Platelet and Coagulation Activation with Vascular Endothelial Growth Factor Generation in Soft Tissue Sarcomas

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ABSTRACT
Angiogenesis and activated blood coagulation are involved in tumor growth and metastasis. Although some have suggested that activation of coagulation in tumors is not linked to activation of platelets, no data exist to either support or refute this concept. However, platelet turnover in cancer patients is often increased, and platelets are carriers of angiogenic growth factors. We hypothesized that platelets are involved in tumor-associated angiogenesis. To obtain evidence supporting this hypothesis, we have studied whether the angiogenic and coagulation pathways and platelets are concomitantly activated in cancer patients with soft tissue sarcomas (STSs) using a novel method to detect activated platelets in tumor specimens. Twelve patients with STS were selected on the basis of having intratumoral accumulation of fluid, which was aspirated. These accumulations demonstrated very high concentrations of vascular endothelial growth factor and coagulation factors (including thrombin-antithrombin-complex). Tumor specimens showed dense vascularization with intense vascular endothelial growth factor expression and the presence of activated platelets. Taken together, these results support the concept that angiogenesis, blood coagulation, and platelets are concomitantly activated in STS and support the hypothesis that platelets contribute to tumor-induced angiogenesis.

INTRODUCTION
Angiogenesis is required for tumor growth and metastasis (1). Tumors stimulate new vessel formation through the secretion of angiogenic factors, e.g., basic fibroblast growth factor and VEGF2 (2). Activation of the coagulation pathway also enhances tumor growth and metastasis (3). Procoagulants involved in angiogenesis include TF and thrombin (4, 5). VEGF, the most potent proangiogenic factor, is an indirect procoagulant; it is capable of inducing vascular hyperpermeability and of increasing TF expression on endothelial cells (6, 7). Vascular hyperpermeability results in leakage of plasma proteins, including prothrombin and fibrinogen, into the extracellular matrix. Prothrombin converted into thrombin by the activated coagulation pathway may result in platelet activation and the production of fibrin from fibrinogen. Fibrin is also a proangiogenic protein, and it provides an ideal matrix for the migration of endothelial cells (8). Clinical observations support the contention that coagulation and angiogenic pathways are important in cancer biology (9, 10). However, it is thought that activation of coagulation in tumors does not result in the activation of platelets (11).

Based on an increased platelet turnover in cancer patients (12), and because it has been recently shown that platelets transport and secrete VEGF on activation (13, 14), we hypothesized that platelets play an important role in tumor-induced formation of new vessels (15). To obtain more evidence for this hypothesis, we have studied whether the angiogenic and coagulation pathways are concomitantly activated in patients with STSs and developed a method to detect activated platelets in tumor specimens. We chose to study STSs because these tumors are highly vascularized (16). Twelve patients with STS and intratumoral fluid accumulation were studied. Aspirated fluid demonstrated high concentrations of VEGF and several activated coagulation factors. Tumor specimens showed dense vascularization with intense VEGF expression. As far as we know, this is the first demonstration of the presence of activated platelets within the tumor vasculature, suggesting that platelets contribute to tumor-induced angiogenesis.

PATIENTS AND METHODS
Patients. We aspirated 50–600 ml of tumor fluid from 12 STS patients treated in our hospital between 1991 and 1998 who had either primary or metastatic disease and had a heterogeneous aspect on MRI suggestive of intratumoral fluid accumulations. These patients were diagnosed with the following subtypes of STS: (a) leiomyosarcoma (five patients); (b) malignant fibrous histiocytoma (two patients); (c) liposarcoma (two patients); (d) gastrointestinal stromal cell tumor (one patient); (e) hemangioendothelioma (one patient); and (f) malignant schwannoma (one patient). Tumor fluids were aspirated under ultra-

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2 The abbreviations used are: VEGF, vascular endothelial growth factor; STS, soft tissue sarcoma; TF, tissue factor; MRI, magnetic resonance imaging; mAb, monoclonal antibody; TAT, thrombin-antithrombin.
sound guidance. Aspirated tumor fluids were aliquoted and immediately frozen at −20°C. Tumor tissue was obtained from patients undergoing radical surgery.

**Tissue Sampling and Immunohistochemical Staining.** Tissues acquired from these patients, from different tumor areas, were histologically analyzed after H&E staining and appropriate immunohistochemistry of formaldehyde-fixed, paraffin-embedded tissues (4-μm sections).

Immunohistochemical staining for VEGF was performed with a polyclonal goat antibody against VEGF (catalogue number AB-293-NA; R&D Systems, Abingdon, United Kingdom) on paraffin slides using a standard procedure. Briefly, after deparaffinization and rehydration, endogenous peroxidase was blocked with 0.3% H2O2 in methanol for 30 min. Nonspecific binding of the secondary antibody was blocked, and subsequent incubation with the primary antibody (20 μg/ml), followed by a biotin-labeled secondary horse-antigoat antibody (1:150), was performed with washing steps in between. A complex of streptavidin and biotinylated horseradish peroxidase was added, and staining was visualized with 3,3′-diaminobenzidine.

Cryostat sections from seven patients (4-μm sections) were fixed in cold acetone. To get an impression of the vascularization of these STSs and discriminate between the intravascular and extravascular location of platelets, we performed a platelet-specific, double immunofluorescence labeling of the αβ3 integrin and an endothelial-specific lectin binding site containing a-linked fucose residues, which binds biotinylated Ulex europaeus agglutinin I (catalogue number FL-1061; Vector Laboratories, Inc). Immunofluorescence labeling of platelets was performed using a mAb against α1β1 integrin (catalogue number M7057; DAKO A/S, Glostrup, Denmark). We used streptavidin-Texas red for the detection of the biotinylated lectin and horse antimouse IgG-FITC for the detection of the α1β3 integrin as secondary fluorescence-labeled antibodies.

A novel method to detect activated platelets was developed, based on the codetection of α1β3 integrin and fibrin/fibrinogen on their surface. The α1β3 integrin, also known as GPIIb/IIIa, is a complex of two major platelet glycoproteins that undergo conformational changes after platelet activation, exposing a high-affinity binding site for fibrin/fibrinogen and other RGD-containing proteins (17). The principle of this double staining for platelet activation is based on the observation that fibrinogen is only bound by activated α1β3 integrin during platelet activation and is not bound by resting platelets. Immunofluorescence labeling of fibrinogen was performed using a rabbit polyclonal antibody (catalogue number A 0080; DAKO) and goat antirabbit IgG-Texas red (both from Vector Laboratories, Inc.). The platelet marker was detected using the same secondary antibody as mentioned above. After preincubation (1% FCS in PBS), slides were incubated with a combination of the primary antibodies, either mAb anti-α1β3 and biotinylated Ulex Europaeus lectin or mAb anti-α1β3 and polyclonal antibody antifibrinogen, and subsequently incubated with the above-mentioned mixtures of the fluorescence-labeled detection agents (antibodies or streptavidin), all in a 1:50 dilution. Thereafter, the slides were mounted with Vectashield antifading medium containing 4,6-diamidino-2-phenylindole as a nuclear counterstaining (catalogue number H1200; Vector Laboratories, Inc.). All slides were examined within 1 week after staining using a Bio-Rad MRC confocal laser scanning unit attached to a Nikon Diaphot inverted microscope (Bio-Rad Microscience Ltd., Hertfordshire, United Kingdom). The α1β3 integrin-stained platelets appeared green, and endothelial cells or fibrin/fibrinogen appeared red, whereas areas of coincident labeling containing colocalized antigens appeared yellow.

**ELISA Assays.** VEGF concentrations were measured with a quantitative sandwich enzyme immunoassay (R&D Systems). If necessary, samples were diluted up to 1000 times. As indicators of coagulation and endothelial activation, we measured TF, TAT-complex, and thrombomodulin concentrations, respectively, in the tumor fluids. TF and thrombomodulin concentrations were determined by ELISA assays (Diagnostica Stago, Asnieres-sur Seine, France). Samples were diluted 1:10 or, if necessary, 1:50. TAT-complex concentrations were determined with an enzyme immunoassay (Enzymnost TAT kit; Behring Diagnostics GmbH, Marburg, Germany).

**Statistical Analyses.** Statistics were performed using SPSS. Correlations were calculated using Spearman’s rank test.

**RESULTS**

**Tumor Fluid Aspirated from STSs.** MRI examination of patients with histologically confirmed STS showed that the majority of patients with tumor diameters > 10 cm had heterogeneous tumor masses, which represent solid elements and fluid accumulations (Fig. 1). We succeeded in aspirating tumoral fluid under ultrasound guidance for 12 patients. H&E light microscopy examination of paraffin sections of the walls of these tumor cavities failed to show either tumor necrosis or epithelial cell lining, but the extracellular matrix appeared very loose with occasional extravasation of blood cells (Fig. 2).

**Angiogenic Phenotype of STS.** An intense vascularization in solid areas of the tumors was demonstrated by immunofluorescence staining. Fig. 3 shows a representative tumor. In
the tumor aspirates of these 12 patients, we found that VEGF concentrations were substantially higher (median, 18 ng/ml; range, 0.3–345 ng/ml) than those in normal plasma (30–40 pg/ml; Ref. 13), whereas basic fibroblast growth factor, another angiogenic factor, was only detectable at low levels (<90 pg/ml) in a minority of the aspirates (data not shown). The median protein concentration of these aspirates was 52 g/liter (range, 33–74 g/liter), marginally below that seen in normal plasma (60 g/liter). Immunohistochemical staining reflected that VEGF was expressed by tumor cells and the endothelial cells (lining the vasculature) in these sarcomas (Fig. 4).

Coagulation Phenotype of STS. Markers of activated coagulation were measured in the tumor aspirates of these 12 patients. TF concentrations were approximately two times greater than those seen in normal plasma, with a median value of 723 pg/ml (range, 400–4998 pg/ml; Ref. 9). The thrombin content, as manifested by TAT complexes, was also high, with a median value of 126 μg/liter (range, 33–184 μg/liter), representing a 63-fold increase in thrombin generation as compared to normal plasma (9). The marker for endothelial activation, thrombomodulin, was 181 ng/ml (range, 39–700 ng/ml) and was only modestly elevated when compared with normal plasma levels (Ref. 18; summarized in Table 1). Spearman rank correlation analyses revealed a significant correlation between increasing aspirate thrombomodulin concentration and that of TF (r = 0.65; P < 0.05; Fig. 5), whereas the other markers did not show significant correlations.

Platelet Activation within Tumor Vasculature. To examine whether platelets are also activated in the tumor vascula-
lature, we performed immunoconfocal microscopy to detect intratumoral activated platelets. The immunostaining confirmed the presence of platelets in the tumor vasculature (Fig. 3). A large proportion of the detected platelets were associated with fibrinogen in aggregates, as demonstrated by double staining of the $\alpha_{11} \beta_3$ integrin and fibrin/fibrinogen in both solid elements and intratumoral cavities (Fig. 6, A and B). The yellow appearance resulting from the double staining of $\alpha_{11} \beta_3$ integrin (green) and fibrin/fibrinogen (red) confirms the presence of activated platelets in these tumors. In addition, most of the extracellular matrices of the tumor tissue showed abundant expression of fibrinogen, which is indicative of leakage of plasma proteins out of the tumor vasculature.

**DISCUSSION**

Contrary to the current view that tumor-induced coagulation does not result in platelet activation, we have shown that platelets do become activated within the tumor vasculature. This finding suggests that platelets contribute to tumor angiogenesis.
through growth factor release upon their activation. It also offers a plausible explanation for the clinically observed increased platelet turnover in cancer patients as compared with healthy volunteers (12).

The involvement of the coagulation cascade in tumor-induced angiogenesis has been described previously (3). In a transgenic murine mouse model of dermal fibrosarcoma, tumors occurred predominantly in areas prone to wounding (19). In a murine fibrosarcoma model, it was demonstrated that the transfection of a TF gene is associated with up-regulation of VEGF and enhanced tumor growth (4). A strong correlation between TF and VEGF expression in breast carcinoma cells in vitro has been established, and a colocalization of TF and VEGF in breast cancer tissue from patients has also been reported (20). In addition, expression of TF was found in active angiogenic sites in invasive human breast carcinoma (21). Through its effect on TF production and vascular permeability, VEGF is an indirect procoagulant. The high VEGF concentration in the tumor aspirates and its high tumor expression lead us to hypothesize that the coagulation cascade may be concomitantly activated in these sarcomas. Our data demonstrate high levels of both VEGF and TF in a human solid tumor, but without any direct correlation, suggesting that they are partly derived from different sources. Of note, VEGF and TF may both be responsible for the high concentrations of each other in these fluids, because it has been reported that stimulation of endothelial cells with exogenous VEGF results in up-regulation of TF (7), whereas transfection of TF results in up-regulation of VEGF (4). The presence of high levels of TAT complexes in sarcoma aspirates provides strong evidence that thrombin, a powerful platelet activator, is generated in these tumors, probably due to TF expression. The proangiogenic activity of thrombin is not dependent per se on fibrin formation (5). Thus, based on the observation that thrombin generation occurs in STS, together with the finding of intratumoral platelet activation, one may speculate that thrombin exerts its proangiogenic effect by activation of platelets. The high intratumoral concentrations of thrombomodulin, a marker of endothelial activation (22) and a physiological anticoagulant, are consistent with previously reported observations (19). The strong correlation between thrombomodulin and TF may be due to the fact that they are both markers of endothelial activation (21, 22). Taken together, these findings show that the angiogenic and coagulation pathways can be concomitantly activated in STS.

It has been described previously that intratumoral fluid accumulation in STS reflects necrosis (23). However, this study provides evidence that cystic areas in STS reflect the biological behavior of these mesenchymal tumors. Our findings, including the dense vascularization, are in accordance with the observation that STSs are highly angiogenic (16) and support the
hypothesis that tumor interstitial fluid has a biological role in tumor growth (24). The exact mechanism for intratumoral fluid accumulation remains to be clarified, but evidence for an important role of VEGF is suggested from this study.

Intratumoral cavities contain proteineaceous fluid with or without cellular elements, suggesting a spectrum from fluid extravasation secondary to endothelial hyperpermeability on the one hand, to frank bleeding into the cavities on the other. The high concentrations of VEGF, which is well known for its ability to induce hyperpermeability (25), suggest that VEGF plays an important role in the formation of these intratumoral cavities. Presumably, some intracavitral VEGF is derived from the STS cells because an intensive immunoreactivity for VEGF was found in STS tissue, and STS cells lines produce high amounts of VEGF in vitro (data not shown). VEGF mRNA levels are dramatically increased under hypoxic conditions (26). It may well be that hypoxia in STS contributes to the abundant VEGF production we observed. In addition, platelets transport VEGF (13), and megakaryocytes synthesize this protein (27). Taken together with the finding that platelets were activated within the STS tumor vasculature and cavities, we propose that the extremely high VEGF levels (levels not previously reported in biological fluids) in sarcoma aspirates are explained by a combination of both tumor- and platelet-derived VEGF.

The immunostaining we used to detect activated platelets also demonstrated that fibrinogen was present in abundance in the extracellular matrix of these tumors. This finding supports the current belief that extravasation of plasma proteins within the tumor is most likely due to the hyperpermeability factor, VEGF (26).

Taken together, the very high concentrations of a number of key coagulation factors and VEGF in these tumor fluids and the evidence for intratumoral platelet activation reflect the importance of both the angiogenesis and coagulation pathways in the biology of STS. Additional studies in a larger number of patients with different types of cancer are presently underway in our institute to define whether intratumoral platelet activation is a general phenomenon in solid tumors.

REFERENCES
