Tissue Factor/Factor VIIa Inhibitors Block Angiogenesis and Tumor Growth Through a Nonhemostatic Mechanism

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ABSTRACT

An association between cancer and thrombosis has been recognized for more than a century. However, the manner by which tumor growth is regulated by coagulation in vivo remains unclear. To assess the role of coagulation on tumor growth, in vivo, we tested coagulation inhibitors specific for either tissue factor (TF)/factor VIIa (FVIIa) complexes or factor Xa (FXa) for antitumor activity. Here, we show that two inhibitors of TF/FVIIa, TF pathway inhibitor (TFPI) and the nematode anticoagulant protein rNAPc2, inhibit both primary and metastatic tumor growth in mice. In addition, we show that rNAPc2 is also a potent inhibitor of angiogenesis. In contrast, rNAP5, a second nematode anticoagulant protein that specifically inhibits FXa, does not exhibit antitumor activity. Because the hemostatic activity of TF/FVIIa is mediated through activation of FXa, these data suggest that proteolytic activity of TF/FVIIa promotes tumor growth and angiogenesis through a novel proangiogenic mechanism and independently of hemostasis.

INTRODUCTION

It has been recognized for more than a century that the presence of a tumor dramatically increases the risk of significant hemostatic abnormalities, such as disseminated intravascular coagulation and venous thromboembolism (1, 2). Studies in animal models have established that the seeding of tumor metastases is a coagulation-dependent process (3–5), although clinical studies testing the anti-metastatic effects of anticoagulant molecules have yielded mixed results (6, 7). It has not been established, though, whether this hypercoagulable environment supports or impedes tumor growth directly. Several clinical studies addressing this issue have been performed to evaluate the role of coagulation on primary tumor progression. Anticoagulant therapy with either warfarin or low molecular weight heparin was demonstrated to delay tumor progression (8, 9), and warfarin treatment after a first episode of venous thrombosis exhibited a small chemoprotective effect (10). These studies are not conclusive, though, because both low molecular weight heparin and warfarin have non-hemostatic activities that could regulate tumor progression (11, 12). However, taken together, these studies suggest that the hypercoagulable state in cancer may sustain and promote tumor growth.

To determine whether the coagulation cascade supports tumor growth, we initially focused our studies on the TF/FVIIa complex, which has been identified as the major procoagulant activity in cancer. Several studies suggest that the TF/FVIIa complex may support tumor growth and angiogenesis (13, 14). We studied the activity of two inhibitors of the TF/FVIIa complex, TFPI and recombinant NAPc2, a factor X-dependent inhibitor of TF/FVIIa, derived from the hookworm Ancylostoma caninum. We show that these specific inhibitors of the TF/FVIIa complex inhibit the growth of primary and metastatic tumors in mice. Interestingly, a second anticoagulant protein from A. caninum, rNAP5, which specifically inhibits FXa but not the TF/FVIIa complex, did not significantly inhibit primary or metastatic tumor growth, suggesting that the activity of the TF/FVIIa complex may be independent of its ability to initiate coagulation. This suggests that the TF/FVIIa complex may have a novel proangiogenic activity that is independent of its role of initiating coagulation and may function as a proangiogenic mechanism.

MATERIALS AND METHODS

Materials. B16 melanoma cells were obtained from the National Cancer Institute-Central Repository (Frederick, MD) certified free of Mycoplasma and other pathogenic murine viruses. LLC cells were obtained from American Type Culture Collection. DMEM, FCS, and l-glutamine were purchased from BioWhittaker (Walkerville, MD). TFPI, purified from human plasma or HepG2 cells, was purchased from American Diagnostica, Inc. (Greenwich, CT), bFGF was purchased from R&D (Minneapolis, MN). C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were kept under a specific pathogen-free conditioned environment and provided sterilized mouse chow (LM485; Harlan Sprague Dawley, Indianapolis, IN) and water ad libitum.

Treatment of Primary B16 and LLC Tumor Growth. Groups of five C57BL/6J mice were injected s.c. with 2.5 × 106 B16 melanoma cells or LLC cells. Treatments were initiated when tumors were 100 mm2 in volume and continued daily for 10–12 days. Animals were treated peritumorally with 0.66 mg/kg human recombinant TFPI or i.p. with increasing amounts of rNAPc2 and rNAP5. Tumor growth was measured on alternate days, and tumor volume was calculated using the following formula: (length) × (width)2/6. Efficacy was calculated as the ratio of tumor size in the treated animals to the tumor size in control animals. Statistical analysis was performed using the Student t test.

Treatment of Experimental Pulmonary Tumor Metastasis. B16 melanoma model. Groups of five C57BL/6J mice were inoculated with 5 × 106 B16 melanoma cells via the tail vein and subsequently treated i.p. with human TFPI, rNAPc2, rNAP5, or equal volumes of diluent control. Treatment was initiated on day 3, post-tumor cell inoculation. Animals were sacrificed and necropsied after 10–14 days of treatment. The lungs were removed, and the number of pulmonary colonies was counted under a dissecting microscope (LLC spontaneous metastatic model). LLC cells (1 × 106) were injected into the hind footpad of 6–8-week-old males. Fourteen days later, the primary tumor was resected by amputation of the rear leg just below the head of the femur. Animals with resected tumors were treated with rNAPc2 daily after ligation. After sacrifice by CO2 asphyxiation, lungs were removed and weighed. Lung weight gain was calculated by subtracting the average lung weights of age-matched normal mice. Efficacy was calculated as above, and results were analyzed for statistical significance using the Student t test.

Matrigel Plug Assay. Groups of 10 animals were injected s.c. at the ventral midline posterior to the xiphoid process with 0.5 ml of Matrigel (Collaborative Research) to which bFGF (final concentration of 2 μg/ml) was added. Control animals were injected with Matrigel lacking bFGF. Animals were treated daily with various amounts of rNAPc2 or buffer control. After 6 days, animals were euthanized with CO2. The Matrigel plug was removed, weighed, and frozen after the addition of 1 ml of water. Angiogenesis was quantified by homogenizing the plug and quantifying the amount of hemoglobin using the Sigma hemoglobin kit (527-A).

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2 The abbreviations used are: TF/FVIIa, tissue factor/factor VIIa; TFPI, tissue factor pathway inhibitor; T/C, treated versus control; LLC, Lewis lung carcinoma; VLDL, very low density lipoprotein; bFGF, basic fibroblast growth factor.
RESULTS AND DISCUSSION

TFPI Inhibits Primary and Metastatic Tumor Growth. To study the regulation of tumor growth by the coagulation cascade in vivo, we first assessed the antitumor activity of TFPI, the primary physiological inhibitor of the TF/fVIIa complex, which is the major procoagulant in cancer. Initially, we assessed its effects in a B16 melanoma primary tumor model. In this model, mice are inoculated with B16 melanoma cells on their hind flank, and peritumoral treatment with TFPI, or control diluent, was initiated after tumor volume surpassed 100 mm³. As shown in Fig. 1A, initially, TFPI inhibited primary melanoma tumor growth by >80%. However, after day 13, tumors begin to exhibit growth kinetics similar to the control treatment. Although this may be attributable to the aggressive nature of the B16 melanoma, we have also found that B16 tumor cells secrete a protease activity that degrades and inactivates TFPI (data not shown), possibly MMP-2 (15).

In an experimental metastasis model, TFPI inhibited B16 melanoma metastatic tumor growth. It has been established in animal models that metastatic tumor seeding is coagulation dependent. For this reason, we initiated TFPI treatment 3 days after inoculation, when tumor seeding is complete. In this way, the potential growth inhibitory effects of TFPI are specifically assessed and not its effect on the earlier steps of metastasis that are coagulation dependent. In this experiment, TFPI significantly inhibited the formation of lung surface metastases (T/C = 0.36, P < 0.02; Fig. 1B). Collectively, these data suggest an important role for the coagulation cascade in the growth of primary and metastatic tumors.

Because TFPI inhibits coagulation by blocking the proteolytic activity of both TF/fVIIa complexes and fXa, we next sought to determine whether inhibition of either of these enzymes alone was sufficient to inhibit tumor growth. We hypothesized that if TFPI’s antitumor activity was caused by inhibition of the coagulation cascade, then inhibitors of either TF/fVIIa or fXa should have identical antitumor activity. We tested this by using two small protein anticoagulants originally isolated from the hematophagous nematode A. caninum, rNAPc2, which is a specific inhibitor of TF/fVIIa (16), and rNAP5, with specificity for fXa (17).

rNAPc2 Inhibits Tumor Growth and Angiogenesis. The antitumor activity of both rNAP5 and rNAPc2 was first assessed in the LLC primary tumor model. In initial experiments, mice were treated with 2.5 mg/kg/day either rNAPc2 or rNAP5 for 7–10 days. Under these conditions, the TF/fVIIa-specific inhibitor rNAPc2 dramatically inhibited the growth of tumors (Fig. 2A; T/C = 0.17, P = 0.02), whereas the fXa inhibitor rNAP5 did not inhibit tumor growth (Fig. 2B; T/C = 0.69, P = 0.22). The antitumor activity of rNAPc2 was dose dependent, with an IC₅₀ of ~0.75 mg/kg/day (data not shown). The absence of antitumor activity for rNAP5 was not attributable to inadequate or incomplete inhibition of fXa, although, since at the highest level of treatment (2.5 mg/kg/day), 40% of the animals died during the dosage period as a result of i.p. hemorrhage. Because of this high mortality, we were unable to test higher doses of rNAP5 for possible antitumor efficacy. The presence of hemorrhage confirms that rNAP5 was functional at the doses assessed and suggests that fXa activity is not necessary for the growth of primary LLC tumors.

Additional studies were performed to assess the effect of rNAPc2 and rNAP5 on metastatic tumor growth in the B16 melanoma model and LLC spontaneous model. In this B16 model (Fig. 3A), 1 mg/kg/day rNAPc2 significantly inhibited the growth of metastatic tumors (T/C = 0.48, P < 0.01). In contrast, rNAP5 inhibited metastases poorly and with marginal significance (T/C = 0.76, P = 0.04). A higher dose of rNAP5 (2 mg/kg/day) also failed to inhibit metastatic tumor growth, although these animals suffered significant mortality (80% by day 10) from peritoneal hemorrhage, suggesting that the levels of rNAP5 achieved were sufficient to block fXa activity and coagulation. Additionally, metastatic tumor growth was not inhibited in the surviving animal (data not shown).

Experiments performed using the LLC spontaneous metastasis model (Fig. 3B) were limited to rNAPc2, because rNAP5 demonstrated no efficacy in either the LLC primary tumor model or the B16 metastatic tumor model. In this model, rNAPc2 exhibited potent inhibition of metastatic tumor growth within the tested range (T/C = 0.32, 0.04 up to 1 mg/kg). These data demonstrate that inhibitors of TF/fVIIa have potent antitumor activity, whereas the fXa inhibitor rNAP5 does not, suggesting that TF/fVIIa may support tumor growth through a nonhemostatic mechanism.

Because TF/fVIIa complexes have been reported to regulate angiogenesis, we next assessed the activity of rNAPc2 in the Matrigel plug assay, an in vivo angiogenesis model. In this assay, rNAPc2 exhibits very potent inhibitory activity in the range of 40–160 µg/kg (Fig. 4). At the lowest dose tested (4 µg/kg), angiogenesis was still inhibited by 57% (data not shown). At doses > 160 µg/kg, rNAPc2 caused significant hemorrhage in the Matrigel plug and adjacent tissue. The results in this model demonstrate that the TF/fVIIa activity plays an important role in stimulating angiogenesis, suggesting a possible
mechanism of action for the \textit{in vivo} antitumor activity of TF/fVIIa complex inhibitors. Previous work from our lab demonstrated that TFPI is a potent inhibitor of human umbilical vascular endothelial cell proliferation and that this activity requires its interaction with the VLDL receptor (18). In contrast, rNAPc2 does not inhibit human umbilical vascular endothelial cell proliferation, nor does it bind to the VLDL receptor (data not shown). Collectively, these studies suggest that the TFPI molecule can regulate tumor growth through two separate mechanisms: (a) through inhibition of TF/fVIIa complexes; and (b) through interaction of its COOH terminus with the VLDL receptor. rNAPc2’s antiangiogenic and antitumor activities appear to be related only to its ability to inhibit TF/fVIIa proteolytic activity. The inactivity of rNAP5 in these experiments suggests that fXa activity may not be required for tumor growth and angiogenesis. Although there are several studies demonstrating that fXa inhibitors can block metastasis (4, 19), they block metastasis by inhibiting tumor cell seeding and not tumor cell growth.

In this study, we demonstrate that TF/fVIIa inhibitors have potent antitumor and antiangiogenic activity, suggesting that the proteolytic activity of the TF/fVIIa complex supports angiogenesis and tumor growth \textit{in vivo}. Surprisingly, although TF/fVIIa activity is vital to these processes, we show that the proteolytic activity of fXa is not required. This suggests that the entire coagulation cascade is not necessary for tumor growth and angiogenesis. Instead, it appears that the TF/fVIIa complex is acting proteolytically through another mechanism, possibly through activation of a proteinase-activated receptor. Proteinase-activated receptors are a family of four G protein-coupled

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**Fig. 2.** Antitumor activity of nematode anticoagulant proteins. Mice bearing primary LLC tumors were treated daily, by i.p. administration, with increasing amounts of either rNAPc2 (○, A), rNAP5 (○, B), or buffer control (●, both panels). Tumor volume is presented as mean +/− SD for groups of 5 animals.

**Fig. 3.** Effect of nematode anticoagulant proteins on metastatic tumor growth. In A, mice bearing B16 melanoma metastases were treated with rNAPc2 (▲), rNAP5 (●), or PBS (○). Metastatic tumor foci were counted and shown for each mouse. Open symbol plus a bar represents the mean +/− SD. In B, mice bearing LLC metastases were treated with rNAPc2. Results show lung mass increase for each animal in the group (4–6 animals/group). The open symbol and bar represents mean +/− SD.

**Fig. 4.** Effect of rNAPc2 on \textit{in vivo} Matrigel angiogenesis assay. Mice bearing Matrigel plugs, mixed with 2 μg/ml bFGF, were treated with increasing doses of rNAPc2 daily. Control mice were implanted with Matrigel plugs lacking bFGF. Groups consisted of 8 animals, and data are presented as means +/− SD.
receptors that signal in response to their proteolytic cleavage by hemostatic enzymes (20) and play an important role in embryonic vascular development and hemostasis. Because these receptors signal in response to activation of coagulation, they provide a very promising target for the nonhemostatic activity of the TF/fVIIa complex (21, 22).

Clinical studies demonstrating potential antitumor effects of coagulation inhibitors have generated renewed interest in developing anticoagulants as antitumor agents. This goal needs to be reassessed in light of our findings, because the protumor activity of TF/fVIIa appears to be independent of its procoagulant activity. Treatment with anticoagulants may decrease TF/fVIIa activity and inhibit tumor growth; however, the level of treatment necessary to block clotting may not be adequate to block the protumor activity of the TF/fVIIa complex. In our studies, the antitumor activity of rNAPc2 was seen at doses significantly higher than necessary for anticoagulant activity in clinical trials (23). Thus, identification of this nonhemostatic pathway of TF/fVIIa activity provides a way to completely separate the protumor and procoagulant activities of TF/fVIIa. Further characterization of this nonhemostatic pathway should allow the development of inhibitors that solely block the proangiogenic activity of TF/fVIIa, without altering its procoagulant activity.

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