Chapter 10 - General Discussion and Summary

Background

In the past 450 million years of evolution, a serine protease-based innate immune system in non-vertebrates developed into a complex blood coagulation system which maintains vascular integrity in vertebrates[1]. Under normal conditions, blood should be kept fluid in order to serve as a transport medium for oxygen, nutrients, hormones, immune cells and degradation products of metabolism. After vascular injury, however, excessive blood loss is prevented by formation of a blood clot. First, vasoconstriction takes place and platelets aggregate in order to make a preliminary hemostatic plug. At the same time, the interplay between full-length tissue factor (flTF) and other proteins of the coagulation system initiates the formation of a fibrin network that stabilizes the platelet plug[2].

Under certain conditions, inappropriate activation of blood coagulation may take place and this eventually leads to thrombosis. More recently it has also become clear that the coagulation system, and in particular aberrant flTF expression on tumor cells, influences cancer progression both directly and indirectly through activation of protease activated receptors (PAR)[3].

In 2003, an alternatively spliced form of tissue factor (asTF) has been identified of which the biological significance is still under study. A role of asTF in coagulation has not been uncovered yet. AsTF is highly expressed in several human tumor tissues and this expression was shown to lead to increased tumor growth in a xenograft pancreatic cancer model[4;5]. A large portion of this thesis describes the non-hemostatic properties of asTF and compares these with those of flTF, predominantly in the context of cancer, but also of atherosclerosis.

Another part of the thesis focuses on flTF and its ligand factor (F)VII(a). We report on the ectopic expression of FVII in human breast cancer and on the association of FVII positivity with prognosis in relatively young breast cancer patients. We further describe an in silico study of the evolutionary conservation of an allosteric disulfide in flTF. The state of this disulfide acts as a post-transcriptional mechanism that controls flTF activity and therefore is relevant in (patho)physiological processes in which flTF is involved.

Alternative spliced tissue factor and angiogenesis

During alternative splicing of the TF pre-mRNA transcript, exon 5 is skipped and as a result, a shift of the open reading frame occurs. This shift leads to a replacement of the

transmembrane and cytoplasmic domains with a unique 40 amino acid C-terminal domain, rendering asTF soluble[4].

Since its discovery, the role of asTF in health and disease has been a matter of debate. Whether asTF behaves as a procoagulant protein appears to depend on the experimental settings, but most studies were negative. In case procoagulant potential was reported, asTF was much less potent than canonical flTF[2;4;6].

The report that asTF expression, rather than flTF, induced larger, more vascularized tumors[5] in a pancreatic cancer model, led us to undertake a functional characterization of asTF. **Chapter 2** is a report on the angiogenic properties of asTF[7]. We identified α6β1 and αVβ3 integrins on endothelial cells as specific targets for ligation by asTF. We also demonstrated in vitro that ligation of these integrins by asTF leads to endothelial cell alignment and migration, and eventually to angiogenesis *ex vivo* and *in vivo*. Blockade of coagulation proteases like factor VII (FVII) and thrombin, or deletion of PAR2 was unable to abolish these effects. This shows that asTF exerts its effects via pathways distinct from flTF. Interestingly, in our *in vivo* and *ex vivo* angiogenesis assays, the angiogenic effect of asTF was similar to that of VEGF, whereas the extracellular domain of flTF, so-called soluble TF (sTF) failed to evoke an angiogenic response. The absence of sTF-induced angiogenesis seems rather unexpected, although it is in concordance with the pancreatic cancer model in which flTF was also unable to promote tumor growth[5]. To date, we have not found a plausible explanation for this finding, although a relative absence of PAR2 and FVII as two conditiones sine qua non may at least partially explain this counterintuitive result.

In our proposed model of asTF-induced tumor angiogenesis, it is hypothesized that asTF is excreted from tumor cells and diffuses into the tumor microenvironment in order to ligate endothelial integrins. To further test this hypothesis we set out to estimate the (patho)physiological relevant concentrations of asTF in tumor tissues. We quantified asTF, as both mRNA and protein, in cervical cancer specimens and protein levels up to 75 nM were found in extracts from tumor specimens, which is in the same range of concentrations of 1-100 nM asTF that induced angiogenesis *ex vivo*. However, the tumor extracts were obtained from whole tumor lysates and this leaves the question whether tumor cells actually secrete rather than store asTF unanswered. Since it is known that asTF can be retained by transfected cells[8], but secreted from stimulated endothelial cells[9], studies on the cellular trafficking of asTF in tumor cells, and release of asTF in the tumor environment are needed to support the hypothesis that tumor-derived asTF drives angiogenesis in a paracrine manner. Another experimental approach would be to avoid use of exogenous asTF and to choose an experimental design that relies on endogenous

asTF production. One approach could be to use of cancer models in which asTF expression is either knocked-down by RNA interference or knocked-in through regulated overexpression. This would allow to more definitely show that tumor-derived asTF drives angiogenesis and eventually tumor progression. In such experiments, the relative contribution of both TF isoforms could also be investigated.

Alternatively spliced tissue factor and monocyte-endothelium interactions

We were also interested in examining whether asTF-induced integrin ligation plays a role in other processes in which angiogenesis is essential, e.g. in wound healing, or in atherosclerosis. As atherosclerotic plaques become more prone to rupture when neovascularization is extensive[10], it is interesting to investigate whether asTF substantially contributes to plaque angiogenesis, and hence may serve as a diagnostic marker or even therapeutic target. In **chapter 3** we speculate on such a role for asTF and, because cells from the monocyte/macrophage lineage are bona fide producers of asTF and play a dominant role in atherosclerosis, we argue that asTF expression in these cells may affect atherosclerotic disease. Influx of monocytes and their maturation to plaque macrophages is a hallmark of atherosclerotic disease, but is of increasing interest in the field of cancer as macrophages determine tumor growth, e.g. through angiogenesis-modulating M2-macrophages[11].

As a first step towards understanding these phenomena, we characterized in chapter 4 the interaction between endothelium and monocytes in the presence of TF isoforms. Ligation of β1 integrins on endothelial cells by both recombinant asTF and sTF induces an upregulation of adhesion molecules, including ICAM-1, VCAM-1 and E-selectin. These changes in adhesion molecule expression eventually result in increased monocyte-endothelium adherence, under both orbital flow as well as laminar flow conditions. AsTF induces a more potent adhesion molecule upregulation under flow conditions when compared to sTF. AsTF further promotes monocyte-endothelial transmigration in the presence of MCP-1. These experiments indicate that the interaction between TF isoforms, especially asTF, and integrins promotes monocyte adhesion to and migration through the endothelium, resulting in an influx of monocytes in atherosclerotic plaques, but also in tumors.

As was the case for the experiments described in **chapter 2**, a disadvantage of this experimental approach is the exogenous administration of recombinant asTF or sTF rather than using a model in which the role of endogenously produced TF isoforms are investigated. Both endothelial cells and cells of the monocyte lineage are able to express TF isoforms, but whether it is endothelium- or monocyte-derived asTF that facilitates

monocyte-endothelium interaction and monocyte influx cannot be concluded from this study.

A possible alternative approach to tackle the problem is to generate tissue specific knock-out models for either myeloid or endothelial cells. That would open the way towards selective knock-out of either monocyte- or endothelium-derived TF isoforms. A next step would be to breed such conditional asTF knock-out mice with an atherogenic mouse model and study the sequence of events in asTF-mediated endothelium-monocyte interaction. This approach enables to investigate atherosclerosis during its progression and to understand whether local inflammatory responses in lipid-laden endothelium induce endothelial asTF secretion or whether systemic inflammation in an atherogenic state makes monocytes produce asTF. But even then, it is possible that other cell types act as a source of TF isoforms, e.g. neutrophils, although it is still unclear whether they indeed are able to express TF isoforms[12;13].

Apart from an experimental approach, it may also be interesting to embark on a more observational study and to investigate expression of asTF in human atherosclerosis specimens and to relate these data to expression of other proteins in plaques and to clinical data. A possible drawback of such a design is that the role of TF isoforms may be less important during stages of advanced atherosclerosis. Similarly to the suggested experiments on asTF in atherosclerosis, future studies on the role of both TF isoforms in cancer as proposed in the paragraph on angiogenesis, may prove helpful to understand the importance of asTF or flTF-mediated macrophage influx in cancer.

Alternatively spliced tissue factor: of mice and men

Murine disease models are increasingly important for the investigation of the pathophysiology and treatment of human disease. Although recombinant human asTF is active in murine *ex vivo* and *in vivo* angiogenesis models, as discussed in **chapter 2**, the field has a preference for syngeneic models. A structural homologue of asTF has been described in mice[14], but it was unclear whether murine asTF (masTF) has functional properties that are similar to human asTF (hasTF).

In **chapter 5** we describe that masTF is expressed in spontaneous, murine breast and pancreatic tumor models, and we show that masTF is present in plaques from atherogenic Ldlr -/- mice. We proceed with experiments that prove that masTF promotes endothelial cell adhesion and migration, and *ex vivo* angiogenesis similarly to hasTF. Moreover, we report that stimulation of murine endothelial cells with asTF induces the expression of a panel of inflammatory and adhesion molecules, which eventually leads to increased

adhesion of monocytes to endothelial cells, regardless whether asTF is murine or human. This is in concordance with our findings in **chapter 4**.

Our experiments confirm (1) that masTF has similar properties to hasTF, (2) that studying the role of masTF in murine disease models may reveal important information on the role of hasTF in human disease, and (3) that asTF plays a role in cancer and atherogenesis, not only through integrin ligation on endothelial cells, but also through modulating the influx of mononuclear cells.

Some differences between human and murine asTF remain incompletely understood. Although hasTF-induced endothelial cell adhesion is mediated through ligation of both β1 and β3 integrins, masTF-induced adhesion of murine endothelial cells only depends on β3 integrins. In contrast, masTF-induced *ex vivo* angiogenesis again was both β1 and β3 integrin-dependent. It is possible that the murine endothelial cell lines used have an abundance of β3 integrins over β1 integrins but this has not been tested. Regarding the *ex vivo* angiogenesis assay, it may be possible that ligands for β1 are overrepresented in matrigel, whereas ligands for β3 integrins are relatively scarce and this also was not investigated. Furthermore, the integrin repertoire needed during angiogenesis may be species specific and perhaps activation of β3 integrins during angiogenesis is more important in mice. Finally, the new C-terminus of masTF is longer and shows no structural similarities with the C-terminus of hasTF. By consequence, the function of either the human or murine C-terminus of asTF is not understood, but the longer neo-terminus may cause a different confirmation of masTF and therefore a different profile regarding integrin ligation.

Tissue factor isoforms have distinct properties in cancer

As has become clear in the paragraphs above, there is ample evidence that asTF may influence the progression of diseases in which angiogenesis plays an important role, e.g. cancer and atherosclerosis. In order to investigate how expression of flTF and asTF are associated with clinical outcome in cancer, we report in **Chapter 6** on a cohort of more than 500 breast cancer patients from whom we obtained primary tumor material that we stained for asTF and flTF.

Both isoforms are expressed in more than 95% of these tumor samples. Normal tissue (adjacent to the tumor cell islands) show limited expression, with asTF and flTF expression in respectively 4% and 38% of the control specimens. A high percentage of asTF-positive tumor cells was associated with higher tumor grade and increased tumor size (as reflected in the so-called T-status), while an increase in flTF tumor cells associated with higher

tumor grade, but not with tumor size. Interestingly, asTF and flTF expression did not affect relapse-free nor relative survival. Additional analyses in **chapter 7**, however, show that in women younger than 55 years old, expression of flTF is associated with a shorter relapse-free and overall survival. This is in contrast to studies in other solid tumors, including lung and breast cancer (reviewed in **chapter 9**).

The samples from the cohort we employed are relatively old and date from a period in which tumors may have been diagnosed in a more advanced stage. Another explanation for the absence of an association between survival and TF isoform expression, is that pre-clinical studies have suggested a role for TF isoforms in early tumor angiogenesis, which is believed to occur at a stage when tumors are still undetectable in humans.

In **chapter 6** we also report peri-nuclear asTF staining in breast cancer samples, which we confirmed by confocal microscopy as well as by Western blotting after sub-cellular fractionation. The nature of this perinuclear localization of asTF remains unclear. The peri-nuclear space as an extension of the endoplasmatic reticulum may be loaded with asTF because cellular secretion is hampered, but we hypothesize that asTF has intracellular properties that may influence the behaviour of tumor cells.

Expression of flTF in tumor samples was associated with aldehyde dehydrogenase 1 (ALDH1), which is an established tumor stem cell marker in human breast cancer. Tumor stem cells have the ability to give rise to a tumor *in vivo* after a single cell injection and tumor stem cells may transdifferentiate into other cell types, e.g. endothelial cells during angiogenesis[15;16]. Although it is an intriguing hypothesis that flTF:FVII:PAR2 signaling directly confers stem cell features to tumor cells, it is perhaps more likely that FlTF:FVII:PAR2 signaling is just one of the numerous properties of tumor stem cells.

As suggested in the paragraphs above, experiments with *in vivo* tumor models with cells engineered to express either asTF or flTF through knock-in or RNAi-mediated knock-out, will increase insight in the relative contribution of each TF isoform. In these experiments, the focus should be on early tumor growth with an emphasis on tumor angiogenesis and monocyte influx. Moreover, investigating the role of the flTF:FVII:PAR2 axis in tumor stem cell biology may prove interesting.

Regulation of TF pre-mRNA splicing in cancer

Alternative pre-mRNA splicing allows a single gene to function as a template for distinct mRNAs, eventually resulting in the production of protein isoforms. More than 95% of the genes produce splice isoforms and some of these alternatively spliced isoforms have

antagonistic properties relative to the canonical isoform. Splicing is tightly regulated by *cis* acting factors, i.e. properties of the nucleotide sequence per se, and *trans* acting factors, which are protein-RNA complexes that form the spliceosome. The interplay between *cis* and *trans*-acting factors controls the final mRNA sequence and eventually the balance between different protein isoforms. Alternatively splicing gained considerable attention in the field of oncology and studies are accumulating that support the notion that characterization of splicing isoforms is valuable for prognostic and diagnostic purposes, and eventually for treatment of cancer itself[17;18].

Splicing may influence cancer progression through altered production and function of established oncogenes and tumor suppressor genes, or genes that display more transcriptional activity in tumors during processes such as epithelial-to-mesenchymal transition (EMT) and angiogenesis[19-22]. Furthermore, evidence comes from hematological malignancies that during oncogenesis genes encoding the spliceosome itself may also become mutated, resulting in an aberrant splicing machinery in cancer cells, resulting in increased numbers of alternatively spliced mRNAs[23].

Potential avenues for treatment may be a blockade at either RNA or protein level of single splice isoforms, but one may also hypothesize that healthy splicing could be restored in cancer cells, thereby restoring the production of canonically expressed proteins.

Increased flTF expression is a general feature in cancer and confers to tumor cells a mitogenic, migratory phenotype, while making them produce angiogenic factors (reviewed in **chapter 9**). asTF has angiogenic properties and expression of asTF in some solid tumors, like flTF, is associated with an unfavorable phenotype. How the changes regarding splicing in tumor cells affect expression of either asTF or flTF is unclear.

The *cis*- and *trans*-regulatory elements that control splicing of TF pre-mRNA have been characterized in peripheral blood monocytes, monocytic cell lines and human umbilical vein endothelial cells (reviewed in **chapter 3**). SF2/ASF, SRp55 and SRp75 are trans regulatory proteins that promote inclusion of exon 5 whereas SC35 and SRp40 promote exclusion of exon 5. At present, the mRNA levels of these splicing proteins have been studied in several types of cancer and, although mRNA levels may be lower for proteins promoting exon 5 inclusion, and higher for those promoting exclusion in some tumors, other tumors display an inverse relationship[22;24-27]. In conclusion, our knowledge on how expression of both TF isoforms is balanced in cancer is still poor and in order to understand how changes in the splicing machinery of tumor cells influence expression of either flTF or asTF, studies in different tumor types are badly needed.

Full-length tissue factor and ectopic FVII

In **chapter 9**, we describe how the TF gene becomes more transcriptionally active in tumor cells, resulting in increased flTF expression on tumor cell membranes through well-defined cellular signaling pathways including EGFR signaling, hypoxia and EMT. The increased expression of flTF facilitates flTF:FVII:PAR2 signaling on tumor cells, directly leading to production of a pro-angiogenic factors that influence the tumor environment in a paracrine fashion and to a pro-mitogenic and pro-migratory phenotype of the tumor cells themselves. Increased flTF expression also leads to coagulation activation and the downstream produced activated coagulation proteins that together with blood platelets, support metastasis through the blood stream. Expression of flTF is increased in most solid human tumors and is associated with adverse pathological and clinical parameters, which confirms the biological relevance of flTF in tumor biology.

flTF is an important modulator of cancer progression, but it is still unclear how flTF:FVII:PAR2 signaling or coagulation activation can take place in tumors that lack blood vessels to obtain liver-derived FVII from the bloodstream. Ectopic production of FVII and simultaneous upregulation of flTF and PARs is detected in several tumor cell lines and it has been hypothesized that ectopic FVII is part of a general mechanism that is involved in promoting tumor angiogenesis[28;29]. In order to investigate the clinical relevance of ectopic FVII expression, we employed the same cohort as studied in **chapter 6**, but now we performed immunohistochemical stainings for FVII (**Chapter 7**). Ectopic production of FVII is present in 39.3% of the tumors in our cohort, whereas no FVII is found in adjacent normal mammary gland tissue. After 10 years of follow-up, 72% of the patients with FVII negative tumors were relapse-free compared to 60% relapse-free patients with FVII positive tumors (P=.02). As flTF serves as the receptor for FVII, we also determined survival in patients with flTF/FVII doubly positive tumors, compared to single positive or negative tumors, which made this difference even more significant (P=.006). In patients aged under 55 years, this effect was more pronounced, with a relapse-free rate of 58% in flTF:FVII positive tumors compared to 82%, whereas the effect of flTF:FVII on relapse-free survival was absent in patients of 55 years and older. Moreover, flTF:FVII positive tumors more often displayed loss of Her2, estrogen and progesterone receptors. These so-called triple negative tumors are known for their aggressive phenotype and poor prognosis. We also tested whether these associations still exist using 65 years as a cut-off, and we still find similar associations with survival. At present, we have no biological explanation for this age-dependent association. One attractive hypothesis is that FVII expression in tumors is a pre-menopausal, hormonal regulated phenomenon, but, since we found a similar association with a cut-off age of 55 years and 65 years, this hypothesis is unlikely to hold

true. Future studies in other solid tumors, e.g. colon cancer, may prove whether the results we report are breast cancer- and gender-specific or rather a general feature of FVII expression in solid tumors.

Full-length tissue factor: evolutionary cysteine conservation

Most flTF resides on the cell membrane in an inactive, "cryptic" form and only gains full coagulant activity after a process called decryption. Cryptic flTF is able to bind FVIIa and to trigger PAR2 signaling[30]. Upon cellular stimulation, flTF can be rapidly decrypted, however, the underlying mechanism for decryption remains a matter of debate[2]. One potential mechanism that decrypts flTF is the exposure of phosphatidylserine at the cell surface, whereas others hypothesize a role for protein disulfide isomerization (PDI)-dependent disulfide formation and breakdown between the cysteines at positions 186 and 209[31;32]. In **chapter 8** we demonstrate in an in silico analysis that the extracellular domain of flTF, including the region surrounding the Cys186 and Cys209, is conserved in vertebrates. Further comparison with other established allosteric disulfide bond-containing proteins led to the identification of a homologous amino acid sequence consisting of a proline, a serine or threonine, an arginine, followed by an asparagine (PSR-N). The PSR-N sequence has a polar and hydrophilic nature and close examination of crystal structures from proteins with an allosteric disulfide revealed that the PSR-N region is solvent exposed. The exact role of this PSR-N sequence is unclear, but an attractive hypothesis is that oxidoreductases may bind to this sequence in order to modify the Cys186-Cys209 disulfide bond. Directions for future research may include a characterization of the redox status of flTF in the tumor microenvironment, the potential role of the PSR-N region and eventually targeting of this region in order to modulate the non-hemostatic events of flTF in cancer.

Conclusion

This thesis describes hitherto unknown non-hemostatic properties of asTF and expands our view on how TF isoforms and integrins interact in disease processes like cancer and atherosclerosis. We further report on associations between expression of TF isoforms and FVII in cancer with clinical outcome and hereby provide new avenues for future studies on non-hemostatic effects of the coagulation system in cancer.

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