td>VWF, % of normal</td>
<td>169±72</td>
<td>209±97</td>
<td>219±106</td>
<td>50–150</td>
</tr>
<tr>
<td>s-E-selectin, ng/mL</td>
<td>86±41</td>
<td>114±68</td>
<td>124±71</td>
<td>30–60</td>
</tr>
<tr>
<td>s-TF, pg/mL</td>
<td>278±163</td>
<td>346±200</td>
<td>411±299</td>
<td>0–200</td>
</tr>
</tbody>
</table>

n=17, *n=14.

TABLE 3. Statistical Analysis of Coagulation and Endothelial Cell Parameters During SU5416 Therapy

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Difference in Levels</th>
<th>Between Days</th>
<th>Mean</th>
<th>SD</th>
<th>95% Confidence Intervals</th>
<th>P</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETP</td>
<td>0–14</td>
<td>12</td>
<td>10</td>
<td>7–17</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0–28</td>
<td>28</td>
<td>36</td>
<td>9–46</td>
<td>0.006</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>s-TF</td>
<td>0–14</td>
<td>69</td>
<td>63</td>
<td>37–101</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0–28</td>
<td>134</td>
<td>161</td>
<td>51–216</td>
<td>0.003</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>vWF</td>
<td>0–14</td>
<td>40</td>
<td>38</td>
<td>21–60</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0–28</td>
<td>50</td>
<td>62</td>
<td>19–82</td>
<td>0.004</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>s-E-selectin</td>
<td>0–14</td>
<td>28</td>
<td>33</td>
<td>11–46</td>
<td>0.003</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0–28</td>
<td>38</td>
<td>35</td>
<td>20–56</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>

P calculated with paired-samples t test.

*P calculated with Wilcoxon signed ranks test.
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