Interplay between cancer and thrombosis; identification of key factors

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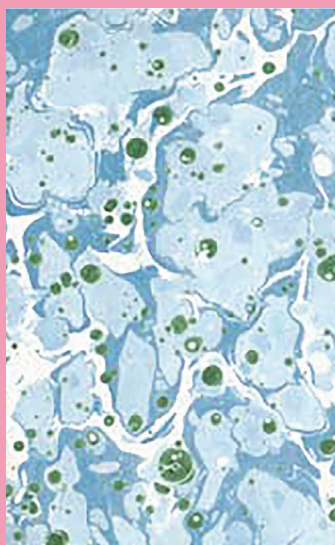

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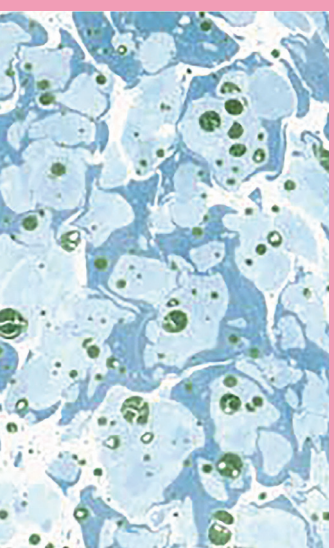

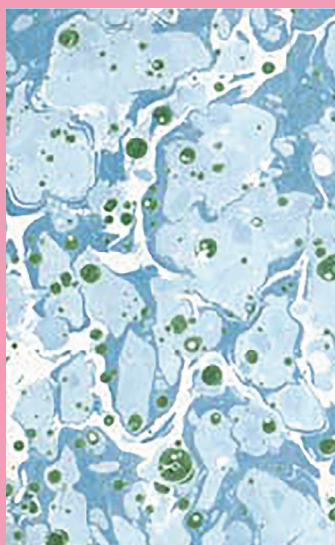

Chapter 5

Development of a spontaneous model for cancer-associated thrombosis

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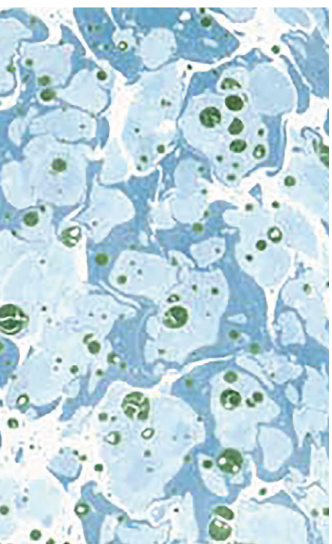

INTRODUCTION

One in eight women will be diagnosed with breast cancer in their life-time. In addition, it is estimated that 250.000 new invasive cases will be reported in 2017 [1]. Despite early detection and improved treatment over the last decade, breast cancer is still ranked among the cancer types with high mortality rates [1]. Survival of breast cancer patients is further reduced when these patients present with cancer-associated thrombosis (CAT). The risk of venous thromboembolism (VTE) in patients with breast cancer is increased by 3-4-fold when compared to women that are not diagnosed with breast cancer [2-4]. In breast cancer patients the relative incidence of CAT is around 1-2% of all breast cancer patients and is therefore generally categorized as a cancer type with a low risk of CAT [5]. However, the absolute incidence of VTE event in breast cancer is high as i) metastasis dramatically increases the risk of CAT and ii) breast cancer is one of the most frequently diagnosed types of cancer. For these reasons, up to 17% of all CAT cases are breast cancer-related [2, 6]. While studies have revealed that most cancer patients have a procoagulant state [7-9] this does not necessarily lead to formation of a thrombus, the reasons for which are unknown. This poor understanding of the mechanism underlying CAT makes it is a challenge to predict which cancer patient will develop CAT and which patient might benefit from prophylactic anticoagulant treatment.

In our laboratory we have developed a preclinical spontaneous thrombosis model relying on siRNA-dependent downregulation of antithrombin (*Serpinc1*) expression in the liver [10]. This imbalance of coagulation causes occlusive venous thrombi and hemorrhages in the head of these mice within days, with consumption of platelets, fibrin deposition in the liver and thrombus formation in the head. However, fibrinogen levels in the plasma are not affected [10, 11]. This study examines the impact of an aggressive breast cancer on thrombus formation in mice using the spontaneous thrombosis model. We demonstrate that the presence of breast cancer alleviates the thrombotic phenotype in mice, when tumor compared to cancer-free mice.

METHODS

Animal experiments

All the animal experiments were approved by the animal welfare committee of the Leiden University Medical Center (LUMC). Orthotopic injections were performed as described previously [12]. In brief, 5x10^5 MDA-MB-231-pcDNA-GFP-lung cells or 50 µl serum free media (Sham) were injected into inguinal fat pads of 6 week-old female NOD-SCID mice (Charles River, Wilmington, MA, USA); as an analgesic 0.1 mg/kg temgesic (Schering-Plough, Kenilworth, NJ, USA) was injected. The tumor dimensions were measured with a caliper and the volume was calculated with the formula V=(LxW^2)/2. When tumors reached ~400 mm^3, the experiment siRNA-mediated silencing was started as previously described [10]. In short, siRNAs targeting antithrombin (***Serpinc1***; cat. #S62673; Ambion, Carlsbad, CA, USA) or control (***NEG***; cat #4404020; Ambion, Carlsbad, CA) were complexed with Invivofectamine 3.0 (Invitrogen, Carlsbad, CA, USA) and 1.2 mg/kg of body weight siRNA was injected intravenously. Citrated blood was collected 1 day prior to xenograftment and siRNA treatment and at the end point (sacrifice).

Four days after tail vein injections, before mice were sacrificed, citrated blood was collected for blood analyses using a hematology analyzer (Sysmex XP-300; Sysmex Corporation, Kobe, JPN). To collect circulating tumor cells [13], 450 µl blood was drawn from the right atrium via heart puncture, after red blood cell lysis, cells were grown ***ex vivo*** for 1 week in 10 cm culture dishes. Furthermore, tumors, lungs and livers were collected, a part was snap-frozen in liquid nitrogen or fixed in 4% formaldehyde. Mouse heads were collected and fixed in 4% formaldehyde.

ELISA

Plasma antithrombin and fibrinogen protein levels were investigated using a commercial murine ELISA kit, according to manufacturer's protocol (Affinity Biologicals). Pooled plasma from mice was set as a reference.

To determine presence of metastasis in organs, a qPCR was performed with primers against the housekeeping genes human GAPDH (Fw: 5'-TTCCAGGAGCGAGATCCCT-3'; Rv: 5'-CACCCATGACGAACATGGG-3') and mouse β-actin (Fw: 5'-AGGTCATCACTATTGGCAACGA-3'; Rv: 5'-CCAAGAAGGAAGGCTGGAAAA-3'). According to manufacturer's protocol total RNA was isolated using Trisure (Bioline; Bio-38033; London, UK) and converted to cDNA using the Super script II kit (Life Technologies, Waltham, MA, USA). SYBR Select (Life Tech-

nologies, Waltham, MA, USA) was used to conduct qPCR on a CFX384 Touch real-time PCR detection system (BioRad, Veenendaal, the Netherlands.

Western blotting

Fibrin deposits in tumor and organs were determined using western blotting as described previously [14]. In brief, fibrin was extracted from tissue specimens, equal protein concentrations were loaded onto 4-12% Bis-Tris Plus Gels (Thermo Fisher Scientific, Waltham, MA, USA) for 20 min at 200V and blotted on 0.2 µm pore size PVDF membranes and blocked in 5% milk in TBST (Tris-buffered saline with Tween-20) for 1 h at room temperature. Blots were incubated with mAb 59D8 (a kind gift from C. Esmon, Oklahoma City, OK, USA) O/N at 4°C, 3 TBST washing steps and incubated with a horseradish peroxidase secondary antibody (Abcam, Cambridge, UK) at room temperature for 1 h. Antigens were visualized with Western Lightning Plus ECL (Perkin-Elmer, Waltham, MA, USA) using the ChemiDoc imaging system (BioRad, Veenendaal, The Netherlands). Band intensity was quantified via ImageJ software.

Statistical analysis

Data are represented as median and range. Non-parametric testing was performed, comparisons between data points were done with Student's *t* test for two conditions, or with 1way or 2way ANOVA for three or more data sets.

RESULTS

To investigate if the presence of an aggressive breast tumor contributes to timing and morphology of thrombus formation, mice were Sham operated or xenografted with the highly aggressive subclone MDA-231-pcDNA-lung, which is a cell line that was previously re-isolated from metastatic lung foci. All mice were sacrificed four days after tail vein injections with siAT (*Serpinc1*) or siNEG (control) siRNAs. To verify knockdown, antithrombin plasma levels were analyzed. Indeed, a >95% reduction of antithrombin levels in plasma was confirmed 4 days post-injection, whereas control siRNA had no effect on plasma levels after tail vein injections (Fig. 1A). The presence of an aggressive breast tumor had no effect on plasma antithrombin concentration. As reported earlier [10], no plasma fibrinogen consumption was detected in this thrombosis model where only *Serpinc1* was targeted, although the presence of a tumor significantly increased overall fibrinogen levels 3-fold (Fig. 1B). Interestingly, white blood cell counts were elevated by 4-fold in mice bearing a

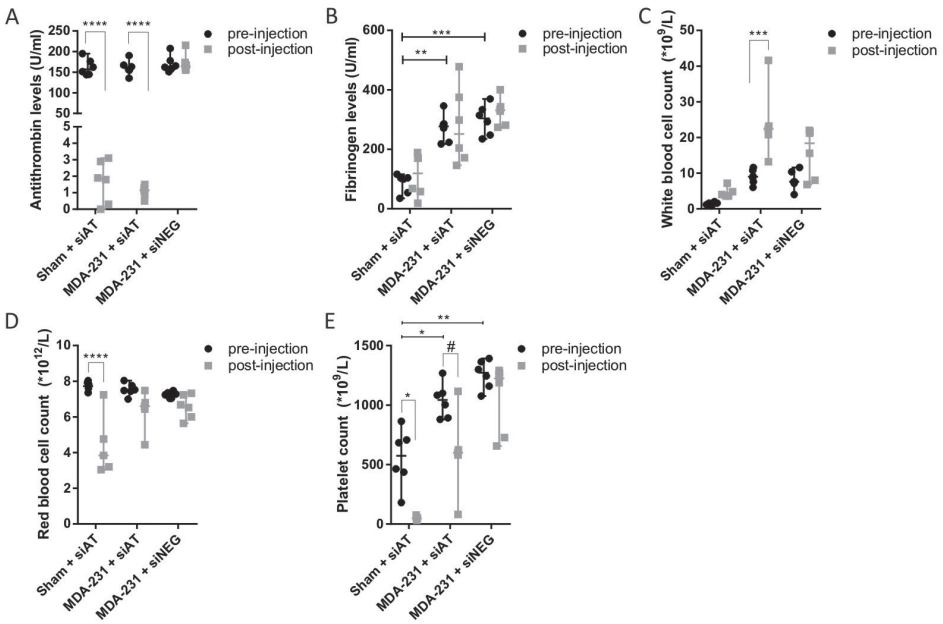

Figure 1 Plasma analysis of NOD-SCID mice before and after siRNA treatment.

(**A**) At 4 days post injection mouse plasma analysis for antithrombin knockdown and (**B**) fibrinogen levels. (**C**) White and (**D**) red blood cell counts in plasma before and after siRNA treatment. (**E**) Platelet levels at baseline (black) and consumed (grey) levels. #P<0.15, * P<0.05, *** P<0.001, **** P<0.0001

tumor when compared to the Sham group. In addition, a significant increase in white blood cells was only observed in tumor-bearing mice after siAT treatment, while in Sham- and control siRNA-treated mice the increase in white blood cells did not reach significance (Fig. 1C). Furthermore, a 2-fold decrease of red blood cells was observed only in mice with siAT injections in the absence of a tumor (Fig. 1D). Platelets were completely consumed in the Sham group, while a trend towards only moderate platelet consumption was shown in mice with a tumor (Fig. 1E). Interestingly, baseline platelet levels were twice as high in mice with a tumor compared to those without cancer. We conclude that an efficient knockdown of antithrombin resulted in platelet consumption while fibrinogen levels remained unaffected.

To investigate whether siRNA treatment had any short-term effects on malignancy, tumor characteristics were studied. No effects of siRNA treatment on tumor volume and weight were observed (Fig. 2A and B). Furthermore, similar numbers of circulating tumor cells were

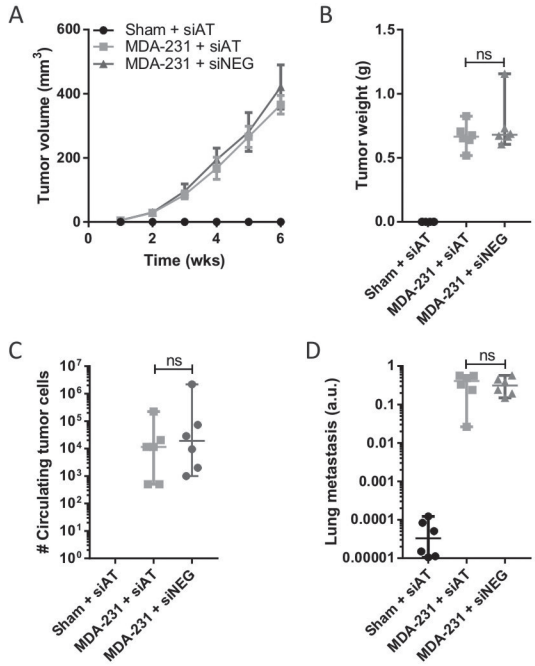

Figure 2 Tumor characteristics of NOD-Scid mice 4 days after siRNA tail vein injections.

(**A**) Sham or tumor cells were orthotopically injected and tumor volumes were monitored until week 6. (**B**) Tumor weight after mice were sacrificed. (**C**) The total amount of outgrown circulating cells *ex vivo* were counted. (**D**) Lungs were collected and metastasis was assessed via qPCR, where human GAPDH expression was normalized to mouse β-actin levels. NS = not significant.

present in mice with breast tumors (Fig. 2C). As expected the Sham group had no circulating tumor cells. As for metastasis to the lungs, no differences were observed in mice treated with siAT or siNEG (Fig. 2D). These data show that siRNA treatment and targeted knock-down of antithrombin in the liver has no short-term effects on tumor progression *in vivo*.

We investigated whether the presence of an aggressive tumor affected the formation of thrombosis *in vivo.* The typical clinical features of the thrombotic coagulopathy after siAT injection were detected in the head with swellings in the head (Fig. 3A). Five out of 6 mice presented clinical signs in the Sham+siAT group, whereas only 3 out of 6 mice had hemorrhages in the MDA-231+siAT condition (Fig. 3B), albeit not statistically significant. As expected, none of the control mice (MDA-231+siNEG) showed signs of bleeding. We finally

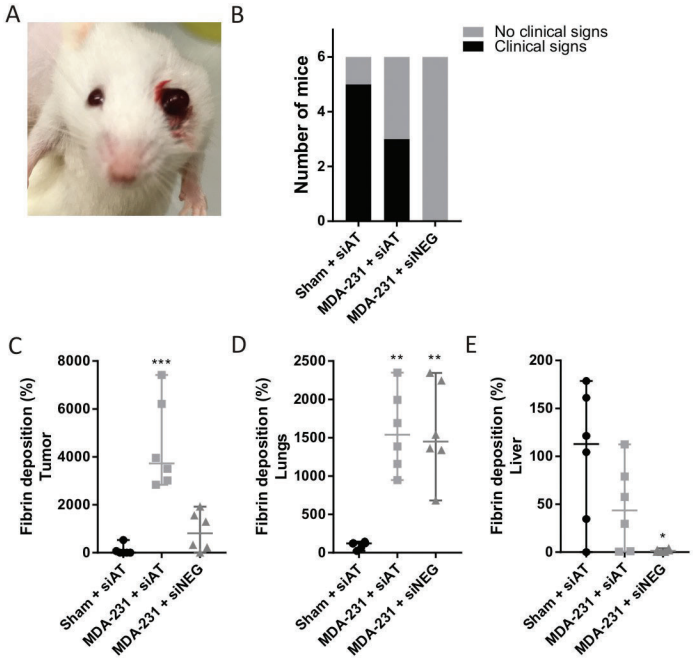

Figure 3 The presence of an aggressive breast cancer tumor improves CAT score. (**A**) Clinical sign of a bleeding phenotype in the left eye of a NOD-Scid mouse 4 days post siAT injection. (**B**) Total number of mice that presented the clinical signs after 4 days of siRNA treatment. (**C-E**) Fibrin deposition in the (**C**) tumor in tumor-bearing mice or mammary fat pad in the Sham group, (**D**) lungs and (**E**) liver were analyzed western blot antigen levels using ImageJ. * P<0.05, ** P<0.01, *** P<0.001

investigated fibrin deposition in various tissues. In Sham mice treated with siAT low levels of fibrin deposition were present in the mammary fat pad (Fig. 3C). However, a dramatic increase in fibrin deposition was found in tumors, 45-fold and 9-fold in siAT and siNEG treated mice respectively. Remarkably, fibrin deposition in the lungs was significantly increased 15-fold in tumor bearing mice when compared to Sham mice, independently of siRNA injection (Fig. 3D). As these mice had lung metastasis, this suggests fibrin formation by the tumor and not siRNA injections. Furthermore, as reported previously [10], high fibrin deposits were present in the liver in Sham+siAT mice and this was reduced in the MDA-231+siAT group (Fig. 3E). Together, these data suggest that the presence of a tumor in combination with antithrombin knockdown has synergistic effects on fibrin deposition in the mammary fat pad, with reduced clinical signs of hemorrhage.

DISCUSSION

Development of thrombosis in cancer patients correlates with poor survival. Unfortunately, the underlying mechanisms remain poorly understood. In this study, we investigated the presence of an aggressive breast tumor on thrombus formation in a spontaneous mouse model for thrombosis. Surprisingly, this study reveals that the presence of an aggressive breast cancer rescues mice from hemostatic abnormalities upon lowering antithrombin levels. In addition, platelet counts were elevated in tumor bearing mice. siRNA treatment on itself had no effect on tumor characteristics. Increased white blood cell counts suggested a pro-inflammatory status in mice with breast tumors that was further elevated after siRNA injections.

Although this ***in vivo*** model is described previously as a spontaneous thrombosis model, based on the data presented it suggests a consumptive coagulopathy that might also reflect a state of disseminated intravascular coagulation (DIC). DIC is one of the most extreme forms of dysregulated hemostasis and is characterized by hemorrhages and/or thrombosis concomitant with decreased platelet count, low fibrinogen levels, prolonged prothrombin time and increased fibrin deposition [15]. This extreme form of hypercoagulation presents in 5% of all breast cancer patients and especially adenocarcinomas of the breast are frequently associated with increased risk of DIC [16]. Although the mechanism behind cancer-associated DIC has remained elusive, it is believed that it may have the same triggers as cancer-associated thrombosis, such as elevated Tissue Factor either on primary tumor cells or extracellular vesicles, and a pro-inflammatory status in patients [17].

In contrast to our hypothesis, in the current model, the presence of a tumor appeared to protect from severe bleeding in the head, albeit not statistically significant. Plasma analysis revealed high platelet counts upon establishment of a tumor before the induction of spontaneous VTE. The platelet counts were above $1000x10^9$ counts/L, which would mimic thrombocytosis in a human setting. Interestingly, thrombocytosis is associated with inflammatory breast cancer [18] and metastasis [19], both being present in our mouse model. As platelets in our model are not completely consumed upon tumor growth, it is tempting to speculate that thrombocytosis produces a phenotype that rescues mice from hemorrhagic bleedings in our preclinical model.

Besides elevated platelet counts, mice with MDA-231 tumors showed upregulated plasma fibrinogen levels, that remained unaltered after siRNA treatment. Several studies have indicated an association of elevated plasma fibrinogen with higher tumor grade and poor

survival [20-23]. In fact, increased fibrinogen levels are considered a marker of systemic inflammation [24]. This is in line with our results, as mice showed 10-fold higher white blood cell counts prior to siRNA treatment. These cell counts were further increased after siRNA injections.

Fibrin deposits in the liver after antithrombin knockdown were increased, as previously reported [25]. Interestingly, in the presence of a tumor less fibrin deposition products were detected in the liver after knockdown of antithrombin, although no significance could be reached. In contrast, a tumor and decreased plasma antithrombin synergistically increased fibrin levels in the mammary glands of mice when compared to Sham+siAT or tumor+siNEG conditions. The reason for this drop in liver fibrin deposition and increase in tumor fibrin deposits remain unclear. Although speculative, this increased fibrin deposition may be formed by the tumor cells as these cells are known to activate platelets and thereby mediate fibrin formation. This latter may - on its turn - prevent attack by natural killer and promote survival of cancer cells [26]. Furthermore, fibrin deposits in the lungs were low in Sham mice, while the lungs of tumor-bearing mice displayed equal fibrin deposits, irrespective of siAT treatment. This might be explained by the selection of the breast cancer cell line for this study. To ensure an aggressive breast cancer phenotype in mice, MDA-MB-231-pcDNA-GFP-lung cells were selected. This cell line was originally derived from MDA-MB-231 cells that stably express GFP and was isolated from lung metastatic foci. Therefore, it has a high preference of metastasis to the lungs, which may directly result in tumor-induced fibrin deposition in the lung.

Tumor expressed TF or TF$^+$ extracellular vesicles are considered to be major players in cancer-associated thrombosis – including DIC. In our study we have used an aggressive breast cancer cell line that overexpresses TF. Based on literature, it was expected that plasma from mice with a tumor would be hypercoagulant, with increased FXa generation rate. Despite high TF content on our tumor cells, plasma analysis showed no differences in FXa presence (data not shown). In contrast with our findings, Hisada et al. reported increased FXa generation in mice bearing orthotopic pancreas tumors [27], although this is the only cancer type that is reported to show an association between TF$^+$ EVs and venous thromboembolism [28]. Therefore, it is unlikely that high amounts of circulating coagulant TF (extracellular vesicles) were present in this mouse model with an aggressive breast tumor.

An increased inflammatory status was observed in mice bearing a tumor, and after siRNA treatment. Unfortunately, the hematology analyzer used in this study did not discriminate

between various types of white blood cells. Therefore, it would be of interest to determine the nature of the immune cells involved in our model.

According to Virchow's triangle three components are of importance in the risk of VTE , i.e.: i) damaged endothelium, ii) disrupted blood flow and iii) changes in blood composition [29]. In this spontaneous model only blood composition is affected; although it might be argued that a tumor, which secretes various (pro-inflammatory) cytokines and has leaky vessels, might contribute to damaged or disrupted endothelium [30]. To reduce blood flow in mice, the stenosis model - partial ligation of the vena cava - might be a sophisticated model to further test our hypothesis.

To our knowledge, this is the first-ever study that investigated the role of a tumor in DIC in preclinical models. This showed that the presence of a tumor in mice with downregulated plasma antithrombin levels has a protective phenotype against DIC. The tumor introduces systemic changes in the blood leading to thrombocytosis, increased fibrinogen levels and white blood cells, all contributing to less formation of clinical bleedings. Unfortunately, the underlying mechanism behind DIC in aggressive breast cancer is still not unraveled. Therefore, we propose further experiments in order to investigate and unravel the pathophysiological mechanism of breast cancer on DIC.

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