Update from the laboratory: mechanistic studies of pathways of cancer-associated venous thrombosis using mouse models

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Cancer patients have an increased risk of venous thromboembolism (VTE). The rate of VTE varies with cancer type, with pancreatic cancer having one of the highest rates, suggesting that there are cancer type–specific mechanisms of VTE. Risk assessment scores, such as the Khorana score, have been developed to identify ambulatory cancer patients at high risk of VTE. However, the Khorana score performed poorly in discriminating pancreatic cancer patients at risk of VTE. Currently, thromboprophylaxis is not recommended for cancer outpatients. Recent clinical trials showed that factor Xa (FXa) inhibitors reduced VTE in high-risk cancer patients but also increased major bleeding. Understanding the mechanisms of cancer-associated thrombosis should lead to the development of safer antithrombotic drugs. Mouse models can be used to study the role of different prothrombotic pathways in cancer-associated thrombosis. Human and mouse studies support the notion that 2 prothrombotic pathways contribute to VTE in pancreatic cancer patients: tumor-derived, tissue factor–positive (TF^+) extracellular vesicles (EVs), and neutrophils and neutrophil extracellular traps (NETs). In pancreatic cancer patients, elevated levels of plasma EVTF activity and citrullinated histone H3 (H3Cit), a NET biomarker, are independently associated with VTE. We observed increased levels of circulating tumor-derived TF^+ EVs, neutrophils, cell-free DNA, and H3Cit in nude mice bearing human pancreatic tumors. Importantly, inhibition of tumor-derived human TF, depletion of neutrophils, or administration of DNAse I to degrade cell-free DNA (including NETs) reduced venous thrombosis in tumor-bearing mice. These studies demonstrate that tumor-derived TF^+ EVs, neutrophils, and cell-free DNA contribute to venous thrombosis in a mouse model of pancreatic cancer.

Learning Objectives

- Understand the role of tumor-derived, TF EVs in venous thrombosis in pancreatic cancer
- Understand the role of neutrophils and NETs in venous thrombosis in pancreatic cancer

Introduction

Cancer patients have an increased risk of all forms of thrombosis, particularly venous thromboembolism (VTE).[1,2] There are many factors that may increase the risk of VTE, including treatment and patient characteristics. In addition, the cancer site has a major influence on the rate of VTE. For instance, breast and prostate cancer have low rates of VTE, whereas pancreatic cancer has a high rate of VTE,[2,3] which suggests that there may be cancer type–specific mechanisms of VTE.

Risk assessment scores have been developed to identify ambulatory cancer patients at high risk for VTE. The Khorana score is the most popular score for the ambulatory cancer population.[4] The Khorana score consists of cancer site, hemoglobin level or use of erythropoiesis-stimulating agents, leukocyte count, platelet count, and body mass index. However, the score is dominated by the cancer site. For instance, a score of ≥2 is deemed high risk, but a score of 2 is given for pancreatic or stomach cancer alone. The biomarkers D-dimer and soluble P-selectin were added to the Khorana score to improve the discrimination between low-, moderate-, and high-risk groups.[5] Recently, a clinical prediction model for a general cancer population was proposed (the Vienna Cancer and Thrombosis Study score) that includes only 2 parameters: cancer site and D-dimer.[6] A recent prospective study showed that fibrin-positive extracellular vesicles (EVs) but not D-dimer was associated with thromboembolic events in patients with colorectal and pancreatic cancer.[7] One study found that the Khorana score performed poorly as a predictor of VTE in pancreatic cancer patients because a similar percentage of patients with intermediate and high scores developed VTE.[8] This suggests that cancer type–specific scores are needed to identify patients at risk for VTE.

Despite the development of risk assessment scores, thromboprophylaxis is not recommended for cancer outpatients, except for those with multiple myeloma.[9] Recently, 2 studies[10,11] determined the effect of factor Xa (FXa) inhibition on VTE in high-risk ambulatory cancer patients (Khorana score ≥2). As expected, the AVERT study showed that the FXa inhibitor apixaban significantly

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reduced VTE but also significantly increased major bleeding.[10] Similar findings were observed in the CASSINI trial with the FXa inhibitor rivaroxaban, although the changes in VTE and major bleeding were not statistically significant.[11] These studies indicate there is a need for new antithrombotics to safely prevent VTE in cancer patients.

Mouse models of cancer-associated venous thrombosis

Mouse models can be used to determine the role of different prothrombotic pathways in cancer-associated thrombosis. However, several decisions need to be made when establishing a relevant mouse model.[12] For instance, either immunocompetent or immunodeficient mice can be used. Immunocompetent mice are superior because they have a functional immune system that may contribute to cancer-associated thrombosis. However, the number of mouse cancer cell lines is limited. In contrast, there are large numbers of human cancer cell lines and patient-derived xenografts that can be grown in immunodeficient mice. Tumors can be grown subcutaneously or orthotopically. Orthotopic tumors grow in a microenvironment that mimics the microenvironment present in cancer patients. However, noninvasive monitoring of orthotopically grown tumors, such as pancreatic tumors, requires expression of reporter genes, such as luciferase. The advantage of subcutaneous tumors is that growth can easily be measured. Finally, there are several different thrombosis models that can be used that include ligation of the inferior vena cava (IVC), ferric chloride treatment of various vessels (including mesenteric vessels), and laser injury of cremaster venules.[13] However, the most common are the IVC stasis and stenosis models.[14] The advantages of the IVC stasis model is that ~100% of the mice develop thrombi, and the thrombi are large and can be analyzed biochemically (~25 mg). The advantages of the IVC stenosis model is that blood flow is maintained and there is minimal damage to the vessel wall. However, there is a variable incidence (~50%) and the thrombi are small (~5 mg). Currently, there is no mouse model of spontaneous cancer-associated thrombosis. At present, the best we can do is to assess a prothrombotic state in tumor-bearing mice by measuring levels of thrombin-antithrombin complexes.

We developed a mouse model of pancreatic cancer that we have used to determine the role of different prothrombotic pathways in venous thrombosis.[15] First, we chose to use immunodeficient mice bearing human pancreatic tumors because of the availability of numerous well-characterized lines. We selected the BxPc-3 cell line because it expresses high levels of tissue factor (TF). Second, we compared the activation of coagulation in mice bearing subcutaneous vs orthotopic HPAF-II or BxPc-3 tumors. Interestingly, we observed activation of coagulation only in mice bearing orthotopic tumors[16] (Y.H. and N.M., unpublished data, 1 June 2018). We introduced the luciferase reporter gene into BxPc-3 cells to allow noninvasive growth of orthotopic tumors using the IVIS Lumina in vivo imaging system.[15] Importantly, we found an association between tumor size and enhancement of thrombus weight. However, there was a threshold tumor size that caused an increase in thrombosis weight and an upper limit of tumor weight from our animal protocol. Therefore, we used mice with 2- to 3-g tumors for all thrombosis experiments. Finally, we chose the IVC stasis thrombosis model because it produces thrombi in almost all mice, and the large thrombi allowed biochemical analysis. The strengths of our model are that (1) tumor-derived human factors can be distinguished from host-derived murine factors, (2) there is an association between tumor size and thrombus weight, and (3) unlike other models we have tried (Hisada and Mackman, unpublished data), nude mice bearing BxPc-3 tumors tolerate the surgery required for ligation of the IVC.

Role of tumor-derived, TF$^+$ EVs in venous thrombosis in pancreatic cancer patients and mice bearing human pancreatic tumors

We and others have analyzed the association between TF$^+$ EVs and VTE in cancer patients. EVs (also known as microparticles or microvesicles) are small membrane vesicles released from activated or apoptotic cells.[17,18] In a study of breast and pancreatic cancer patients, higher levels of EVTF activity were observed in patients with VTE compared with those without VTE[19] (Table 1). Additional studies have measured EVTF activity at the time of diagnosis and then observed the patients for symptomatic VTE. One study measured the level of EVTF activity in pancreatic, stomach, colorectal, and brain cancer.[20] Pancreatic cancer patients had the highest levels of EVTF activity among these 4 cancer types.[20] Moreover, there was a borderline significant association between EVTF activity and VTE and a significant association between EVTF activity and mortality in pancreatic cancer patients but not in patients with the other types of cancer (Table 1). However, it should be noted that the follow-up time was 2 years, which is a long time, particularly for pancreatic cancer patients who have a relatively short survival. A second study with pancreatic and biliary cancer patients found that EVTF activity was associated with thromboembolism and mortality in a 6-month follow-up period[21] (Table 1). Another study used a TF-dependent fibrin generation assay to measure plasma TF activity in patients with esophagus, lung, colon, pancreas, breast, stomach, ovary, prostate, or bladder cancer[22] (Table 1). Patients with high plasma TF activity had a significantly higher rate of VTE than patients with low plasma TF activity. Pancreatic cancer patients had the highest hazard ratio for the association between plasma TF activity and VTE in a 6-month follow-up period. Finally, we found that EVTF activity was associated with mortality in a general cancer population[23] (Table 1).

Longitudinal studies have also been performed to determine the relationship between EVTF activity and VTE in cancer patients (Table 1). Longitudinal studies are superior to single time-point studies because one can determine whether a given biomarker exhibits a stepwise increase before an event such as VTE. We found that EVTF increased in a step-wise manner before VTE in 2 pancreatic cancer patients, whereas no increases in EVTF activity were observed in 8 pancreatic cancer patients without VTE over a 20-week period.[24] We performed an independent longitudinal study with patients who had pancreatic or colorectal cancer and documented symptomatic and asymptomatic VTE. We found a stepwise increase in EVTF activity in 1 of 13 pancreatic cancer patients, and this patient developed a symptomatic VTE (Raj Kasthuri, Y.H., Anton Ilich, Nigel Key, and N.M., unpublished data, 1 March 2019). We observed VTE in 5 of 22 (4 symptomatic and 1 asymptomatic) colorectal cancer patients. However, there was no increase in EVTF activity in any of the patients. Tumors, particularly pancreatic tumors, are the most likely source of circulating TF$^+$ EVs. Taken together, these studies indicate that EVTF activity is associated with mortality in general cancer patients and with VTE in pancreatic cancer patients. This suggests that EVTF activity might be a good biomarker for predicting VTE in pancreatic cancer patients. Is TF a good target for preventing VTE in pancreatic cancer patients? The answer is no, because TF plays an essential role in hemostasis. Indeed, a recent study reported a large increase in major bleeding (65% vs 19%) in pancreatic cancer patients treated with an FVIIa inhibitor.[25] An alternative strategy would be to selectively inhibit TF expression in the tumor to reduce VTE in pancreatic cancer patients. Multiple signaling pathways and proteins regulate the release of

Table 1. Association between EV or plasma TF activity and VTE and mortality in cancer patients

Reference	Tumor type	No. of patients with VTE/total no. of patients	%	Assay	VTE	Mortality
19	Pancreatic	5/23	22	EVTF activity	Higher EVTF activity in patients with VTE vs patients without VTE*	↑EVTF activity associated with mortality
	Breast	2/27	7			
24	Pancreatic	2/10	20	EVTF activity	2 patients with serial increases in EVTF activity had VTE	ND
20	Pancreatic	12/60	20	EVTF activity	Hazard ratio (per doubling of EVTF activity) pancreatic, 1.6; stomach, 0.7; brain, 0.9. All nonsignificant (borderline for pancreatic).	↑EVTF activity associated with mortality in pancreatic cancer but not in other cancers
	Stomach	6/43	14			
	Colorectal	12/126	9			
	Brain	19/119	16			
21	Pancreatic and biliary†	52/117	45	EVTF activity	↑EVTF activity in patients (3.07 ± 5.2) with VTE vs patients without (1.4 ± 1.5) ($P = .01$)	↑EVTF activity associated with mortality
23	17 different types of cancers	11/60	11	EVTF activity	EVTF activity was not associated with VTE.	↑EVTF activity associated with mortality
22	9 different types of cancers	40/648	6.1	TF-dependent fibrin generation	↑Fibrin generation associated with VTE.	ND
NP	Pancreatic	1/13	8	EVTF activity	1 pancreatic cancer patient with ↑EVTF activity had VTE	2 pancreatic cancer patients with ↑EVTF activity died
	Colorectal‡	5/22	23			

ND, not determined; NP, not published.
*Samples collected after VTE.
†Thromboembolism.
‡Asymptomatic and symptomatic.

TF^+ EVs from cancer cells. These include protein activated receptor 2,[26,27] mitogen-activated protease kinases,[28] and filamin A.[29] Inhibition of release of TF^+ EVs could be an additional way to reduced cancer-associated thrombosis.

Mouse models can be used to study the mechanisms of cancer-associated thrombosis.[12] Human and mouse tumors grown in mice release TF^+ EVs into the circulation.[13,16,30,31] Most studies on the role of tumor-derived TF^+ EVs have used mice bearing murine and human pancreatic tumors (Table 2). C57BL/6 mice bearing Panc02 mouse pancreatic tumors had shortened occlusion times in ferric chloride–injured mesenteric vessels.[13] Tumor-derived EVs from Panc02 tumors also accumulated in the laser-injured cremaster venules.[13] In addition, Panc02-bearing mice had an increase in incidence and size of thrombi in an IVC stenosis model.[32] We found that nude mice bearing HPAF-II human pancreatic tumors had a shortened occlusion time in a saphenous vein injury model but did not have larger thrombi than controls in an IVC stenosis model.[16] However, nude mice bearing BxPc-3 human pancreatic tumors had increased thrombi area in the IVC stenosis model and increased thrombi area and weight in the stasis models.[15,33] Moreover, we could detect human TF in thrombi formed in the IVC, which suggests that tumor-derived, TF^+ EVs are incorporated into thrombi. Importantly, we found that inhibition of human TF with a species-specific monoclonal antibody significantly reduced the weight of thrombi in mice bearing BxPc-3 tumors.[15] Taken together, these studies indicate that pancreatic tumors release TF^+ EVs into the circulation, and these EVs enhance venous thrombosis.

Role of neutrophils and NETs in venous thrombosis in cancer patients and mice tumors

The coagulation pathway contributes to the innate immune response by forming a clot that limits the dissemination of pathogens, such as

Table 2. Mouse studies on tumor-derived, TF^+ EVs and venous thrombosis

Reference	Mouse type	Cancer cell type	Tumor site	Thrombosis model	Observation
13	C57BL/6	Panc02	SC	Mesenteric venules $FeCl_3$	Shortened time to occlusion
13	C57BL/6	Panc02	SC	Cremaster venules laser injury	Accumulation of tumor-derived TF^+ EVs at the site of injury
32	C57BL/6	Panc02	SC	IVC stenosis	Increased incidence and weight of thrombi
16	Nude	HPAF-II	Orthotopic	IVC stenosis	No enhancement of thrombosis
16	Nude	HPAF-II	Orthotopic	Saphenous vein $FeCl_3$	Shortened time to occlusion
33	Nude	BxPc-3	Orthotopic	IVC stenosis	Increased thrombus area using ultrasound but no increase in incidence of thrombosis
15	Nude	BxPc-3	Orthotopic	IVC stasis	Increased weight of thrombus

SC, subcutaneous.

Table 3. Mouse studies that investigated the contribution of neutrophils and NETs to venous thrombosis

Reference	Mouse type	Cancer cell type	Tumor site	Thrombosis model	Observation
41	BALB/c	4T1	SC	NA	Increased neutrophil count
39	BALB/c	4T1	Orthotopic	NA	Increased neutrophil count and percentage of H3Cit high neutrophils. Increased NET formation after PAF stimulation
39	C57BL/6	LLC	SC	NA	Increased neutrophil count. Increased NET formation after PAF stimulation
42	BALB/c	4T1	Orthotopic	Rose Bengal jugular vein	Shortened time to occlusion. DNAse I reduced thrombosis in tumor-bearing mice
43	Nude	BxPc-3	Orthotopic	IVC stasis	Increased neutrophil count and plasma H3Cit, increased H3Cit in thrombus, reduced thrombus weight after neutrophil depletion or DNAse I treatment

LLC, Lewis lung carcinoma; NA, not applicable; PAF, platelet activating factor.

bacteria.[34] For instance, activated monocytes express TF. Neutrophils release neutrophil extracellular traps (NETs) that facilitate killing of bacteria.[35] NETs promote thrombosis by capturing platelets, erythrocytes, and EVs and enhancing fibrin formation.[36] Peptidylarginine deaminases (PADs), such as PAD4, citrullinate histones, and this leads to de-condensation as a step in NET formation. Citrullinated histone H3 (H3Cit) is widely used as a biomarker of NET formation. Moreover, PAD4$^{-/-}$ mice have smaller venous thrombi compared with those in control mice.[37]

A recent study analyzed the association of levels of plasma H3Cit with VTE in a variety of cancer types.[38] The strongest association between H3Cit and VTE was observed in pancreatic cancer with a significant but weaker association in lung cancer. In contrast, there was no significant association between H3Cit and VTE for breast, brain, or colon/rectum cancer and lymphoma.[38] These data suggested that the role of NETs in cancer-associated thrombosis might be cancer type specific.

Some studies showed that granulocyte colony-stimulating factor (G-CSF) in tumor-bearing mice activates neutrophils to form NETs. For instance, neutrophils isolated from BALB/c mice bearing 4T1 breast tumors treated with an anti-G-CSF antibody showed significantly less NET formation compared with those from tumor-bearing mice treated with a control antibody.[39] In addition, administration of exogenous G-CSF to mice bearing murine melanoma B16 tumors that do not produce G-CSF increased levels of H3Cit in tumors in a PAD4-dependent manner.[40] These data suggest that G-CSF plays an important role in forming NETs in these tumor-bearing mice.

Mouse studies have analyzed the role of neutrophils and NETs in cancer-associated thrombosis (Table 3). An early study observed an increase in neutrophil count in BALB/c mice bearing 4T1 breast tumors.[41] Two additional studies with mice bearing 4T1 tumors reported increased levels of neutrophils, plasma cell-free (cf) DNA, myeloperoxidase, H3Cit, and NET formation in neutrophils.[39,42] In addition, Demers et al[39] observed increased neutrophils and NET formation in mice with Lewis lung carcinoma. Importantly, mice bearing 4T1 tumors had a shortened occlusion time compared with controls in a photochemical injury model of the jugular vein.[42] Furthermore, administration of DNAse I reversed the shortened occlusion time in the tumor-bearing mice but did not affect the occlusion time in control mice.[42] Taken together, these data suggest that neutrophils and cfDNA (including NETs) contribute to venous thrombosis in mice bearing breast tumors.

We investigated the role of neutrophils and NETs in mice bearing human pancreatic tumors. We found that mice bearing BxPc-3 tumors had an increased neutrophil count and increased plasma levels of G-CSF, neutrophil elastase, cfDNA, and H3Cit.[43] In addition, we observed increased levels of the neutrophil marker Ly6G and H3Cit in thrombi from tumor-bearing mice compared with thrombi from control mice.[43] Finally, we found that either depletion of neutrophils or administration of DNAse I reduced thrombus weight in tumor-bearing mice but not control mice.[43] Our data with control mice is consistent with data from Leal et al[42] showing that DNAse I did not affect basal thrombus formation. In addition, another study found that depletion of neutrophils did not affect thrombosis in control mice in the IVC stasis model.[44] Taken together, these data suggest that neutrophils and NETs contribute to venous thrombosis in pancreatic cancer patients and mice bearing human pancreatic tumors.

In conclusion, elucidation of prothrombotic pathways that drive cancer-associated thrombosis may reveal new biomarkers that can be used to identify patients at risk of VTE and new targets for the development of safe antithrombotic drugs for cancer patients. Cancer patients may have common prothrombotic pathways and cancer type–specific pathways that drive thrombosis. For instance, tumor-derived TF^{+} EVs may contribute to VTE in pancreatic cancer but not other cancer types whereas neutrophils and NETs may contribute to VTE in both pancreatic and lung cancer.

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