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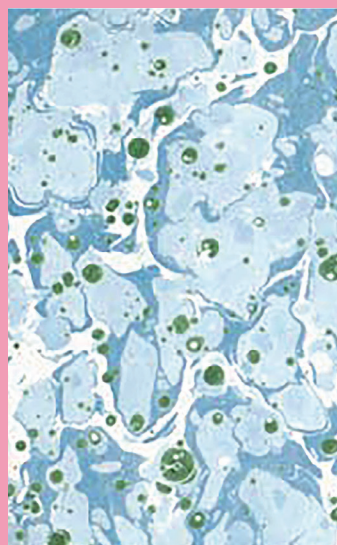


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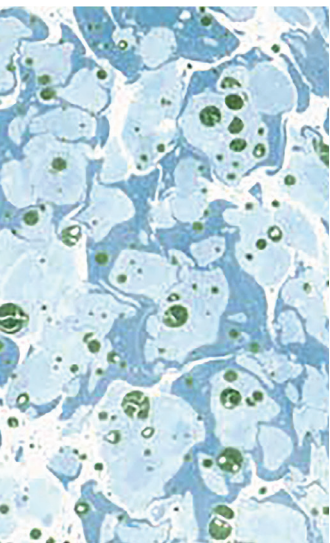
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Chapter 8

General discussion and summary



BACKGROUND

The human body is a complex system and regulates various processes, from synthesis of tiny molecules to complete organ function. The vascular system allows the blood to circulate through the body, deliver nutrients, oxygen and immune cells to the tissue and transport metabolic degradation products away from the cells. The hemostatic system keeps the vascular system in equilibrium between liquid (bleeding) and solid (clotting) phase. Hemostasis consists of a number of independent biochemical and cellular processes. Upon wounding, primary hemostasis i.e. platelet binding, activation and aggregation is initiated [1]. In addition, secondary hemostasis, i.e. the blood coagulation cascade is initiated by the principal activator of coagulation, tissue factor (TF). According to current cellular models, TF – expressed on subendothelium – activates extrinsic coagulation after it comes in contact with the blood. There, it binds and activates its natural ligand VII (FVII) to generate activated factor X (FXa), that on its turn generates low levels of thrombin. Via feedback loops, previously known as the intrinsic coagulation cascade, these levels of thrombin lead to activation of clotting factors IX, XI, FV, FVIII and FX on the surface of activated platelets. As this is a relative slow process, it contributes to clot stability [1]. Deficiencies in components of this pathway results in increased bleeding risk [2]. Finally, FX in complex with FV generates thrombin levels that are sufficient to convert soluble fibrinogen into fibrin. The fibrin-rich blood clot further stabilizes the previously formed platelet plug [1]. In disease settings, the balance of blood coagulation is disturbed. This may lead to excessive bleeding e.g. in case of coagulation deficiency such as in hemophilia or to venous thromboembolism (VTE) in case of a hypercoagulant state.

In 2003 an alternatively spliced isoform of TF (asTF) has been described, that lacks a transmembrane domain [3]. Therefore, asTF is a soluble protein and has a unique C-terminal tail as a consequence of a frameshift. It has been shown that asTF induces angiogenesis by binding to endothelium-expressed $\alpha v \beta 3$ integrins to promote endothelial cell migration and $\alpha 6 \beta 1$ integrins to form capillaries [4]. In a cancerous setting, asTF promotes tumor growth after ligation to $\beta 1$ integrin [5]. Additionally, in breast cancer it synergizes with the estrogen receptor pathway to further promote breast cancer progression [6]. Interestingly, in pancreatic ductal adenocarcinoma asTF increases coagulant activity on cells and microvesicles (MVs) [7], however, whether asTF has coagulant properties has been a matter of debate. Although the N-terminal part of the protein is identical to full-length TF (flTF) and has a binding site for FVII, asTF lacks the binding site for FIX and FX. Nevertheless, asTF is present in occlusive thrombi [8]. To further elucidate whether asTF has coagulant properties,

the first part of this thesis is dedicated to the interplay between asTF and flTF in the initiation of coagulation on endothelial cells.

In patients with cancer a hypercoagulant state is often observed and accordingly these patients are at increased risk of cancer-associated thrombosis (CAT). CAT is the second leading cause of death in cancer patients, after cancer itself, but diagnosis of VTE in these patients is also associated with increased morbidity and mortality that cannot be explained by the occurrence of VTE itself. This reduced survival may be influenced by increased activation of cellular and circulating coagulation factors that activate transmembrane receptors. In addition, while circulating coagulation factors are usually synthesized by the liver, tumor cells are also able to ectopically express procoagulant factors that promote tumor-associated processes like tumor growth, angiogenesis and metastasis [9, 10]. The second part of the thesis deals with the contribution of coagulation factors to tumor progression. Specific focus is placed on the role of TF signaling in metastasis in breast cancer, since TF is considered the linking pin in cancer and thrombosis. The final part of the thesis addresses genomic and non-genomic key players that may contribute to CAT. We describe a novel mouse model to investigate the effects of tumor development on thrombus formation. Furthermore, a proof-of-principle study is presented that elucidates molecular mechanisms and biological pathways behind CAT in an unbiased manner.

1. Alternatively spliced Tissue Factor has no function in hemostasis

Since its discovery 15 years ago, the contribution of asTF to blood coagulation has remained unclear. Despite the lack of a functional FX binding site, asTF has minimal coagulant activity in the presence of phosphatidylserine [3]. Yet, asTF has been found at the edge of growing thrombi in mouse models [8], which suggests a role for asTF in blood clotting. However, studies that had been performed so far were lacking, as no asTF protein could be detected in endothelial cells or supernatant after cytokine stimulation [11] or experiments were performed in the absence of flTF [12, 13], while asTF expression does not occur in the absence of flTF [14]. Therefore, conclusions from these studies focusing on direct or indirect asTF coagulant activity should be taken with caution [11, 12]. Hence, to address the interplay between asTF and flTF in hemostasis we co-expressed these proteins in endothelial cells and studied changes in coagulant activity of flTF in **chapter 2**. With relative low concentrations of asTF no alterations on flTF-dependent clotting was observed, neither on cells nor on MVs. In fact, when asTF and flTF were co-expressed, these isoforms did not co-localize and were found in different cellular compartments.

Based on the results in **chapter 2** it may be concluded that asTF has a limited role in coagulation activation, with minimal coagulant activity and no regulatory activity towards flTF. Nevertheless, it is possible that asTF plays a role in hemostasis. Thus far, asTF was detected in thrombi [8, 15], and associates with platelets, where it may increase coagulant potential. asTF ligation to $\beta 1$ integrins also induces interactions between endothelial cells and monocytes [3, 16], the latter being one of the known players involved in initiation and propagation of a thrombus [17]. In support of the view that asTF may modulate monocyte function, treatment of microvascular endothelial cells (MVECs) with asTF increases expression and activation of cell adhesion molecules like E-selectin, VCAM-1 and ICAM-1 to further support monocyte binding [18]. Although extremely speculative, asTF on endothelial cell membranes and/or MVs might further support thrombus formation acting as a kind of 'Velcro'. Collier et al. previously reported that endothelial cells can internalize TF⁺ MVs and recycle TF to the cell membrane thus increasing coagulant activity on these cells [19]. asTF might facilitate fusion of TF⁺ MVs to endothelial cells via ligation to integrins. Alternatively, asTF may mediate integrin-dependent TF⁺ MV shedding. Although no increase in TF⁺ MVs was observed in our study, asTF did increase procoagulant MVs shedding in a pancreatic cancer model in a $\beta 1$ integrin-dependent manner, while flTF levels remained unchanged [7]. Furthermore, $\beta 1$ integrin on TF⁺ MVs increases coagulant activity as it preserves the FVII binding site [19], a similar mode-of-action may apply to asTF. Therefore, it would be of interest to test effects of an asTF blocking antibody, RabMab1, in VTE *in vivo* models to understand the contribution of asTF to thrombus formation. Finally, determining associations between asTF plasma levels in VTE patients and controls appears warranted.

One other aspect of our study was that excessive asTF expression led to decreased flTF expression in the non-raft membrane. This reduction of flTF at the cell surface resulted in decreased coagulant activity as measured with FXa generation assays. It has to be noted that supraphysiological levels of asTF induced endoplasmic reticulum stress, as reflected by increased BiP levels, and asTF was subsequently degraded in a proteasome-dependent manner. Thus, excessive asTF expression might also result in recognition of 'incorrect' folded TF and as a result, degradation of flTF.

2. Coagulation factors contribute to tumor progression

The contribution of coagulation factors to tumor progression has been extensively investigated. Not only do coagulation factors facilitate tumor growth via diffusion from the blood into the tumor milieu due to leaky vessels, it has also been reported that tumors cells can express clotting factors ectopically. Roles of blood-derived and tumor-derived clot-

ting factors in tumor progression are extensively reviewed in **chapter 3**. It becomes clear that coagulation factors noticeably contribute to two specific hallmarks of cancer: angiogenesis and metastasis. As mentioned before, asTF induces angiogenesis in a $\beta 1$ integrin dependent manner. Angiogenesis is also promoted via intracellular signaling triggered by fITF/FVIIa and thrombin that activate members of the Protease-activated Receptor (PAR) family. Activation typically leads to increased expression and secretion of IL8, VEGF and angiopoietin-1, and thereby supports tumor growth [20]. Upon entering the bloodstream, tumor cell survival becomes critically dependent on fITF-dependent coagulation activation to form a fibrin/platelet-rich shield around the tumor cell that provides protection against the immune system and shear stress. Recently, the role of von Willebrand factor (VWF) was demonstrated to further support successful metastasis. Tumor cells can activate endothelial cells in order to secrete VWF strings. Even under shear stress, these VWF strings can activate and bind platelets, which creates a docking-site for the tumor cell to escape the bloodstream and invade in the tissue at a metastatic site [21, 22].

Tissue Factor signaling supports metastasis

For successful metastasis, tumor cells must undergo epithelial-to-mesenchymal transition (EMT) and gain cancer stem cell (CSC) properties. As others already had reported that TF influences migration of tumor cells and influences cancer stemness [23, 24], we decided to study for the first time the nature of the mechanism linking TF to these processes in **chapter 4**. We show that the antibody Mab-10H10, which inhibits TF signaling, but does not influence the coagulant properties of TF, decreases breast cancer metastasis by i) regulation of EMT-associated markers to maintain an epithelial phenotype, ii) decreasing tumor-initiating capacity through maintenance of CSCs and iii) regulating integrin function to further suppress a mesenchymal state. Furthermore, treatment of breast cancer cells with the antibody Mab-10H10 shifted TF complex formation with $\alpha 3\beta 1$ integrin to $\alpha 6\beta 1$ and $\alpha 6\beta 4$, that dictates focal adhesion kinase activation and recruitment. This finally induced an epithelial morphology with decreased tumorigenic properties and metastasis.

In our research, we used the breast cancer cell line MDA-MB-231-mfp, which expresses the asTF isoform, albeit at relative low levels. We did not assess asTF function in metastasis in **chapter 4**, nor its potential synergy with fITF. Thus far, abundant expression of asTF has been found in pancreatic and breast cancer, where it – in a $\beta 1$ integrin-dependent manner – promotes angiogenesis, proliferation in both tumor types, and metastasis in pancreatic cancer [7, 25, 26]. In breast cancer asTF is mainly expressed in ER⁺ tumors, and a synergy with the ER signaling pathway led to induced cell proliferation [5, 6]. Interestingly, when

$\beta 1$ integrin was blocked with an antibody, asTF-dependent proliferation could not be completely inhibited [5]. This would suggest another integrin binding partner for asTF in breast cancer progression. As we have shown elevated $\alpha 6\beta 4$ integrin expression after treatment with Mab-10H10 in **chapter 4**, it is plausible that asTF can ligate to and signal via $\alpha 6\beta 4$ integrins to further contribute to the epithelial-like morphology in cancer, as increased expression and secretion of cell adhesion molecules was observed in MVECs in the presence of asTF [18]. Thus far, interactions between asTF and $\beta 4$ integrin to influence cell adhesion, capillary formation and migration of endothelial cells in a physiological context has been ruled out [4], but $\beta 4$ integrin and asTF in cancer has not been addressed yet.

It is unlikely that in the breast cancer model used in this thesis asTF plays a dominant role in cancer-associated angiogenesis; we have previously shown that asTF affects proliferation, but not angiogenesis, in the MDA-MB-231 breast cancer model [5]. In contrast, asTF induces angiogenesis - in the absence of flTF - in MCF-7 breast cancer cells, with similar results in pancreatic cancer [7, 13, 27]. In pancreatic cancer it was shown that asTF increases angiogenesis in an integrin-dependent manner, i.e. via ligation to $\alpha 6\beta 1$. A number of explanations for this apparent contradiction may be postulated: i) asTF expression levels in MDA-MB-231-mfp cells are low in comparison to the rather artificial overexpression cell lines used in other studies; ii) asTF might be involved in physiological angiogenesis rather than pathological angiogenesis in the presence of flTF and/or iii) flTF sequesters all $\beta 1$ integrin molecules at the cell membrane, thus preventing asTF from binding integrins and induce intracellular signaling. It would be of interest to further study the above-mentioned possibilities in order to elucidate the potential interplay between asTF and flTF in tumor-associated angiogenesis.

Thus far, the focus of this thesis was mainly on changes within the tumor cell prior to metastasis. One other contributor to metastasis that has not been studied in the context of this thesis is MVs. According to Stephen Paget's 'seed-and-soil' hypothesis in which he compared metastatic cells to plant seeds, tumor cells can only grow at metastatic sites that constitute a favorable microenvironment to these tumor cells [28]. In recent years it has been shown that MVs can dictate organ-specific metastasis by preparing the pre-metastatic niche. Of note, MV-directed pre-metastatic niche formation is dependent on the MV integrin expression profile [29, 30]. $\alpha v\beta 5$ integrin positive MVs predominantly mediate liver-specific metastasis, while $\alpha 6\beta 1$ and $\alpha 6\beta 4$ integrin positive MVs direct homing of circulating tumor cells to the lung [29]. Extensive research has been performed on TF⁺ MVs with respect to tumor progression [31, 32]. Da Rocha Rondon et al. recently showed by using

CRISPR/Cas9 approaches, that knockout of TF in MDA-MB-231 cells decreased MV shedding [31]. Furthermore, phosphorylation of TF at Ser253 and Ser258 has been shown to be an 'on-off switch' for the incorporation of TF into MVs in endothelial cells [33]. We propose that TF signaling inhibition with Mab-10H10 may lead to a similar reduction in MV shedding, especially as Mab-10H10 also prevents cytoplasmic phosphorylation of TF by PAR2 [34, 35]. In addition, it would be interesting to study if TF influences integrin incorporation into MVs, and thereby influences organotropic metastasis.

3. Molecular mechanism underlying cancer-associated thrombosis

Cancer-associated thrombosis is a frequent complication in cancer patients and contributes to high morbidity and mortality. Unfortunately, as the mechanisms underlying CAT are incompletely understood, it is a challenge to predict those patients with elevated risk and those who might benefit from thromboprophylaxis. Therefore, the third part of this thesis focusses on CAT. In **chapter 5** a mouse model is used to investigate the interactions between cancer and spontaneous thrombosis. Thus far, established VTE *in vivo* models are invasive as the vessels needs to be exposed, or do not mimic the pathological nature of thrombosis. The ferric chloride model relies on the induction of oxidative stress to the vessels. Although a relative simple procedure, it does not reflect the clinical setting of thrombus initiation [36]. In the inferior vena cava (IVC) stenosis model the vena cava is partially ligated while preventing vessel injury. In this stenosis model, development of a VTE is dependent on immune cells like leukocytes, neutrophils, which can form neutrophil extracellular traps, thus better mimicking a DVT as in a human setting [36, 37]. Unfortunately, the incidence of thrombus formation is variable and the thrombus propagates in a direction opposite to the blood flow. Therefore, we have recently developed a new non-invasive spontaneous VTE model in which antithrombin is silenced in the liver, via siRNA injections into the tail vein. Downregulation of antithrombin leads to an imbalance in coagulation factors resulting in venous thrombosis and hemorrhages in the head of mice [38, 39]. We observed that mice with breast tumors were partially rescued from these hemostatic abnormalities, while this treatment had no effect on short-term tumor characteristics, such as tumor growth and metastasis. Furthermore, the presence of a tumor induced elevated platelets counts, fibrinogen levels and a systemic pro-inflammatory status, all of which were unaffected by antithrombin knockdown. Closer examination of the organs showed increased fibrin deposition in the livers of non-tumor-bearing mice, while tumor-bearing mice presented with high fibrin deposits in the tumor. Interestingly, macrovascular thrombosis in the large

veins of the head of these mice was less frequently observed, the latter being a phenotype that is typically associated with antithrombin knockdown in mice.

Thus, in contrast to the hypothesis, a protective phenotype was observed in tumor-bearing mice. This might be explained by elevated platelet counts in the plasma of these mice. We hypothesize that the protective phenotype is caused by lack of a complete consumption of platelets in mice with breast cancer. The elevated platelet counts found in these mice would represent thrombocytosis in patients. In support, thrombocytosis is associated with metastasis and inflammatory breast cancer [40, 41]. Furthermore, the phenotype presented in mice with tumors would suggest disseminated intravascular coagulation (DIC), which is defined by e.g. low antithrombin levels, fibrin products, platelet consumption, venous thrombosis and/or hemorrhages [42]. DIC occurs in 5% of breast cancer patients and is an extreme form of hypercoagulation [10]. As this extreme form was presented, it would be of great interest to combine MDA-MB-231 breast cancer model with the classical IVC stenosis model. In murine pancreatic cancer models, larger venous clots were observed in tumor-bearing mice when compared to tumor-free mice, and that this clot formation was dependent on TF⁺ MVs [43]. However, in our breast cancer model, no TF-dependent hypercoagulant plasma was observed, which warrants studying CAT in a TF⁺ MV independent manner.

A different approach to study involvement of key factors and biological pathways in CAT is to investigate tumor gene expression and to link these expression profiles to VTE. The study described in **chapter 6** is the first ever study to report differential gene expression profiles in tumors from patients with both colorectal cancer (CRC) and VTE compared to those from patients with CRC only. A pro-inflammatory status was shown in the tumors of patients with CAT, and elevated levels of fibrin deposits were present in the tumors from patients with VTE. Increased fibrin products could serve as a matrix for tumor vessel formation, or promote metastasis, which may contribute to the decreased survival in CAT patients [44-46]. When patients with VTE were subdivided into two groups of VTE before (max 1 year) and around (max 3 months) diagnosis, altered expression profiles were observed. This might be induced by cancer-related treatment, as metabolism related pathways were up-regulated in the 'VTE around diagnosis' group.

In order to fully understand the pathophysiologic mechanism of CAT, irrespective of cancer treatment, the top 3 genes that were found to be differentially expressed in CRC patients with VTE before cancer diagnosis are of interest for further investigation, especially since

links can be found with coagulation-related factors. Reg4 can bind to the naturally occurring anticoagulant heparin in the absence of calcium [47] and Spink4 is a serine protease inhibitor of the Kazal type [48] and might inhibit coagulation factors. Furthermore, co-expression of Reg4 and Spink4 are tightly regulated in inflammatory bowel disease [49], indicating a (chronic) pro-inflammatory status that might increase TF expression and thereby further contribute to VTE [1, 50]. One in five patients with inflammatory bowel disease get diagnosed with cancer, furthermore they are 2-3 fold at increased risk of VTE [51-54]. In addition, elevated Reg4 expression is associated with tumor progression, metastasis and a poorer survival [55-57]. A more direct link with coagulation regulation is constituted by *SERPINA1*, which encodes α 1-antitrypsin (A1AT). A1AT can directly and indirectly stimulate thrombus formation by inactivation of several coagulation factors such as FXa, FXII and activated protein C; and complex formation of A1AT with elastase may increase systemic neutrophil activation that triggers VTE via neutrophil extracellular traps [58-60]. Additionally, A1AT prolongs clot-clearance as it inhibits neutrophil elastase-mediated fibrinolysis [58, 61, 62]. Furthermore, A1AT expression was found in fibrin-rich blood clots, suggesting a direct role for this protein in VTE [58]. In relation to cancer, A1AT overexpression has been found in several tumor types such as brain [63], colorectal [64] and gastric cancer [65], or elevated A1AT plasma levels were observed in patients with breast, malignant melanoma and gastric cancer [66]. In the latter study, cancer patients with elevated risk of VTE were included, although no remarks have been made on confirmed VTE in these patients. Therefore, no direct associations between A1AT plasma levels and VTE could be established.

Unfortunately, the majority of studies that have attempted to find biomarkers for CAT have focused on coagulation factors, with TF being in the center of cancer-associated thrombosis. More importantly, scientists and clinicians have, perhaps unjustified, attempted to extrapolate classical VTE risk factors to CAT, resulting in a low success-rate of biomarkers that accurately predict CAT. Several risk assessment tools have been developed to predict which cancer patients are at elevated risk of VTE, unfortunately with low prediction accuracy. In **chapter 7** we plea to stop using classical VTE risk factors as predictors for CAT, especially as a recent study by Mohammed et al. showed different plasma protein profiles in patients with CAT, when compared to plasma from those diagnosed with either cancer or VTE [67]. Furthermore, we discussed the link between mutations and elevated risk of CAT. As it is a very biased and time-consuming approach to investigate all and every gene on CAT, therefore, we propose an unbiased approach by performing molecular profiling in patients with CAT, as was described in **chapter 6**.

Conclusion and further directions

This thesis describes i) the function of asTF in hemostasis, ii) the contribution of coagulation factors on cancer progression, and iii) expands our view on cancer-associated thrombosis. Inhibition of TF signaling with Mab-10H10 resulted in decreased EMT- and CSC associated transcription program, tumor initiating capacity and metastasis in a triple negative breast cancer (TNBC) cell line. Since this is a tumor type that is difficult to treat, and has high relapse-rates, it would be of interest to target TF signaling. Dual treatment of TNBC with conventional chemotherapy and Mab-10H10 could result in a positive treatment strategy as both highly proliferative and cancer stem cells are targeted.

Furthermore, we provided a proof-of-principle study to search for novel biomarkers in CAT patients in an unbiased manner. Expansion of this study to validation cohorts and other tumor types will give insights in the underlying molecular mechanism of cancer-associated thrombosis. Eventually, this will aid a better prediction model to select those cancer patients with high risk of VTE and those who might benefit from thromboprophylaxis.

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