The role of blood coagulation in cancer, inflammation and embryonic development
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Citation for published version (APA):
Bruggemann, L. W. (2008). The role of blood coagulation in cancer, inflammation and embryonic development
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Blood coagulation is a complex process aimed at preventing blood loss after vascular injury. The sub-endothelial matrix of the damaged vessel wall is rapidly covered by a platelet- and fibrin-containing clot thereby stopping bleeding and allowing the repair of the damaged vessel. Concurrently, a cascade of proteolytic reactions of coagulation factors results in the formation of fibrin strands which strengthen the platelet plug. Until several years ago, it was generally believed that the individual coagulation factors represented a group of relative passive mediators only involved in the linear transduction of the coagulation cascade. Nowadays, it is however generally believed that these coagulation factors actively engage target cells, thereby fulfilling critical functions in a wide variety of (patho)physiological phenomena. Indeed, coagulation factors play important roles in cancer biology, inflammatory processes and during vascular or embryonic development. Consequently, the primary objective of this thesis is to further understand the role of blood coagulation factors in cancer, inflammation and during (vascular) development.

Chapter 1 provides a general overview of blood coagulation and its potential role in (patho)physiology. Chapters 2-6 address the interplay between blood coagulation and tumor cell metastasis. First, we subjected factor V Leiden and factor VIII deficient mice to a murine model of experimental lung metastasis to test the hypothesis that congenital prothrombotic disorders, like factor V Leiden (FVL), facilitate metastasis whereas bleeding disorders like hemophilia impede metastasis. As described in chapter 2, both hemi- and homozygous factor VIII deficient mice were indeed protected against lung metastasis compared to wildtype littermate controls. In contrast, homozygous FVL mice developed more metastases than wildtype littermates, whereas heterozygous carriers showed an intermediate number of pulmonary foci. These data indicate that congenital susceptibility to either bleeding or thrombosis modify the metastatic capacity of tumor cells in the bloodstream and suggest that procoagulant phenotypes are a risk factor for tumor metastasis. Next, we assessed the risk of rFVIIa for promoting tumor metastasis as rFVIIa is nowadays a well accepted treatment option for patients suffering from serious bleeding complications, such as hemophilia, intracerebral hemorrhage, obstetrical and gynaecological haemorrhage, and thrombocytopenia. In chapter 3 we show that a rFVIIa bolus injection has no effect on pulmonary metastasis of B16F10 melanoma cells in mice, whereas site-inactivated rFVIIa did reduce metastasis. This suggests that FVIIa is an important mediator in metastasis but that increased levels of FVII do not promote tumor metastasis, indicating that FVIIa is not rate-limiting. Long-term follow-up studies of patients treated with rFVIIa might give more definitive answers in this respect.

It is well known that thrombin inhibitors, like hirudin, inhibit metastasis most efficiently in murine models. However, hirudin has a rather narrow therapeutic window, compromising its safety when translated to the clinical setting. Consequently, novel direct thrombin inhibitors are being developed. One of these novel thrombin inhibitors is the orally active reversible inhibitor, ximelagatran. In chapter 4, we aimed to assess the anti-metastatic activity of
ximelagatran in an experimental murine pulmonary metastasis model. To this end, mice received ximelagatran containing food pellets or normal control pellets for 7 consecutive days before tumor cell injection. Quite surprisingly, ximelagatran treatment was shown to be detrimental as it increased the number of pulmonary tumor foci. Follow-up studies showed that the effect was not specific for ximelagatran but that long-term anticoagulant treatment with hirudin was also detrimental in contrast to short-term hirudin treatment. Antithrombotic therapy might under certain circumstances promote tumor metastasis and anticoagulant treatment of cancer patients should therefore be pursued with great care.

In **Chapter 5** we set out experiments in which we tested the general applicability of anticoagulant treatment as treatment option for cancer patients. Therefore, we assessed whether LMWH treatment was effective in diminishing liver metastasis of colon cancer cells. Although during LMWH treatment anticoagulant activity was in the therapeutic range, no difference in liver weight, number and size of metastases were found between LMWH-treated mice and controls. Similar doses of LMWH are known to protect against metastasis in the pulmonary melanoma model as used in chapters 2-4, indicating that colon cancer metastasis is not dependent on the activation of the blood coagulation cascade and therefore is likely not susceptible for the anticancer effects of anticoagulants. Our next goal was to understand the underlying mechanism which causes melanoma cell metastasis to the lung to be coagulation dependent and colon cancer metastasis to the liver to be coagulation independent. Therefore, we compared the effect of hirudin on the B16F10 melanoma cell line with the K1735 melanoma cell line and the CT26 colon carcinoma cell line. Hirudin only inhibited metastasis of B16F10 cells but not that of K1735 and CT26 cells. This differential effect was not explained by pro-coagulant activity but may be explained by differential expression of the surface proteins PAR-1 and CD24. Future experiments should however further clarify this intriguing finding.

**Chapters 7-10** focus on the role of blood coagulation in inflammation. Inhibition of blood coagulation appears to be an important therapeutic strategy to improve the outcome in sepsis. However, the beneficial effect of anticoagulant treatment in sepsis is solely based on experimental data using inhibitors of the extrinsic coagulant pathway, whereas the role of the intrinsic pathway of coagulation in the pathogenesis of sepsis has not been explored. In **Chapter 7**, we determined the role of FVIII on host defence against bacterial peritonitis. The injection of E. coli led to growth and dissemination of bacteria and provoked an inflammatory response as evident from elevated cytokine levels, increased cell influx, liver necrosis and endothelialitis resulting in mortality. The FVIII genotype reduced bacterial outgrowth but had no effect on inflammation and survival. In addition, FVIII deficient mice showed profound activation of coagulation, thereby improving the hemophilic phenotype. Overall, FVIII deficiency slightly modifies host defence in septic peritonitis in mice, but does not influence the final outcome of peritonitis. In the subsequent chapter, **Chapter 8**, we determined the effects of the FVLeiden (FVL) phenotype in peritonitis. The rather high prevalence of the FVL mutation in the general population prompted speculation about a potential survival benefit for individuals carrying the FVL allele. Our murine experiments did however not provide evidence for this notion as the
FV Leiden allele has no beneficial effect in mouse septic peritonitis.

In the traditional view of blood coagulation, vascular injury leads to the exposure of extravascular membrane bound tissue factor (TF) to the blood stream, thereby initiating blood clot formation. Essential in this model of haemostasis is that TF is normally not in contact with blood as it resides in the adventitial lining of blood vessels. However, a soluble TF variant, which is derived by alternative splicing of the TF mRNA, has been described in humans. This alternatively spliced TF (asTF) lacks the transmembrane domain, possesses a unique 3' peptide sequence, is present in plasma and seems biologically active albeit in high concentrations. The existence of (active) asTF is raising controversy in the field of haemostasis because active TF within the bloodstream would lead to massive intravascular thrombosis. In chapter 9, we show that mice also express a soluble TF variant lacking exon 5 and thus the transmembrane region. Murine asTF is expressed in lung tissue, in which it is induced by Streptococcus Pneumoniae infection. Furthermore, murine asTF is present in plasma and can be found throughout arterial blood clots induced by FeCl₃. The fact that mice produce asTF, which is induced by S. pneumoniae and is omnipresent in blood clots, strongly suggest an important role for asTF in (patho)physiology. Ongoing studies should prove or refute this notion.

In chapter 10 we studied the effect of hyperglycemia on arterial thrombosis and we assessed whether inflammation would enhance diabetes-induced thrombotic effects. Indeed, hyperglycemia accelerated the rate of thrombus formation. This effect was associated with increased thrombin generation and could not be explained by changes in vessel wall TF activity. Surprisingly, inflammation reduced the rate of thrombus formation and this reduced rate of thrombus formation was attenuated by hyperglycemia. From this chapter we concluded that there seems to be a discrete, but clear contribution of hyperglycemia in experimental arterial thrombosis.

The final chapters of the thesis focus on the role of TF in development. Chapter 11 describes studies in mice chimaeric for tissue factor expression. The major arteries and veins (aorta, vena cava inferior and superior) in the chimaeric mice contained approximately equal numbers of wildtype and TF knock-out cells. Furthermore, we did not observe any difference in TF protein expression between chimaeric and wildtype animals. We conclude that TF chimaeric mice are vital and develop without vascular malformations. Moreover, the capacity to produce TF on every single cell is not required for normal embryogenesis, but TF seems essential for the development of a number of specialized structures during embryogenesis.

The studies described in chapter 11 did not provide solid evidence for a role of TF in vascular development. In chapter 12, we further address the role of TF in the formation of blood vessels. In this chapter, we determined the capacity of wildtype and TF deficient embryoid bodies to differentiate into endothelial and smooth muscle cells. Embryoid bodies differentiated into smooth muscle cells and endothelial cells irrespective of the genotype. Endothelial and smooth muscle cells derived from both wildtype and TF deficient embryoid bodies were positioned in close contact and able to form vessel-like structures. These studies did not provide any evidence that the TF genotype is involved in vascular development.