The role of blood coagulation in cancer, inflammation and embryonic development
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Chapter 1

General overview and outline

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Coagulation

To prevent blood loss after vascular injury, the damaged vessel wall is covered by a platelet- and fibrin-containing clot thereby stopping bleeding and allowing the repair of the damaged vessel. During so-called primary hemostasis, platelets adhere to the subendothelial matrix to form a hemostatic plug at the site of injury. Simultaneously, during secondary hemostasis in a cascade of proteolytic reactions coagulation factors form fibrin strands which strengthen the platelet plug [1-2].

The coagulation cascade (figure 1) was traditionally divided into an “intrinsic” and an “extrinsic” pathway, which converge in a “common” pathway. The “intrinsic” pathway of coagulation is initiated when blood comes into contact with negatively charged surfaces. A series of proteolytic reactions results in the subsequent activation of prekallikrein, factor (F)XII, FXI and FIX, and the cleavage of high molecular weight kininogen, leading to FX activation [3]. The “extrinsic” pathway is initiated by tissue factor (TF) expression upon vascular injury and after complex formation with FVII also results in activation of FX [4]. Activated FX (FXa) activates in the “common” pathway prothrombin, leading to thrombin formation, finally converting fibrinogen into fibrin. This concept of the “intrinsic” and “extrinsic” pathways served for many years as a useful model for coagulation, but recent evidence shows that the pathways are highly interconnected. For example, high levels of TF in complex with activated FVII (FVIIa) directly activate FX, while at low TF concentrations, FX activation also involves FIXa of the “intrinsic” pathway [5-6]. Furthermore, thrombin directly

Figure 1: Schematic representation of the coagulation cascade. Tissue factor (TF) and activated FVII (FVIIa) form a complex to activate FIX and FX. FXa converts prothrombin into thrombin, which on its turn cleaves fibrinogen into fibrin. Thrombin also activates FVIII and FV, which are co-factors for FIXa and FXa, respectively, and FXI, which activates FIX. Thrombin generation is inhibited by the natural anti-coagulants tissue factor pathway inhibitor (TFPI), anti-thrombin (AT) and activated protein C (APC). APC is formed by the activation of protein C by thrombomodulin (TM) in the presence of thrombin and/or endothelial cell protein C receptor (EPCR).
activates FXI [7-8], whereas clinical practice learns that patients with severe FVII deficiency may bleed even though the “intrinsic” pathway is intact [9]. Moreover, the severe bleeding complications associated with deficiencies of FVIII or FIX would not be expected if the “extrinsic” pathway alone would be sufficient to achieve normal haemostasis.

The current model of blood coagulation is therefore that vascular injury leads to TF exposure to the blood and subsequent complex formation between TF and FVII (see figure 1). Subsequently, the TF/FVII complex activates FX either directly (“extrinsic” pathway) or indirectly via activation of FIX (“intrinsic pathway”), resulting in FXa mediated cleavage of prothrombin into thrombin. Thrombin cleaves fibrinogen into fibrin, fibrin monomers form polymers and a fibrin clot is formed. In addition, thrombin activates FXI, FVIII and FV, resulting in enhanced production of FIXa and FXa, thereby augmenting thrombin production [7-8].

To prevent thrombotic complications due to excessive or untimely fibrin formation, several regulatory mechanisms exist. Procoagulant factors are diluted in and removed from the circulation and are additionally controlled by natural antithrombotics such as heparins [10-11]. Furthermore, several anticoagulant proteins are active in the bloodstream to terminate the clotting cascade. The most important anticoagulant proteins are the circulating enzyme inhibitors TF pathway inhibitor (TFPI), antithrombin (AT) and protein C [12]. TFPI binds to and inhibits FXa. Subsequently, the so-formed TFPI-FXa complex interacts with the TF/FVIIa complex thereby inhibiting the activation of both FX and FIX. The second enzyme inhibitor, AT, inhibits FIIa, FXa, and thrombin. The anticoagulant effect of AT is modified by heparin binding, which increases the affinity of AT for thrombin but also for its other substrates. The final anticoagulant protein, protein C, circulates as an inactive zymogen. In the presence of low thrombin concentrations, protein C is activated, this activation involves two endothelial cell receptors: i.e. thrombomodulin (TM) and the endothelial protein C receptor (EPCR). TM is a cofactor for thrombin and changes thrombin’s specificity from procoagulant to anticoagulant by activation of PC [13]. Recruitment of PC to EPCR enhances its activation by thrombin/TM. Once activated, activated PC (APC) proteolytically degrades the factors FVIIIa and FVa [14], using protein S as a cofactor, thereby limiting thrombin formation [15].

For decades it had been thought that coagulation factors represented a group of relative passive mediators only involved in the linear transduction of the coagulation cascade (as described above). Scientific progress in the last decade has taught us, however, that these factors actively engage target cells thereby fulfilling critical functions in a wide variety of pathophysiological phenomena. For example, coagulation factors play (in a coagulation independent manner) important roles in cell migration and proliferation [16], tumor neovascularisation [17], metastasis [18] and the development of embryonic blood vessels [19]. The major objective of the studies described in this thesis is to further understand the ‘coagulation-independent’ role of blood coagulation factors in cancer, inflammation and during embryonic development.
Blood coagulation and Cancer

Armand Trousseau, a French physician, is often considered to have been the first to describe the association between cancer and venous thrombosis [20]. However, a careful literature analysis revealed that already in 1823 Bouillaud described three cancer patients with deep venous thrombosis [21-22]. Dr Bouillaud described a peripheral oedema in the legs of cancer patients and suggested that the oedema resulted from obstruction of the veins by fibrin clots (‘caillot fibrineux’) which were induced by the cancerous process. Trousseau outlined in detail the relationship between cancer and venous thromboembolism (VTE) in his often quoted book from 1865, thus 42 years after the first report of Bouillaud [20]. Trousseau named the syndrome “phlegmasia alba dolens” (acute white and painful inflammation). The underlying mechanism of the so-called “Trousseau’s syndrome” likely includes all aspects of Virchows triad: stasis of blood, trauma or pathology of the endothelium and hypercoagulability of the blood itself [23-24]. Since the publications of Bouillaud and Trousseau numerous studies investigating the relationship between cancer and VTE have been performed and from these studies it is now clear that the chance of having cancer at the time of being diagnosed with VTE is somewhere between 4 and 12% [25]. The diagnosis of cancer is most often made during the first 60 days after an unprovoked episode of VTE [26]. After a year the risk gradually decreases [27] and after 4 to 6 months the observed VTE equals again the expected number [22].

Also deep venous thrombosis (DVT) is associated with a significantly higher frequency of malignancy during the first six months after diagnosis of the DVT. The incidence of malignancy was higher in the patients with thrombosis than in the patients without thrombosis (11 (upto 25) versus 7.5 %) [28-29].

More recently, the inverse idea that cancer cells might ‘abuse’ a hypercoagulable state to more efficiently metastasize has gained attention [30-31]. Based on the notion that activation of the coagulation cascade plays a detrimental role in cancer outcome, several studies explored the beneficial effect of anticoagulant therapy in patients with cancer. From a clinical viewpoint, the first report on a possible beneficial effect of anticoagulants in cancer progression dealt with vitamin K antagonists (VKA) in the 1960’s. However, a systematic literature review showed that there is not enough evidence to support long-term therapy with VKA for prolonging survival in cancer patients [32].

More recent data from clinical trials in cancer patients have suggested a beneficial effect on survival from low molecular weight heparins (LMWHs) compared to unfractionated heparins or placebo. Overall, dalteparin (fragmin) administration did not significantly improve 1-year survival rate in patients with advanced malignancy [33]. However, subgroup analysis revealed that patients with a better prognosis at entry of the study do live longer suggesting a potential modifying effect of dalteparin on tumor biology. In addition, Klerk and colleagues showed that a brief course of subcutaneous LMWH (nadroparine = fraxiparine) favorably influences the survival in patients with advanced malignancy [34]. Again, patients with better prognosis at the inclusion date show the most prominent survival benefit. The potential anti-tumor effect of LMWHs can be attributed to a wide variety of features: (1) inhibition of angiogenesis, (2)
enhancement of immune attack on tumors, (3) a direct effect on tumor cells including inhibiting expression of oncogenes like c-myc and c-fos, (4) altered enzymatic activity, including the inhibition of tumor heparanase that mediates invasion and metastasis and the inhibition of matrix-degrading enzymes, (5) anti-oxidant effects, (6) the modification of growth factor activity, (7) the inhibition of blood coagulation activation, (8) the inhibition of cell migration, (9) the inhibition of tumor cell adhesion to endothelial cells, (10) the inhibition of multi-drug resistance and (11) the suppression of coagulation proteases in the tumor environment [35-43].

In support of the results from the the clinical trials, experimental animal models have shown that blood coagulation factors might affect cancer outcome. In a murine pulmonary metastasis model, it has been shown that a minute concentration of thrombin (not reducing platelet count) enhanced metastasis [44]. In addition, thrombin helped tumor cells to adhere more avidly to platelets, fibronectin and endothelial cells, and thrombin-treated tumor cells undergo enhanced experimental pulmonary metastasis [45]. Thrombin had also a significant stimulatory effect on angiogenesis via VEGF production [46] and thrombin induced tube formation of endothelial cells in a matrigel membrane system [47]. Applying as little as 0.05-0.1 U/ml thrombin to a chorioallantoic chick membrane stimulated angiogenesis about 2 to 3-fold, thus establishing thrombin-induced angiogenesis in a more relevant model. Finally, the importance of endogenously generated thrombin for tumor metastasis was established employing the highly potent and specific inhibitor of thrombin, hirudin. Hirudin given at various dosing regimens before tumor inoculation dramatically reduced pulmonary metastasis [48].

**Blood coagulation and Inflammation**

*In vitro* and *in vivo* data have provided abundant evidence for cross-talk between coagulation and inflammation [49]. Inflammatory mediators influence the coagulation cascade through upregulation of coagulation factors like TF, thrombin and fibrin, and via inhibition of the fibrinolytic system. Endotoxemia studies in human volunteers and/or chimpanzees have demonstrated that endotoxin-induced activation of the extrinsic coagulation system [50] appears to be mediated by pro-inflammatory cytokines like tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) and IL-6 [51]. TNF-α administration to healthy volunteers elicited rapid activation of coagulation which was similar to that evoked by endotoxin [52]. Moreover, intervention with monoclonal IL-6 antibodies [53] or IL-1 receptor antagonists [54] attenuated endotoxin-induced coagulation.

Evidence for the role of coagulation factors in inflammation is also derived from sepsis and/or endotoxemia models. For instance, inhibition of the TF/FVIIa complex with anti-TF antibodies, [50] tissue factor pathway inhibitor (TFPI) [55-56] or active-site inhibited FVIIa (DEGR-FVIIa) [57] prevented disseminated intravascular coagulation (DIC) and increased survival in baboons intravenously injected with *Escherichia (E.) coli*. In addition, several years ago, a phase III study showed that adjuvant treatment with activated protein C (APC) decreased mortality in patients with severe sepsis [58-59]. Kerschen recently showed that APC’s efficacy in reducing mortality in LPS-induced sepsis was predominantly based on EPCR-
and PAR-1-dependent cell signaling, since APC variants with normal cell signaling but reduced anticoagulant activities retain the efficacy while reducing the risk of bleeding [60]. APC has anti-inflammatory [14, 61] as well as anti-apoptotic effects on endothelial cells [62].

Not only APC is considered to have functions outside of the coagulation cascade (as already mentioned above). Thrombin, for instance, has pro-inflammatory capacities, since it acted as a chemotactic factor for neutrophils and was associated with an increase in adhesion molecule expression [63]. In addition, TF may have important biological functions independent from its well-established role in blood coagulation. TF may be important for processes like embryogenesis, [64-65] tumor progression and neovascularization, [66] and chemotaxis [67]. However, with regard to sepsis, TF’s proposed role in cell adhesion might be more relevant. During inflammation, mononuclear phagocytes cross the lymphatic endothelium in the basal-to-apical direction (i.e. reverse migration), a process dependent on the expression of TF on the surfaces of these cells [68]. Taken together, these studies suggest that coagulation factors play an important role in inflammation.

### Blood coagulation and embryonic development

Most genes involved in blood coagulation have been knocked out in mice. It has been known for several years that knocking out the murine TF gene leads to embryonic lethality between embryonic day 9.5 and embryonic day (ED) 11.5 of gestation [64-65, 69]. As TF-deficient blood vessels lack a proper muscular wall, they are too fragile and have a tendency to leak when blood pressure rises [64-65, 69]. Thus, early embryonic death in TF-deficient mice probably results from haemorrhage and leakage of blood from both extra-embryonic and embryonic vessels [64]. Although TF knock-out mice can survive to birth if delivered by Caesarean section, they die after just 2–3 weeks as a result of major bleeding [65]. Interestingly, very low TF levels (less than 1% of wild-type levels) seem sufficient for embryonic development and survival in mice [70], although mice with low TF typically die at around 8 months of age from cardiac fibrosis and left ventricular dysfunction caused by haemorrhage from cardiac vessels [71]. Consistent with the severe bleeding phenotype and consequent embryonic lethality of TF deficiency in mice, no TF deficient individuals are known, implying that TF is essential for human life.

Rather surprisingly considering the lethality of TF deficiency, mice deficient for the TF ligand, i.e. FVII, do survive until birth [72]. In contrast to the defects in TF deficient embryos, no vascular defects were seen in the yolksac or in the embryo itself. However, more than 50% of the FVII deficient embryos died within the first 24 hours after birth because of fatal intra-abdominal bleeding. To explain the seeming discrepancy between TF and FVII deficient mice, it has been hypothesized that the survival of FVII deficient embryos might be due to the transfer of small amounts of maternal FVII (and/or FX) to the deficient embryos. Indeed, crossing conditional FVII knock-out females with FVII+/- males enabled the generation of FVII deficient embryos in mothers that produce very low levels of FVII and therefore have markedly reduced potential for maternal transfer of FVII [72, 73]. Remarkably, such a cross
produced no viable FVII deficient embryos beyond embryonic day 12.5, whereas younger embryos show malformations that resemble TF deficient embryos. Apparently, in the normal situation, maternal FVII rescues FVII deficient embryos and this implies that, FVII, like TF, plays an important role in vascular development [74].

Deficiencies of coagulation factors more downstream in the coagulation cascade seem less deleterious for proper development. FX deficiency causes only partial embryonic lethality between ED 11.5-12.5. Only 15% instead of the expected 25% of the offspring of heterozygous deficient FX breeding pairs was homozygous deficient for FX. The embryos that did succumb, died because of severe bleeding complications. The phenotype of FX deficiency resembles that of FV and prothrombin deficiency, suggesting that thrombin provides a critical embryonic function at midgestation although it is not essential as are TF and FVII [74].

Outline of the thesis

As already indicated, the primary objective of this thesis is to elucidate the role of blood coagulation factors in cancer, inflammation and during embryonic and vascular development. Chapters 2-6 address the interplay between blood coagulation and tumor cell metastasis. We first determined the effect of congenital coagulation disorders (FV Leiden and Hemophilia A) (chapter 2), high FVIIa levels (chapter 3) and specific thrombin inhibitors (chapter 4) after which we showed that the effects of anticoagulantia might be constricted to certain types of cancer (chapter 5 and 6).

In the next series of chapters (7-10), we studied the role of blood coagulation in inflammatory disease. To this end, FVIII deficient (chapter 7) or FV Leiden (chapter 8) mice were subjected to E. coli peritonitis. Subsequently, we determined the (patho)physiological relevance of a recently identified splice variant of tissue factor, i.e. alternatively spliced tissue factor (asTF). We first identified the murine variant and subsequently we subjected wildtype mice to a Streptococcus pneumoniae model and asTF levels were determined (chapter 9). Furthermore, we determined the presence of asTF in FeCl₃ induced blood clots. Chapter 10 focuses on the causal relationship between hyperglycemia and arterial thrombosis. We determined whether inflammation (LPS) influenced hyperglycemia-induced thrombus formation.

Chapters 11 and 12 aim to elucidate the role of TF in embryonic vascular development. First, we generated chimeric mice from wildtype and TF deficient embryonic stem cells (chapter 11) and subsequently we used these embryonic stem cells to generate embryonic bodies. Chapter 12 describes the capability of these embryonic bodies to form vascular structures.
References

Chapter 1
