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CHAPTER 6

General Introduction and Outline
VENOUS AND ARTERIAL THROMBOSIS

Thrombosis is the formation of a blood clot inside a blood vessel which can lead to the obstruction of blood flow in the circulatory system, and is a major cause of death worldwide (World Health Organization, www.who.int). Thrombosis can be divided into two categories, either venous or arterial thrombosis, depending on the presenting location (1). Venous thrombosis affects 1-3 per 1000 individuals per year, and mainly occurs in the leg, where it can cause local hypoxia (2-4). When the thrombus detaches and is guided by the bloodstream via the heart towards the lungs, an individual can develop a pulmonary embolism. This complication can be fatal due to right ventricular dysfunction and hypoxia (5, 6). Arterial thrombosis occurs when atherosclerotic lesions (also, plaques) in the arteries rupture, and it is the most common cause of death in the human population (7, 8). Rupture of a plaque leads to the exposure of thrombogenic material from underneath the plaque to the bloodstream, leading to the formation of a thrombus. Arterial thrombi in an artery can disrupt blood flow locally, which causes ischemia in the distal organs. Moreover, thrombi can migrate towards other organs, such as the brain (leading to a cerebrovascular accident), which can result in sudden and fatal hypoxia (9, 10).

The pathophysiology of venous and arterial thrombosis is relatively well-understood, and for several decades multiple therapies have been introduced in an attempt to treat the diseases. However, patients receiving these therapeutics show bleeding as a side effect. In order to better understand the diseases and to improve the current generation of therapeutics, an important question remains: what is the exact trigger for a thrombotic event at a certain point in time? The pathophysiology of both diseases can be studied in animal models, which have contributed to new insights in the initiation and progression of the diseases and the development of new and improved medicines. However, the nature of thrombosis in animal models is not spontaneous: complex invasive procedures, such as damaging the endothelial wall or ligating an entire vessel, are required to provoke experimental thrombosis (11-15). In this thesis, we induced spontaneous thrombosis in mice without invasive procedures to study the pathophysiology of the disease. Regarding venous thrombosis, we studied the importance of novel players for spontaneous venous thrombosis in mice. With regard to arterial thrombosis, we assessed the role of natural anticoagulants in mouse atherothrombosis. Remarkably, a transient depletion of natural anticoagulants led to spontaneous atherothrombotic events, a condition which we used to study the influence of several factors associated with the disease.

In the following part of the introduction of this thesis, the coagulation system, which plays a central role in thrombosis research, is briefly outlined. Subsequently, animal models to study venous thrombosis and the novel concept of immunothrombosis are introduced. Following, the role of inherited factors for venous thrombosis and the discovery of a novel risk gene for the disease are addressed. Finally, the pathogenesis of atherosclerosis and arterial thrombosis and currently used mouse models for this disease are shortly outlined.

THE SYSTEM OF COAGULATION

Hemostasis is the balance between maintaining blood flow throughout the body and protecting against incidences of vascular damage. Blood should be able to flow in closed vessels to fulfill its transport function, and in the event of a damaged vessel the wound must be closed to minimize the risk of blood loss. The complex process of coagulation is involved in creating a protective barrier at the place of injury.

Coagulation (here, the biological process of fibrin formation) can be initiated ex vivo in plasma by two coagulation pathways: the extrinsic or intrinsic pathway (1). Although both pathways consist of serine proteases that can consecutively activate one another and converge in the common pathway, the trigger to start coagulation via the extrinsic or intrinsic pathway is different. The extrinsic pathway, or tissue factor (TF) dependent pathway, is initiated when zymogen factor VII (FVII), which is present in the blood, is converted to activated FVII (FVIIa). This process is strongly catalyzed in the presence of TF, which is expressed on the membrane of non-endothelial cells. Upon vascular damage, TF and FVIIa for a complex and convert coagulation factor X (FX) to FXa, which promotes the activation of prothrombin to thrombin. Finally, thrombin converts the soluble blood-dissolved protein fibrinogen to fibrin, which forms a network of long strands. These strands can capture different types of blood cells, thus providing a barrier to prevent blood loss (16).

Coagulation via the intrinsic pathway, also known as the contact-activation pathway, is initiated by the conversion of coagulation factor XII (FXII) to FXIIa. FXIIa activation is triggered by a wide variety of particles which contain a negative surface, such as DNA, RNA, polyphosphates, and collagen (17, 18). Additionally, it has been suggested that FXII can auto-activate (19). FXIIa can activate coagulation factor XI (FXI) to FXIa, which subsequently activates coagulation factor IX (FIX). Together with activated cofactor VIII (FVIII), FIXa converts FX to FXa, which is the first component of the common pathway. Eventually, FXa leads to fibrin formation, as described previously.

Activation and propagation of the coagulation pathways are tightly controlled by natural anti-coagulants, which prevent unwanted clotting (20). Natural anticoagulants provide a balance between an overactive coagulation system and unwanted blood loss due to dysfunctional coagulation. Antithrombin predominantly inhibits thrombin, as the name implies, but it can also inhibit serine proteases FVII, FIX, FX, and FXI (16). Protein C is another important natural
anticoagulant, which can inactivate cofactors Va and Vila. Protein C is activated by thrombin, a process in which protein S serves as a cofactor (21). A third major anticoagulant is TF pathway inhibitor (TFPI), which can inhibit both FXa and the TF-FVIIa-FXa complex (22).

Interference with the conserved balance of pro- and anticoagulants can lead to pathological situations. Disturbing the pro-coagulants axis can lead to excessive blood loss (bleedings). On the other hand, an overactive coagulation system can cause the formation of a blood clot in undesired situations i.e. thrombosis (1). From a mechanistic point of view, thrombosis can occur when one of the three elements of the triad of Virchow is disturbed (16). This triad was originally described by the German physician Dr. Rudolph Virchow in the 19th century (23, 24). He postulated that thrombosis can occur when 1) blood flow is interrupted (stasis), 2) the vessel wall becomes injured (endothelial damage), or 3) the constituents of the blood are altered (hypercoagulability). Although the exact interpretation of the three specific conditions has changed over time, the paradigm itself is still applicable to how researchers and clinicians approach thrombosis prevention (25).

VENOUS THROMBOSIS AND MOUSE MODELS

Venous thrombosis is ranked as the third cause of cardiovascular death in the world (2). Current therapeutics are effective in combating the disease by shifting the coagulation balance, by means of procoagulant inhibition or anticoagulant stimulation (anticoagulant therapy (26)). An undesired consequence of anticoagulant treatment is bleeding, which remains a clinically relevant issue (27). Since patients treated with the current generation of therapeutic anticoagulants have bleeding episodes, a new generation of anticoagulants with reduced bleeding risk is desired. To gain better insight into the pathophysiology of venous thrombosis and to identify possible novel drug targets, animal models are used to mimic the initiation and progression of the disease (13, 14). Studying venous thrombosis in animals is mainly done in mice, because of their highly conserved coagulation system, low costs of maintenance, access to inbred strains, and possibility for genetic manipulation. Mice have proven value in coagulation research and have contributed to the development of new therapeutics. In preclinical studies, agents which inhibit mouse coagulation factors also inhibit their human counterparts (28-30).

It is striking that mice never develop venous thrombosis without surgical, chemical, or mechanical manipulations; Mice appear to be resistant to the formation of a thrombus. Due to this resistance, venous thrombosis has to be initiated artificially by disturbing one of the elements of the triad of Virchow in the venous vasculature (13, 14). Vascular damage results in exposure of non-endothelial cells to thrombogenic components (e.g. TF and collagen) in the blood, and the exposure of these components catalyzes a thrombotic phenotype. The notion that vascular damage induces thrombosis in mice is widely used in many different variations, e.g. by chemical, electric, or laser injury (31-36). Although injury-induced experimental venous thrombosis consists of some components which are equivalent to surgery-associated venous thrombosis in humans, it is not applicable to every pathological setting of venous thrombosis in humans. The need for additional preclinical mouse thrombosis models remains.

More recently, it was shown that venous thrombosis can also be induced in mice when another element of Virchow’s triad is disturbed: The blood flow (37, 38). Mouse models where stasis is induced have provided additional insights for (mouse) venous thrombosis pathophysiology. Here, platelets, neutrophils, and FXII are key players in the initiation of venous thrombosis (39). Conceptually, these components are thought to be involved in the process of immunothrombosis (40). As the name implies, the concept directly links venous thrombosis to immune cells. This link has been established in mouse venous thrombosis, and there are clues that a similar event occurs in humans (41, 42). In short, a stasis mouse model of venous thrombosis without vascular injury showed that platelets are recruited to the venous vessel wall and are subsequently activated. Activated platelets can serve as a platform for the initiation of thrombus formation via the intrinsic pathway of the coagulation cascade (39). Activated platelets can release polyphosphates, which create a negatively-charged surface suitable for FXII activation to FXIIa. Moreover, activated platelets can attract and activate neutrophils, a subset of leukocytes primarily involved in innate immunity (43). Activated neutrophils can produce neutrophil extracellular traps (NETs) (44). NETs are formed and secreted upon decondensation of the nucleus of the neutrophil, and they consist of several nucleic components such as DNA and histones. Besides their immunological function (NETs contain antimicrobial proteins with a high affinity for DNA), NETs can also form a negatively-charged surface for FXIIa activation (41, 45, 46).

Platelets, neutrophils, and FXII have been introduced as interesting potential therapeutic targets (47). Platelets are crucially involved in primary hemostasis, which consists of the formation of a platelet plug to quickly close a wound before the coagulation system can become active. Interestingly, neutrophils or FXII are not related to normal in vivo hemostasis (41, 48), which suggests that targeting neutrophils or FXII for therapeutic purposes would not result in bleeding. This would make both of them an interesting novel target for venous thrombosis therapy, as compared to the current anticoagulant therapy where bleeding is a major side effect.

The final element in Virchow’s triad is the composition of the blood. Mice can be genetically manipulated resulting in the deficiency for genes involved in the coagulation cascade. These mice can serve as animal models for patients with hemophilia or thrombophilia. For instance, comparable to hemophilia A patients, mice deficient for FVIII are prone to bleedings (49).
Regarding thrombophilia, mice deficient in anticoagulant genes (antithrombin, protein C, or TFPI) will die perinatally due to severe coagulopathy (50-52). Mice with a less severe genetic “thrombophilic” modification, such as factor V Leiden or TFPI heterozygosity, will survive birth and will not develop spontaneous venous thrombosis, despite having a subclinical prothrombotic phenotype (52-54). The lack of venous thrombotic events in mice without surgical interventions precludes research on Virchow’s triad third element: The blood composition. In the research described in the current thesis, we attempted to overcome this problem by transiently lowering natural anticoagulants to induce a prothrombotic state in mice.

GENETIC RISK FACTORS FOR VENOUS THROMBOSIS

Venous thrombosis can be induced in animal models in order to better understand the pathophysiology of the human disease. Factors which may contribute to venous thrombosis, such as neutrophils and FXII, can be identified and investigated to understand their importance in the initiation and progression of the disease. Another approach to obtain a better insight in venous thrombosis is to study the human population. Risk factors which predispose to venous thrombosis can be identified within the diverse human population by comparing individuals with and without the disease (55). The current paradigm for venous thrombosis is that the actual event is an accumulation of different risk factors leading to a certain threshold (56). Identifying these risk factors can aid to understand venous thrombosis pathophysiology, explain thrombotic events, and ideally predict the disease. Roughly, thrombotic risk factors can be divided into two groups: Environmental and genetic. Some examples of the most pronounced risk factors for venous thrombosis are obesity, bed rest, and oral contraceptives (environmental), and antithrombin/protein C/protein S deficiency, and the factor V Leiden and prothrombin G20210A mutation (genetic) (2).

At present the most common risk factors in the human population have been identified and their role in venous thrombosis has been elucidated. Genetic risk factors are directly linked to the coagulation cascade; deficiencies in natural anticoagulants (e.g. antithrombin, protein C) predispose to venous thrombosis, while deficiencies of a procoagulant proteins (e.g. FIX, FVIII) result in bleeding (57). Moreover, a specific gain of function mutation in a procoagulant gene leads to an increased risk of venous thrombosis (e.g. factor V Leiden, which leads to a form of coagulation factor V that cannot be inactivated by anticoagulant protein C (58).

Identifying novel risk factors using the human population is still of interest. Unidentified factors are likely not useful to target or to screen in a population, due to their low ratio for increased risk for venous thrombosis. However, they can help us to improve our understanding of the pathophysiology of the disease. For this purpose, large international consortia have been established to compare genomes of venous thrombosis patients with those of healthy controls, to investigate in which genomic loci the two groups differ (59). In two of these large studies, a novel risk gene was identified: SLC44A2 (60, 61). This gene was not associated with thrombosis before, nor involved in the classical coagulation cascade (16). In this thesis, we investigated the role of SLC44A2 in immunothrombosis.

ATHEROTHROMBOSIS AND ANTICOAGULATION

Diseases resulting from arterial thrombosis (also, atherothrombosis), such as a cerebrovascular accident and myocardial infarction, are the most frequent cause of death in the human population (World Health Organization, www.who.int). A common theme to both arterial and venous thrombosis is the formation of a clot in the bloodstream, however, they are the result of a different pathophysiology (5, 7).

The initiation process leading to atherothrombosis, called atherosclerosis, is characterized by chronic thickening of the arterial vessel wall. In short, activation of endothelium in vessels with increased shear stress causes the retention of low-density lipoproteins (LDL). Increased levels in the circulation of these particles, due to e.g. a diet rich in fat, are associated with an increased risk for arterial thrombosis (62). Activated endothelial cells cause monocytes to migrate through the endothelium towards the lipid particles. Monocytes clear the lipid particles and become “foamy” macrophages, because of their high lipid content. These macrophages get trapped under the endothelium. As a result, smooth muscle cells migrate and proliferate towards the endothelium to restore the damaged tissue, covering the foamy macrophages in the process. The foamy macrophages become necrotic and eventually form a large mass of prothrombotic material underneath the vulnerable endothelial wall. Over time, the atherosclerotic necrotic core causes calcium deposition and hardening of the artery (63). The vulnerable atherosclerotic vessels are prone to atherothrombosis; the rupture of an atherosclerotic lesion, which leads to the exposure of prothrombotic (necrotic) material to the blood. This results in the rapid formation of a blood clot (thrombus). Within minutes, the thrombus can block the blood supply to vital organs, which causes acute clinical problems and can be fatal (7, 64).

Atherosclerosis, the initial process leading to atherothrombosis, can be studied in vivo in transgenic mice with a modified lipoprotein metabolism (65, 66). When these mice are fed a “Western-type diet”, a diet containing high levels of cholesterol and/or fat, they develop atherosclerotic lesions (also, plaques) in the arterial vasculature. Preferentially, atherosclerosis occurs in areas with disturbed flow patterns, such as the aortic root and in and around
branches of large vessels (67). Although mouse models mimicking human atherosclerosis have helped us to study the pathophysiology of the disease, mice never develop atherothrombosis spontaneously; a mouse with an unprovoked heart attack has never been described. The lack of atherothrombotic events in mice precludes in vivo studies on atherothrombosis. Moreover, the actual value of atherosclerosis mouse models to better understand the processes which occur in the human disease is unclear (68, 69). The reason for the absence of atherothrombotic events in mice is currently unknown; however species differences between mice and humans related to this matter have been described, such as differences in size, metabolism, heart rate, vessel anatomy, plaque composition, and many more (70).

One of the main species differences between human and mice is that mice have a more potent anticoagulant system than humans. When prothrombin is activated in plasma, thrombin activity is inhibited approximately nine times faster in mouse plasma compared to human plasma (71). This implies that the natural anticoagulant potential of mice is significantly higher than in humans, which is likely the result of higher levels of circulating natural anticoagulants. For this reason, we hypothesized that mice with severe atherosclerosis will not give rise to atherothrombosis due to a more potent anticoagulation system, although plaques may be damaged or rupture, of which evidence has even been reported (72-74).

**OUTLINE OF THE THESIS**

Within this thesis, we aimed to introduce a transient imbalance in the coagulation profile of mice. Introducing a transient imbalance circumvented the use of invasive methods to introduce thrombosis, such as by damaging or inducing stasis of blood vessels. This allowed us to study the pathophysiology of thrombosis in a setting that mimics human scenarios for thrombosis, where an imbalance (e.g. genetic profile or obesity) is a major contributor.

As a tool to introduce an imbalance of the coagulation profile of mice, we used RNA interference (RNAi; also, small interfering RNA; siRNA). siRNA consist of short stretches of RNA, and is used to inhibit the transcription of specific mRNAs. When siRNAs enter a cell, they can interact with their mRNA complementary counterpart. This specific interaction leads to mRNA breakdown and thus decreased protein production (75). In contrast to its use in in vitro studies, RNAi as a research tool to study gene function in vivo is a relatively new concept (76). Currently, siRNA in vivo studies are restricted to genes expressed in the liver by hepatocytes, which happens to be the cell type that produces the majority of the coagulation factors. This means that RNAi thus can be used to study the function of hepatic genes in vivo, when genetically modified animals deficient in a specific gene are not viable. Moreover, inhibition of genes by RNAi can be achieved much faster and cheaper than genetically modifying animals.

RNAi is not only used for research purposes; RNA therapeutics or oligonucleotides (of which siRNA is a subclass) have been introduced to treat a wide variety of diseases (77). In chapter 2, the molecular background of oligonucleotides and current applications in several animal models in the context of pharmacotherapy of coagulation (thrombosis and hemophilia) is summarized. Moreover, the perspective for oligonucleotides as therapeutic modalities within the fields of thrombosis and hemophilia is discussed.

In chapter 3, we investigated the influence of platelets, neutrophils, and FXII in mouse spontaneous venous thrombosis. Here, venous thrombosis is induced by the acute siRNA-dependent inhibition of natural anticoagulants antithrombin and protein C. We showed that platelets are crucial for the onset of spontaneous venous thrombosis, while neutrophils were not rate-limiting. Remarkably, acute siRNA-mediated depletion of FXII even seemed to exacerbate the thrombotic phenotype. This counter-intuitive observation around FXII function is extensively investigated and discussed in chapter 4.

Based on studies in which single nucleotide polymorphisms (SNPs) were investigated, the gene SLC44A2 was identified as a risk factor for venous thrombosis (60, 61). In chapter 5, we investigated whether the SNP most significantly different between venous thrombosis patients and healthy controls (the “top” SNP) could be linked to altered levels of thrombosis plasma biomarkers.

In chapter 6, we used siRNA to study the role of the natural anticoagulant protein C in the progression of mouse atherosclerosis towards atherothrombosis. Protein C-low mice spontaneously developed a unique atherothrombotic phenotype; Structured fibrin-rich thrombi on atherosclerotic lesions in the aortic root were identified, albeit at a low frequency. In chapter 7, we aimed to reproduce the unique low protein C-induced atherothrombotic phenotype in mice. Moreover, in order to gain more insight in the pathophysiology of atherothrombosis, we attempted to increase the development of atherothrombosis by transiently increasing blood pressure. Also, atherothrombosis was redirected to a predefined vascular site of atherosclerosis.

In chapter 8, the thesis is discussed, and chapter 9 is a Dutch summary of the findings.


