Tissue factor isoforms and factor VII in cancer progression

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Chapter 2 – Anticoagulants versus Cancer

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Abstract

Venous thromboembolism (VTE) and cancer are strongly associated, and present a major challenge in cancer patient treatment. Cancer patients have a higher risk of developing VTE, although the differs widely among tumour type. VTE prophylaxis is routinely given to cancer patients, in the form of vitamin K antagonists (VKA) or low molecular weight heparin (LMWH). Several studies have reported that cancer patients receiving anticoagulants show prolonged survival and this effect was more pronounced in patients with a good prognosis, although the mechanism is poorly understood. Tissue Factor (TF) is the initiator of extrinsic coagulation, but its non-haemostatic signalling via protease activated receptors (PARs) is a potent driver of tumour angiogenesis. Furthermore, coagulation activation is strongly implicated in tumour cell migration and metastasis. This review described the literature on anticoagulants and whether they inhibit cancer progression in patients, as well as inhibition of tumour cell proliferation, angiogenesis, and metastasis in both *in vitro* and *in vivo* models. Inhibition of TF signalling shows great promise in curbing angiogenesis and *in vivo* tumour growth, but whether this translates to patients is not yet known. Furthermore, non-haemostatic properties of coagulation factors in cancer progression are discussed, which provide exciting opportunities on limiting oncologic processes without affecting blood coagulation.

Introduction

Ever since the 19th century an association between cancer and venous thromboembolism (VTE) has been observed, presenting a major challenge in the treatment of cancer patients.[1] Malignancy increases the risk of VTE 4.1-fold, which increases even further to 6.5-fold in patients receiving chemotherapy.[2] VTE incidences differ per cancer type, with pancreatic, brain and haematological malignancies resulting in the highest incidences.[3] Treatment of VTE has consisted of unfractioned heparin (UFH), now largely replaced by low-molecular-weight heparins (LMWH) or vitamin K antagonists. Cancer patients that develop a VTE and receive LMWHs or

VKAs are at an increased risk of recurrent VTE as well as bleeding complications.[45] Extensive studies have been performed to investigate how VTE incidence can be reduced and whether VTE treatment might have any influence on cancer progression and overall patient survival.[6,7,8]
The development of experimental animal models in the second half of the 20th century allowed for the investigation of anticoagulants on experimental tumour development. Several *in vivo* studies have shown that tumour progression in these models is hampered by vitamin K antagonists (VKAs).[9]
VTE is caused by the activation of the blood coagulation cascade. Multiple risk factors (besides active cancer) such as surgery, immobility and age have been identified.[10] Extrinsic coagulation is initiated when Tissue Factor (TF), a transmembrane glycoprotein expressed on the subendothelium of vessels, comes into contact with Factor VII (FVII), which is activated upon binding. The TF:FVIIa complex can bind and activate Factor X (FX), which can activate Prothrombin (FII) to thrombin. Intrinsic coagulation involves subsequent activation of Factors XII, XI, IX and X, resulting in thrombin generation. Nevertheless, it must be mentioned that *in vivo*, TF is the sole activator of the coagulation cascade with the intrinsic pathway serving as an amplifier or thrombin generation.[11] Thrombin activates platelets as well as fibrinogen, leading to stable fibrin clots. Coagulation activation also leads to activation of coagulation inhibitors to limit the scope and duration of clot formation. Antithrombin, a serine protease inhibitor, is able to inactivate thrombin, FXa and FIXa, and this can be accelerated by heparin sulphate proteoglycans. LMWHs, routinely used for VTE prophylaxis, accelerate the ability of antithrombin to inactivate FXa, thus inhibiting coagulation.[12]
Tissue Factor Pathway Inhibitor (TFPI) is an inhibitor of both the TF:FVIIa complex as well as FXa.[13] Carboxylation of Factors II, VII, IX and X is dependent on vitamin K, which is oxidized in the process. Vitamin K epoxide reductase (VKORC) reduces vitamin K, thus repleting vitamin K available for coagulation. VKAs block the reduction of vitamin K, leading to a shortage of carboxylated coagulation factors, inhibiting coagulation.
In this review inhibitors of coagulation and their effects on cancer progression in *in vitro* and *in vivo* models as well as in patients is discussed.

VTE in cancer

A multitude of cancer subtypes, such as pancreatic and brain cancers, are associated with an increased risk of VTE in patients. The risk of recurrent thrombosis is also increased in cancer patients compared to VTE patients without cancer,[5] and this risk itself is associated with the severity of the cancer; a higher grade confers an increased VTE incidence.[14] Conversely, patients with an unexplained VTE have a significantly increased risk of having an as of yet undiscovered malignancy.[15]

The reported odds-ratios of developing VTE among cancer patients vary from 4.1[2] to 6.7[16], and in the latter study the odds ratio increased to 58.0 when distant metastases were present. This leads to a 4.3% VTE incidence per year in cancer overall, but certain subtypes show a higher VTE incidence, namely pancreatic (11%), brain (8%), lung (4.5%), hematologic (4%) and colorectal cancer (3%).[17] Interestingly, when tumour subtypes are split in high or low grade, the cumulative probability of VTE is doubled in high grade tumours.[18]

As cancer patients have an increased risk of (recurrent) venous thrombosis, patients presenting with a VTE episode receive more specialized treatment. Cancer patients with an acute VTE receiving the LMWH Dalteparin have a 50% lower risk of developing a recurrent VTE, as compared to patients receiving oral anticoagulant treatment, while the rate of major bleeding remained similar.[19] A more recent study showed that patient with advanced or metastatic cancer benefited from ULMWH Semuloparin administration during chemotherapy, reducing the VTE incidence significantly.[20]

Effects of anticoagulants on cancer progression in patients

Besides reducing VTE incidence in cancer patients, there is a large body of evidence that anticoagulant therapy, in the form of LMWH or vitamin K antagonists, influences tumour progression and patient survival (Table 1). In studies as early as 1984 warfarin therapy was associated with prolonged survival in small cell lung carcinoma[7]. A study published in 1992 showed

increased survival in patients given LMWH versus un-fractioned heparin.[21] In a 1999 meta-analysis, LMWH administration resulted in a three month survival benefit over UFH in cancer patients.[22] Importantly, this effect remained consistent in subgroups of specific types of cancer and was not the result of VTE-related deaths.

In a study of cancer patients with advanced solid malignancies but without VTE, the effect of subcutaneous nadroparin administration on patient survival was tested and a prolonged survival was observed.[23] In another study, the use of heparin reduced death by metastatic disease by 50% three years after undergoing surgery for cancer[24]. When dalteparin is administered in combination with chemotherapy in small cell lung cancer patients, tumour response and survival improves significantly.[25]

Regular aspirin use has been associated with a long-term benefit regarding cancer incidence and metastasis, at least for a subset of cancer types. A large meta-analysis by Algra *et al.* shows a decrease in colorectal cancer incidence in long-term aspirin use, with an odds-ratio of 0.62 (95% CI 0.58-0.67, 17 studies combined).[26]

Effects of anticoagulants on patients with a good prognosis

In the FAMOUS study by Kakkar *et al.*, the effect of chronic administration of LMWH in cancer patients without VTE was studied. Dalteparin did not influence the survival of advanced cancer patients, although a subgroup of patients with a relatively good prognosis had a significant survival advantage after two and three years.[27] In a study with cancer patients without metastatic disease, a subcutaneous dose of dalteparin resulted in improved survival compared to oral anticoagulant treatment.[28] A study tracking cancer incidence in a large population based study comparing long-term VKA use to a control group showed a 0.88 hazard ratio of cancer incidence (95% CI 0.8-0.98, $P < 0.015$). When looking at specific types of cancer, only the prostate cancer group showed a significantly reduced hazard ratio when VKA use was compared to control.[29] Non-metastatic prostate adenocarcinoma patients undergoing radiotherapy and using

anticoagulants (warfarin, clopidogrel, and/or aspirin) showed a reduced metastasis rate.[30] These data suggest that long-term anticoagulant treatment might have an influence on early tumour development, because the effects do not appear to impact survival of patients with high-grade or metastatic disease.

Most patient studies investigating cancer and anticoagulant treatment focus on VTE incidence rather than cancer progression as a primary outcome. This has limited the possibilities to study the effects of anticoagulants on tumour progression. A recent meta-analysis by Sanford *at al.* found no significant survival benefit for cancer patients without VTE receiving LMWH.[31] Furthermore, the studies mentioned either use warfarin or LMWH, that may not have an effect on the TF:FVIIa complex. Especially TF:FVIIa has recently been found to influence tumour growth in vitro and in vivo.[32–34] Several drugs targeting this complex are currently under clinical investigation, such as Alt-836[35] and HuMax-TF-ADC,[36,37] both targeting TF in locally advanced or metastatic tumours. It remains to be seen whether these compounds will have clinical benefits, as well as possible adverse effects on haemostasis.

Effects of anticoagulants on tumour development *in vitro* and *in vivo*

A multitude of anticoagulants have been tested *in vitro*, as to whether they can inhibit tumour cell proliferation. The effects of LMWH on tumour cell proliferation have been obscure. In a melanoma study, UFH and LMWH did not influence tumour cell proliferation.[38] In an osteosarcoma cell model, exposure to thrombin increased cell proliferation, and this effect was attenuated when LMWH were added, suggesting that inhibition of thrombin generation using LMWH might be beneficial for attenuating primary tumour growth. Indeed, LMWH was able to suppress primary tumour growth *in vivo* using TF-expressing osteosarcoma cells.[39] In another study, MDA-MB-231 and 4T1 breast cancer cells were exposed to dabigatran but this did not influence cell proliferation or viability.[40] Overall,

these data do not point to an anti-proliferative effect of anticoagulants on tumour cells.

Apart from anti-thrombotics, a multitude of specific inhibitors of coagulation proteins have been studied for their ability to reduce tumour growth both *in vitro* and *in vivo* (Table1). Tissue Factor Pathway Inhibitor (TFPI) is a potent endogenous inhibitor of the extrinsic coagulation pathway by blocking the TF:FVIIa complex via its Kunitz domains. The loss of TFPI-2 correlates with a higher grade in human glioma cell lines and tumour samples[41], and when TFPI-2 expression is restored in a glioma cell line this reduced the size and number of colonies in an *in vitro* colony assay[42]. Furthermore, TFPI-2 inhibited matrigel invasion of glioblastoma cells in a dose-dependent manner.[41]

The thick-derived coagulation inhibitor Ixolaris, which contains two Kunitz-like domains, is able to form a quaternary TF:FVIIa:FXa:Ixolaris complex similar to TFPI[43]. Ixolaris was able to inhibit both TF:FVIIa and TF:FVIIa:FXa complex signaling on breast tumour cells *in vitro*.[44].. *In vivo*, it showed potency to decrease primary tumour growth in human glioblastoma and mouse melanoma models. [45]

rNAPc2, a nematode anticoagulant protein that specifically inhibits TF:FVIIa, significantly suppresses tumour growth in mice in both a lung[33] and colorectal cancer model[46]. Interestingly, the specific FXa inhibitor rNAP5 had no effect on tumour growth[33] suggesting that it is not necessarily coagulation activation that drives tumour growth. And indeed TF signaling has been shown to promote tumour growth independently of it's coagulant function[32]. Therefore direct TF signaling inhibitors might have the most benefit when used to restrain tumour growth.

In vivo tumour growth is limited by both cell proliferation as well as angiogenesis. Activation of the coagulation cascade leads to increased angiogenesis via the protease activated receptors (PARs), i.e. PAR-1 (activated by thrombin and TF:FVIIa:FXa) and PAR-2 (activated by TF:FVIIa and TF:FVIIa:FXa) as shown in Figure 1. PAR-dependent angiogenesis mainly promotes enhanced expression of pro-angiogenic factors by tumour cells[47], activating the surrounding endothelium. The effects of

anticoagulants on *in vivo* tumour growth might thus be effective because they limit angiogenesis rather than cell proliferation.

Non-haemostatic signaling of TF-dependent PAR-2 activation leads to increased mRNA levels of VEGF[48], IL-8 and CXCL-1[32,47] and promotes angiogenesis.[49] PAR-2 activation leads to phosphorylation of the cytoplasmic domain of TF, which activates cell signalling via mitogen-activated protein (MAP) kinases.[50] Blockade of TF or PAR-2 by specific antibodies can effectively attenuate this PAR-2 dependent IL-8 upregulation, tumour growth and density of CD-31^{+} tumour vessels.[32] In MDA-MB-231mfp breast cancer cells this effect can be inhibited using Ixolaris and effects are similar to those observed after PAR-2 inhibition with a blocking antibody.[44] Interestingly, alternatively spliced Tissue Factor (asTF) can also induce angiogenesis, though in a PAR-2 and FVIIa independent manner.[51] AsTF is a soluble, non-coagulant isoform of TF, and is widely expressed in breast[52] and pancreatic[53] cancer. AsTF promotes cell proliferation *in vitro* as well as tumour growth *in vivo*, both of which can be inhibited by a specific antibody[52].

Tissue Factor pathway inhibitor (TFPI) was shown to inhibit endothelial cell formation, as well as blocking phosphorylation of vascular endothelial growth factor (VEGF) receptor 2, suggesting that TFPI can inhibit angiogenesis via its carboxyl terminus.[54]

The LMWH nadroparin was shown to inhibit angiogenesis in a rodent skinfold chamber model.[55] LMWH bemiparin and ultra (U)LMWH RO-14 inhibit angiogenic effects on endothelial cells when exposed to tumour cell-conditioned medium from leukemia, breast, and lung cancer cells in tube formation and migration assays.[56] Another study showed that LMWH affects fibrin matrices *in vitro*, leading to impaired capillary formation using human micro vascular endothelial cells, as well as attenuating their proliferation.[57] LMWHs are able to reduce capillary tube formation, an effect not observed when UFH was used.[58] The phosphorylation of VEGF-mediated KDR (VEGF receptor-2) in human umbilical vein endothelial cells (HUVECs) was diminished after the LMWH fraxiparin administration, as well as vessel formation in an *in vivo* matrigel plug assay.[59]

Inhibition of experimental metastasis by anticoagulants

The rationale behind anticoagulants as possible inhibitors of cancer metastasis is based on evidence that tumors cells benefit from activation of the coagulation cascade: clot formation by tumor cells facilitates attachment to the endothelium[60], clots protect tumor cells from vascular shear stress[61] and facilitates evasion of immune surveillance.[62,63] Furthermore, platelet interaction with tumour cells drives epithelial-mesenchymal transition (EMT) via the TGFβ/SMAD and NF-κB pathways[64], and both platelets and P-selectin promote experimental liver metastasis.[65]

Metastatic spread of tumour cells is the leading cause of mortality, and LMWH shows a limited, but positive effect on cancer patient survival as described above. The effects of heparins on experimental metastasis models has been reviewed in detail by Niers *et al;*[66] in 14 out of 17 reviewed studies heparins reduce either primary or secondary metastasis. A common *in vivo* model for metastasis is the subcutaneous injection of tumour cells such as the B16 melanoma cell line and quantifying lung metastases after a given time period. Several groups have shown that LMWH administration can significantly limit lung seeding of melanoma cells.[38,67] *In vitro,* UFH and LMWH pre-treatment significantly decreased melanoma cell migration and matrigel invasion[38].

Thrombin is capable of enhancing metastasis by platelet activation and fibrin formation, as well as triggering tumour cell signalling via PARs. Activation of coagulation by TF on cancer cells leads to platelet clots around the tumour cells, whereby macrophages are attracted, leading to increased survival of metastatic cells.[68] Platelet aggregation around tumour cells also limits natural killer (NK) cells in targeting the tumour cells.

Besides anticoagulants used for VTE treatment, other specific coagulation inhibitors have been tested for their ability to inhibit metastasis. The Heparin-like heparin sulphate 6-O-sulphotransferase-2 was shown to reduce TGF-β-induced IL-11 expression *in vitro* as well as in *in vivo* tumour progression in bone.[69] In an *in vitro* human umbilical cord vein model, S-

NACH-a modified heparin lacking inhibition of FXa and FIIa-, as well as the LMWH tinzaparin, inhibited adhesion and invasion of MPanc96 human pancreatic tumour cells.[70] S-NACH was even shown to be more potent in reducing surgically induced metastasis compared to tinzaparin, and S-NACH treatment led to reduced E-cadherin expression in pancreatic cancer cells.[71]

Hirudin is a potent thrombin inhibitor naturally occuring in leech saliva. It was shown to diminish B16 melanoma tumour cell induced lung tumours, possibly acting through PAR-1[72]. The potent TF/FVIIa inhibitor rNAPc2 diminishes lung metastases in the B16 melanoma mouse model,[33] and the specific FXa active site inhibitor rAcAP reduced pulmonary metastasis after tail vein injections of LOX human melanoma cells in SCID mice.[73] Furthermore, active site-inhibited FVIIa (FVIIai), which competes with FVII for binding of TF, reduces the number of tumour foci on the lungs of mice injected with B16F0 melanoma cells.[74]

Future directions

The association between increased risk for VTE and a malignant state has been widely established over the last century. Whether treatment of VTE in cancer patients is also beneficial for the treatment of malignancy is less clear. The use of LMWHs only shows increased survival in certain subgroups of patients, namely patients with a better prognosis. Another limitation is the lack of studies in which the effect of anticoagulants on cancer progression is measured as the primary outcome, which limits the conclusions that can be drawn from observed effects of survival of VTE patients with cancer. LMWHs affect the angiogenic behaviour of tumour cells both *in vitro* and *in vivo*, which would confirm the beneficial effects found in patients.

Recently, novel oral anticoagulants (NOACs) have shown great promise in clinical trials, and both direct thrombin (dabigatran) and FXa inhibitors (apixaban and rivaroxaban) are now approved for use in the U.S.[75] As the role of both thrombin and PAR-2 signalling (activated by both the TF:FVIIa

and the TF:FVIIa:FXa complex) have been clearly established as potent contributors to cancer progression we may expect a benefit for cancer patients in curbing both VTE and cancer progression. Recently, the safety and efficacy of NOACs has been described in cancer patient cohorts: use of dabigatran and warfarin resulted in comparable VTE recurrence and bleeding.[76] A meta-analysis showed that dabigatran as well as FXa-inhibitors are effective and safe for use in cancer patients.[77] As the use of NOACs becomes more accepted it will soon become clear whether systematic thrombin or FXa inhibition will also slow cancer progression. Lastly, a striking benefit of blood coagulation research is the identification of non-coagulant peptides and proteins that do show promise in attenuating oncogenic processes. S-NACH, a sulphated non-anticoagulant heparin, showed higher potency in attenuating metastasis in experimental models compared to tinzaparin, a LMWH.[71] This suggests that it is not necessarily the anticoagulant effects of heparins that influence metastasis. Recent publications have moved asTF out of the realm of a non-coagulant and somewhat misunderstood protein to a potent and widely occurring oncogene.[51,53,78] Furthermore, TFs non-haemostatic signalling via PARs is now firmly accepted as a pro-angiogenic pathway.[32,79] Although these insights steer away from blood coagulation, they do provide exciting angles for future research in understanding cancer metastasis.

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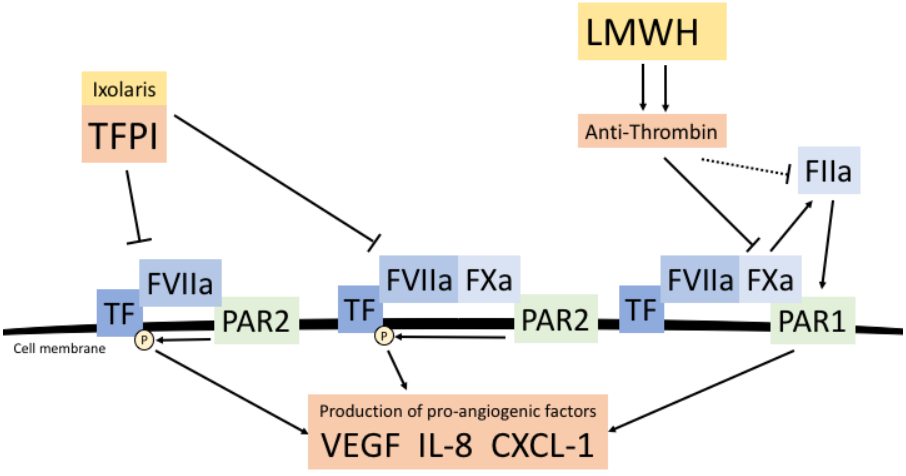

Figure 1. TF:FVIIa signalling promotes angiogenesis. TF:FVIIa and TF:FVIIa:FXa can both activate PAR2. PAR1is activated by TF:FVIIa:FXa as well as FIIa/thrombin. TFPI and ixolaris inhibit the TF:FVIIa and TF:FVIIa:FXa complexes. Anti-thrombin (AT) inhibits both FXa and FIIa, and LMWHs greatly accelerate the ability of AT to inhibit FXa.

Table 1. Overview of studies reporting benefit of anticogulants on cancer progression

	patients	*In vitro*	*In vivo*
	References		
Cancer Incidence			
Aspirin	26		
VKA	29		
Patient Survival			
Warfarin	7		
Heparin	24		
LMWH	21,22,23,25,27,28		
Proliferation/Tumour Growth			
LMWH		39	39
TFPI-2		42	
Ixolaris			45
rNAPc2			46
TF antibody			32
asTF antibody		52	52
Dabigatran			40
Angiogenesis			
LMWH		56,57,58,59	55,59
TFPI		54	
Ixolaris		44	
TF antibody		32	32
Metastasis			
Warfarin	30		
clopidogrel	30		
aspirin	30		
UFH	66	38	
LMWH	66	38,70	38,67
TFPI-2		41	
6-O-sulphotransferase-2		69	69
S-NACH		70,71	71
Hirudin			72
rNAPc2			33
rAcAP			73
FVIIai			74
Dabigatran			40