CHAPTER 1

General introduction

Part of this chapter is based on:
THROMBOSIS: A MAJOR CAUSE OF DEATH AND GLOBAL DISEASE BURDEN

Thrombosis is the intravascular formation of a blood clot (thrombus) by a process of platelet activation and blood coagulation, which leads to occlusion of the blood vessel causing a disruption of the blood flow. Thrombotic processes can occur both in arteries and veins and are the leading cause of death worldwide. In the United States and Europe, over 2,200 and 10,000 patients die each day respectively due to cardiovascular disease, with the vast majority suffering from thrombus formation in coronary or cerebral arteries leading to myocardial infarction and stroke, respectively. Besides arterial thrombosis, venous thromboembolism (VTE), comprising deep vein thrombosis (DVT) and pulmonary embolism (PE), is a major cause of morbidity and mortality worldwide. PE is a life-threatening consequence of DVT that occurs when the thrombus dislodges from deep veins and migrates into the lungs. VTE affects approximately 1 per 1000 adults of European ancestry annually and accounts for approximately 12% of all deaths in European countries and the United States. Additionally, despite novel antithrombotic and prophylactic strategies, the annual incidence of VTE has increased during the past decades and is predicted to increase further due to the aging population. Therefore, thrombosis is an important healthcare issue. Furthermore, (microvascular) thrombosis can occur secondary to many other conditions, such as sepsis, organ and tissue transplantation, major surgery, trauma, and hypothermia.

Unfortunately, antithrombotic treatments affect both pathological thrombosis as well as physiological hemostasis, which is the biological process that prevents bleeding after vessel injury. Consequently, current therapies reduce the risk of thrombosis while increasing the risk of unwanted bleeding as exemplified by the majority of emergency hospitalization due to adverse drug events being from anticoagulant and antiplatelet therapies (6.4-17.3% and 8.7-10.4% of cases respectively). Improving our understanding of natural hemostatic and antithrombotic mechanisms may identify novel ways or improve current strategies to enable specific inhibition of arterial and/or venous thrombosis while maintaining normal hemostasis.

NORMAL HEMOSTASIS

When damage occurs to a blood vessel, either accidentally or surgically, its endothelial barrier is disrupted, enabling contact of blood components with the subendothelial and extracellular matrix (Figure 1). Blood now passes through the vessel wall until the bleeding is stopped physiologically (by hemostasis) or artificially (e.g. by a tourniquet or surgical clamp). The word hemostasis is derived from the Greek αίμα/hema (=blood) and στάσις/stasis (=halt), literally the stopping of blood. Although a myriad of processes occurs simultaneously, hemostasis is generally divided into several phases, of which each phase can be divided in sequential steps (Figure 1).
Primary and secondary hemostasis

**Primary hemostasis** starts the moment circulating platelets make contact with damaged endothelium or subendothelial tissue (Figure 1A). Platelets are the smallest of blood cells; they are anucleated cells budded off from megakaryocytes, their large multinuclear mother cells mainly residing in bone marrow. Human platelets average 2-5 µm in size, but despite their small size they play a major role in hemostasis, inflammation, bacterial defense, wound regeneration and cancer metastasis 19, 21-23. Platelets are activated by a whole range of molecules present at the site of a damaged blood vessel, e.g., extracellular matrix (ECM) proteins, such as Von Willebrand Factor (VWF) or collagen, and soluble factors such as thrombin, adenosine di-phosphate (ADP) and adrenaline 24, 25. Damaged endothelial cells release the contents of Weibel-Palade bodies, which are granules filled with coagulation and inflammation enhancing and modulating compounds, such as VWF and various cytokines 26, while subendothelial smooth muscle cells and fibroblasts express tissue factor (TF), a potent activator of the plasmatic coagulation system (initiating secondary hemostasis) 27. Activated platelets express several membrane (glyco)proteins, amongst others GPⅡb-Ⅸ-V and P-selectin that bind to activated endothelium or subendothelial collagen directly or via intermediate adhesion factors, such as VWF 24. Similar to endothelial cells, platelets degranulate upon activation, thus releasing a myriad of molecules enhancing platelet activation, plasmatic coagulation, inflammation, tissue regeneration and bacterial killing 19, 21. Upon activation, platelets change shape by increasing surface area with membrane extensions thus enabling quicker adherence to other platelets and cells. Consequently, more platelets are now recruited to the site of injury (Figure 1B). Platelets stick to the subendothelial ECM and to each other, forming the hemostatic plug, while platelet thromboxane A2 released from granules induces vasoconstriction to prevent further blood loss. Once the platelet hemostatic plug covers the damaged site completely, it is further strengthened via secondary hemostasis by the plasmatic coagulation system.

**Secondary hemostasis**, occurring simultaneously with primary hemostasis, creates a fish-net like fibrin network to trap red and white blood cells and further strengthen the hemostatic platelet plug, reducing the bleeding risk. Secondary hemostasis occurs via plasmatic coagulation cascades which are classically divided into either two pathways, the intrinsic and extrinsic pathway of coagulation, or three phases according to a widely used current model 28 (initiation, amplification, and propagation phase, Figure 1B). During the **initiation phase**, low amounts of active coagulant factors are generated. This starts with exposure and binding of TF to plasma coagulation factor VII, which forms a TF/VIIa complex. The TF/VIIa complex proteolytically activates factor IX and X, creating a prothrombinase complex with Va that converts prothrombin (factor II) into thrombin 25. Thrombin slowly accumulates during the **amplification phase**, activating platelets and platelet derived factor V, amplifying the prothrombinase activity. Thrombin also activates factor XI and VIII, the latter acting as cofactor to IXa on the surface of activated platelets, generating more factor Xa. Thus, the amplification phase boosts the level of active coagulation factors (Vα, VIIIa, IXa and XIa) 25. Factor Xa initiates the **propagation phase**, by activating factor IX that associates with VIIIa. Factor VIII and IX are crucial in the coagulation cascade, since their (near-)absence leads to severe bleeding disorders with hemorrhagic complications (hemophilia A and B, respectively). On procoagulant membranes of activated platelets, the IXa/VIIIa complex stimulates Xa and Xa/Va complex formation, subsequently propagating thrombin formation. The increase in thrombin generates gross amounts of fibrin fibers from fibrinogen, which are cross-linked yielding an elastic, polymerized fibrin network and clot that strengthens the hemostatic plug 25. The initiation phase is classically referred to as **extrinsic pathway** (Figure 1C), which can be assessed in vitro by measuring the prothrombin time (PT). The **intrinsic pathway** of coagulation overlaps with the amplification and propagation phase, but can also be triggered independently by collagen, polyphosphates secreted by platelets, neutrophil extracellular traps (NETs), and artificial material such as glass.
leading to activation of factor XII, XI and kallikrein and the subsequent downstream coagulation factors (Figure 1C) \(^{25, 30}\). The intrinsic pathway can be assessed \textit{in vitro} by measuring the activated partial thromboplastin time (APTT). Both PT and APTT determine the time it takes to form a fibrin clot, partially depending on the common pathway of coagulation and either extrinsic or intrinsic pathway of coagulation, respectively.

**Counterbalancing clot formation**

Under normal conditions, endothelium constantly prevents unwanted thrombus formation by actively producing and excreting anticoagulant compounds, preventing platelet adhesion and coagulation cascade activation. Such anticoagulants are supported by plasmatic anticoagulant factors produced by the liver, such as protein C, protein S and antithrombin, which inhibit specific procoagulant factors. An important physiological process following hemostasis is the recovery of blood flow due to degradation of the formed clot by a process called \textit{fibrinolysis} (Figure 1D). The cross-linked fibrin network is enzymatically degraded by plasmin, which is formed from plasminogen by tissue plasminogen activator (t-PA). t-PA is slowly released by damaged endothelium enabling a gradual degradation of fibrin after the bleeding has stopped and tissue regeneration has started. Fibrin is cleaved into fibrin degradation products, of which amongst others D-dimer can be detected in plasma and is commonly used in the diagnosis of venous or arterial thrombosis.

**PATHOLOGICAL HEMOSTASIS**

In pathological thrombotic conditions, the balance between thrombus formation on one hand and the inhibition of clotting with clot lysis on the other hand tips towards clot formation, leading to thrombi and/or emboli and subsequent organ damage (briefly outlined in Figure 2). Contrarily, if the balance tips towards less clotting, bleeding may be the result. To date, many patient characteristics for an increased risk of thrombosis are known. Although the etiology of arterial and venous thrombosis is somewhat different, several shared risk factors are: age, overweight/obesity, smoking and thrombophilia (inherited or acquired procoagulant disorders) \(^{31-34}\), although age and body mass index are not consistently associated with increased VTE risk in literature \(^{35}\). Moreover, there are many conditions that can provoke both arterial and venous thrombosis, such as hyperhomocysteinemia, antiphospholipid antibodies, malignancies, infections and the use of hormonal therapy \(^{36}\). Classical risk factors for arterial thrombosis include smoking, overweight, hypertension, diabetes and hypercholesterolemia, these are also characteristics that can be found in patients with atrial fibrillation \(^{37}\). Specific risk factors for VTE are deficiencies in anticoagulant factors (antithrombin, protein S, protein C), increased level or activity of procoagulant factors (e.g. factor V, VIII, IX, fibrinogen, prothrombin), hospitalization, cancer and surgery \(^{3, 18}\), but also \(>4\) hours of travel \(^{39, 40}\), immobility \(^{41}\), oral contraceptive use and pregnancy \(^{2, 18}\).

Arterial and venous thromboembolism have long been considered as distinct pathophysiological conditions with arterial thrombosis due to platelet activation on atherosclerotic plaques on one hand and coagulation cascade activation in VTE on the other hand. However, an overlap in pathophysiology also exists, for instance coagulation cascade activation resulting in fibrin-rich thrombi also occurs in arterial thrombosis, specifically in atrial fibrillation and myocardial infarction \(^{36}\). The key role of coagulation in the formation of arterial thromboembolism, hence beyond VTE, is supported by anticoagulant drugs which are also highly effective in preventing arterial embolism in atrial fibrillation \(^{42}\) and can be used in addition to antiplatelet drugs to increase the effectiveness in treatment of established coronary artery disease \(^{43}\). Furthermore, patients with hemophilia (less functional coagulation cascade) have an 80% reduced risk of myocardial infarction \(^{44}\). Thus, arterial thromboembolism is not only due to platelet activation but also due to coagulation cascade activation. Similarly VTE comprises both coagulation activation and platelet activation, several examples support this notion. For example, during early venous thrombus formation aggregated platelets attach to endothelium \(^{45}\) and excrete granular content \(^{46}\). Inhibiting platelet adhesion to endothelium by blocking P-selectin reduces venous thrombus formation \(^{47}\) and inhibiting platelet function by clopidogrel or aspirin reduces experimental venous thrombus formation and PE mortality, respectively \(^{48, 49}\). Thus, these studies demonstrate the role of platelets, besides the already known role of coagulation cascade, in the development and consequences of VTE. Moreover, inhibiting platelets by aspirin after a first unprovoked VTE can reduce the recurrence of VTE in patients by 42% \(^{50}\). Antiplatelet drugs are therefore effective in the prevention of VTE although to a lesser extent than anticoagulant drugs \(^{51, 52}\).

Consequently, platelets play a role in both arterial and venous thromboembolism \(^{53-56}\) and both primary and secondary hemostasis are crucial in the development of diseases such as myocardial infarction, stroke, deep vein thrombosis and pulmonary embolism. Additionally, pathological hemostasis is implicated in other conditions like sepsis and accidental hypothermia \(^{4, 15, 16, 57, 58}\).
inflammatory system. Subsequent activation of coagulation cascade and platelets occur and the ensuing thrombus formation leads to complete or partial occlusion of the vein critically reducing blood flow. C) When part of a thrombus breaks, an embolus is formed which travels from either the venous or arterial thrombus with the remaining blood flow until it occludes a subsequent blood vessel in an organ. D) Potentially lethal occlusive diseases in different organs. Arterial thromboembolism can induce occlusion of blood supply to for instance the brain or heart, leading to stroke or myocardial infarction. Venous thrombosis may lead to deep vein thrombosis of legs and arms and emboli moving through the venous system into the right heart subsequently occluding pulmonary arteries leading to pulmonary embolism.

HEMOSTASIS IN HYPOTHERMIA

Hypothermia is a condition wherein the body has lost heat faster than it can produce, resulting in a lower than normal (~37°C) body temperature. Accidental hypothermia can lead amongst others to arrhythmias, central nervous system depression and respiratory failure, eventually leading to death 16, 59, 60. Moreover, hypothermia may induce pathological activation of the hemostatic system. The ensuing disseminated intravascular coagulation (DIC) of hypothermic patients may result in ischemia and necrosis of organs and eventually result in death 16, 57, 59. Besides these thrombotic complications, DIC may provoke hemorrhage due to consumption of clotting factors and platelets, thereby leaving a hypocoagulopathy to favor bleeding 61. Consequently, hypothermia is associated with a hypocoagulated state with prolonged PT and APTT already when temperatures drop below 35 °C 62, 63. Low temperature in vivo has also been shown to increase activation, aggregation, and sequestration of platelets 64, 65. Low temperatures of the extremities have been implicated to ‘prime’ platelets for activation at these sites most susceptible to bleeding throughout evolutionary history, which also leads to increased clearance of these platelets from circulation 66. Furthermore, both accidental and therapeutic hypothermia are associated with a reduction in platelet count (thrombocytopenia) 60, 67-72. Whether this thrombocytopenia can be reversed quickly by rewarming is still not clear.

Ex vivo cooling of platelets induces platelet shape changes similar to activation of platelets 73-76 and several studies described low temperature to increase degranulation of activated platelets and activation products of platelets in plasma 65, 77. Moreover, cooled platelets demonstrate an increased tendency to aggregate 78. Furthermore, cold (4°C) stored platelets are rapidly cleared from circulation after transfusion 65, 79, 80. Therefore, platelets are stored at 22-24°C room temperature before transfusion which increases risk of bacterial contamination and thus limits shelf-life to only 5-7
days, compared to 40 days for cold stored red blood cells. Enabling cold storage of platelets without their activation may reduce monetary losses from discarded and expired platelet concentrates, improve logistics and limit bacterial contamination. Besides provoking prothrombotic effects, low temperature has also been described to induce anticoagulant mechanisms, for instance lowering the enzymatic coagulation reactions, prolonging bleeding time in cold skin and diminishing thromboxane A2 release from platelets. Taken together, the effects of temperature on hemostasis are still incompletely understood, specifically since hypothermia is associated with both prothrombotic and hypocoagulant effects, of which the latter can be secondary to the consumption of coagulation factors and platelets in cases of DIC, however this cannot explain all anticoagulant effects studied so far. Further unraveling the temperature effects on hemostasis may yield improved knowledge on hemostasis and potentially new pathways for drug development focused on novel antithrombotic strategies.

MAMMALIAN HIBERNATION: A UNIQUE NATURAL MODEL OF SUPPRESSING HEMOSTASIS

Hibernation is an energy conserving behavior, which in small rodents consists of repetitive cycles of torpor and arousal. During torpor, metabolism, body temperature, heart and respiratory rate as well as other physiological processes reduce to a minimum and revert during each short period of arousal (Figure 3). Torpor lasts several days to weeks, whereas arousals last several hours to a day. Contrarily to this ‘deep torpor’, some mammals perform ‘daily torpor’ to save energy, entering torpor for a few hours while remaining normothermic the rest of the day.

Hibernators also embody several risk factors for thrombosis compliant with the triad of Virchow for thrombotic risk - by stasis of blood, hypercoagulability, and endothelial activation/injury. Specifically, hibernation entails periods of prolonged immobility with low blood flow (stasis) in veins and atria, increased blood viscosity (hypercoagulability), cycles of hypoxia-reoxygenation and cooling-rewarming with signs of endothelial activation. Additionally, at entrance of the hibernation season, hibernators are generally grossly overweighted/obese. Remarkably, despite these risk factors for thrombosis, hibernators do not demonstrate signs of organ damage due to thrombosis during hibernation or upon arousal in spring. Moreover, despite the frequent periods of dramatically reduced body temperatures, hibernators emerge apparently unharmed from hibernation and seem to escape from the potential fatal consequences of hypothermia as well.

Therefore, it seems that hibernators developed mechanisms to prevent amongst others pathological activation of the hemostatic system. Alterations in components of primary hemostasis, secondary hemostasis and fibrinolysis may play a crucial role to diminish the risk of thrombosis during hibernation as some of these alterations have been disclosed in different hibernating species, amongst others in brown bear, ground squirrel species, hedgehog, and Syrian hamster. However, mechanisms governing the opposite, i.e. the rapid normalization of hemostasis in arousal to prevent bleeding, is less well documented. Additionally, platelets from hibernators resist exposure to low temperatures for a prolonged time and ex vivo cold storage of hibernator platelets still allows transfusion in summer animals without rapid platelet clearance.

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Thus, hibernation features a potentially lifesaving natural anticoagulant mechanism to prevent thrombosis in times of increased risk. Unlocking this mechanism for
AIM OF THIS THESIS

The main goal of this thesis is to elucidate the regulation of key modulators of hemostasis in hibernation and establish to what extent these are present in non-hibernating mammals. Therefore, Chapter 2 describes the effects of hibernation and hypothermia on circulating platelet dynamics in hibernating and non-hibernating mammals. Chapter 3 presents an overview of alterations in the components of primary and secondary hemostasis as well as in the fibrinolytic pathway in the hibernating Syrian hamster. The underlying mechanism of platelet dynamics in hibernating hamsters is further assessed and described in Chapter 4. Since the findings in Chapter 2 demonstrated that the platelet dynamics are temperature dependent and applicable in non-hibernating mammals, the study in Chapter 5 further assessed the application of hypothermia induced suppression of hemostasis via a thrombocytopenia in non-hibernating mammals, amongst others by (intravital) imaging studies to elucidate the underlying mechanism in rat and mouse. Additionally, in Chapter 6 the role of cytoskeletal rearrangements was explored in underlying shape changes of platelets associated with hibernation and a comparison was made with shape changes of platelets from human and other non-hibernating species. Lastly, in Chapter 7 we summarize and discuss the obtained data in our experimental chapters, review the literature on hemostasis in hibernation and provide future perspectives.
REFERENCES


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