Experimental, clinical, and meta-analytical studies of antithrombotic therapies in venous and arterial thrombosis
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Chapter 1

GENERAL INTRODUCTION AND OUTLINE OF THE THESIS

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General introduction and outline of the thesis

Arterial thrombosis is mostly associated with atherosclerosis, clinically manifested as myocardial infarction, stroke and peripheral arterial occlusive disease (PAOD) causing a high mortality and substantial morbidity. In addition, the incidence of venous thromboembolism due to either acquired or genetic prothrombotic states, immobility, cancer, trauma, and infection adds considerably to the burden of vascular disease. To improve the clinical management of these disorders, thrombosis research has focused its efforts over the last two decades on establishing clinical, meta-analytical and experimental methods to allow a better comprehension of risk factors, treatment efficacy, and pathophysiological principles of thrombosis formation. The first part of this thesis concentrates on the questions of arterial and venous bypass occlusions in patients suffering from PAOD, who were treated with either balloon-angioplasty or an infrainguinal bypass implantation. In both clinical settings, reocclusion of the re-established patency of the occluded arterial segment represents a considerable complication. Therefore, the effect of administering antithrombotic or antiplatelet agents as secondary prophylaxis to prevent reocclusion in these patients, has become a main focus of many clinical and meta-analytical studies. Meta-analysis has gained increasing acceptance as a tool, to objectively quantify the overall clinical effects in patients receiving a specific drug compared to a placebo or another treatment. We performed three meta-analyses in collaboration with the Cochrane Peripheral Vascular Disease Group, assessing the effect of antiplatelet and antithrombotic agents in PAOD patients treated with either angioplasty or bypass surgery.

The second part of the thesis focuses on two new strategies of an antiplatelet and an antithrombotic agent, which were first investigated in vitro and subsequently applied in an in vivo venous and arterial animal thrombosis model. A review on the usefulness and limitations of animal models in arterial and venous thrombosis gives an overview of the different models that have been available in the last four decades.

In section three of this thesis, thrombosis formation is studied with the help of a transgenic mouse model. A review on genetically modified mouse models used in thrombosis and hemostasis research describes available models and the present state of the art. The murine model used in subsequent studies is a mouse with a functional deficiency in the thrombomodulin gene, leading to a prothrombotic state. Whether such a deficiency has an impact on thrombosis formation in the carotid artery was studied. The same mouse type was also used in an Escherichia coli peritonitis model, to evaluate the crosstalk between the coagulation and inflammation system. Thus, a wide spectrum of anticoagulant strategies and pathophysiological mechanisms in thrombosis research is covered in this thesis.
Part I

Antiplatelet agents

Platelets, the smallest circulating cells in blood, have the capability of adhering and aggregating to the injured endothelium of the arterial and venous wall. This process is useful to avoid bleeding. However, when the lesion of the endothelium is due to atherosclerosis, platelets will adhere and aggregate as well, inducing clot formation and eventually complete vessel occlusion. This process is, of course, undesired, and therefore medical treatment focuses on the prevention of clot formation in this context. Platelet aggregation is triggered by cell activation through various proteins present in the blood. Thromboxan A₂ (TxA₂), thrombin and collagen, for example, are able to activate platelets, which leads to the secretion of multiple vasoactive substances contained in the thrombocyte granula.

The crucial mechanisms triggering platelet activation and aggregation are the Arachidonic Acid (AA) pathway and the Adenosin-Diphosphate (ADP) pathway leading to the activation of the fibrinogen receptor on the platelet membrane. AA is released by the cell membrane as a precursor of various eicosanoids and among those it is Thromboxan A₂ (TxA₂) that is synthesized in the platelet cell by the enzyme Cyclooxygenase-1 (COX-1). Thus, COX-1 represents a target for the development of agents capable to inhibit platelet activation/aggregation. The most widely used drug having this property is acetylsalicylic acid (ASA). Extracted initially from willow bark at the beginning of the last century and later on synthesized artificially as the first drug in the world, it was used as an antipyretic and antiinflammatory drug initially. It was only later in the 20th century that its platelet inhibiting potency was detected. Its present use as a platelet inhibitor concerns all diseases related to atherosclerosis.

The platelet membrane contains receptors for fibrinogen (Fb) which belongs to the acute phase proteins. After proteolytic cleavage by thrombin, it forms soluble fibrin, which in turn will be converted to solid fibrin by coagulation factor XIII, thus contributing to clot formation. On the other hand, Fb binds to its receptor, a glycoprotein complex GPIIb/IIIa, on the activated platelet membrane with the capability to link two platelets together, thus consolidating the growing clot. Increased levels of Fb are found in patients suffering from progressive atherosclerosis, as in unstable angina, myocardial infarction, stroke, and PAOD. The Fb-receptor is, therefore, another target for antplatelet drugs like for example small peptides containing the amino acid sequence -R-G-D- (Arg-Gly-Asp; RGD-peptides), which is identical to the binding site of Fb. These small peptides bind to the Fb receptor on the platelet cell reversibly and competitively. Irreversible binding is also achieved by another category of antplatelet agents, which blocks the Fb receptor GPIIb/IIIa (glycoprotein) like abciximab (Reopro), which are chimeric antibodies.
The third target of clinical interest to inhibit platelet activation/aggregation is the receptor for ADP on the platelet membrane. Thienopyridin derivatives such as Clopidogrel and Ticlopidine are drugs with this capacity. Clopidogrel is a substance that is being evaluated in combination with ASA in different settings of atherosclerotic diseases and has recently been shown to increase the efficacy of ASA by 20% in the CURE trial in patients with acute coronary syndromes. Dipyridamole, a phosphodiesterase inhibitor delaying the breakdown of cyclic AMP (cAMP), which has a platelet inhibiting effect, has often been used in combination with ASA in PAOD patients, and now shows a revival in the treatment of stroke patients, based on the European Stroke Prevention Study II, which also revealed an additional effect.

**Antithrombotic agents**

There are mainly two different approaches to achieve anticoagulation. The first is by preventing the synthesis of vitamin K dependent coagulation proteins in the liver, such as is effected by coumarins and coumadin (acenocoumarol and phenprocoumon or warfarin). These drugs are administered in an oral form.

The second approach is by preventing coagulation proteins from being activated or by blocking activated proteins by a specific drug. Inhibitors of thrombin and factor Xa, like unfractionated heparin (UH) and low molecular weight heparin (LMWH) are most widely used. The disadvantages of these agents are, that they have to be administered intravenously or by subcutaneous injection. Newer agents have been or are being evaluated in clinical trials as for example pentasaccharides, a molecule that is identical with the antithrombin binding site found in heparin, or fibrinogen-inhibitors (ancrod), direct thrombin inhibitors (hirudin), or tissue factor-factor VIIa (TF/FVIIa)-inhibitors (NAPc2). Some of these agents show promising first results such as in the prevention and treatment of venous and arterial thrombotic disease.

**Reocclusion after infragingual graft implantation and peripheral angioplasty**

Symptomatic PAOD has a prevalence of 2 to 3% in men and 1 to 2% in women above 60 years of age in Western Europe and North America. Among these, 70 to 80% will not get worse or even improve their state of disease, while 20 to 30% will suffer from progression of the disease within 5 to 10 years, necessitating an intervention. The treatment options are, firstly, conservative by walking exercise, secondly, a percutaneous transluminal balloon-catheter therapy (angioplasty, PTA), and thirdly, a surgical treatment by either thrombendarterectomy or implantation of an infrainguinal bypass. A number of patients, undergoing PTA or graft-implantation will suffer from reocclusion within one to five years. The patency rates of the dilated or revascularized arterial segments vary depending on the presence of risk factors, such as the localisation and length of the occlusion to be treated or of the implanted graft, the condition of the lower leg arteries (out-flow), graft-material, and the presence of other conditions such as
diabetes, gender (women have higher risks), compromised inflow (occlusion in the iliac arteries)\(^*\). Consequently, the question is, whether secondary prophylactic treatment with either antithrombotic or antiplatelet agents could improve patency rates of the treated arterial segments.

The three meta-analyses presented in Chapters 2, 3, and 4 that were performed in collaboration with the Cochrane Peripheral Vascular Disease Group, include all available randomised controlled trials comparing different antithrombotic or antiplatelet agents on the outcome of graft occlusion and reocclusion following PTA, but also amputation, cardiovascular death, and side effects.

**Part II**

*Two New Compounds in an Arterial and a Venous Thrombosis Model*

The second part of the thesis concentrates on two new compounds applied in *in vitro* models of arterial and venous thrombosis in the rabbit.

The first is a conjugate of a LMWH linked to autologous erythrocytes. By introduction of a sulfhydryl group to LMWH, the molecule gains the capability of binding to an amino group located on the erythrocyte membrane. This compound was tested *in vitro* with rabbit red blood cells and in an acute experiment of venous thrombosis in the jugular vein and for the evaluation of the half-life in rabbits. Evidence for its antithrombotic activity *in vivo* is presented in Chapter 6.

The second compound is a cyclic RGD-peptide conjugated to human albumin (HSA), which was developed in order to prolong its half-life (Chapter 7). RGD-peptides are potential inhibitors of platelet activation. The conjugate did not lose its antiplatelet inhibition potency *in vitro* and exhibited a 30fold prolongation in half-life *in vitro* compared with the free peptide. In addition, its platelet inhibitory property in an acute experiment of carotid artery thrombosis was as potent as ASA (Chapter 8).

**Part III**

*The influence of a functional thrombomodulin deficiency on coagulation and immunity*

This part of the thesis is dedicated to the study of a functional thrombomodulin deficiency as achieved in a genetically modified mouse. The animals present a dysfunction in the capability of binding thrombomodulin (TM) to thrombin. TM is a transmembrane glycoprotein of the endothelium, with a predominant presence in the microvasculature. The TM/thrombin complex accelerates the activation of protein C. Activated protein C by a proteolytic cleavage inactivates coagulation factors Va and VIla leading to impaired thrombin generation. The described mouse
model (TM<sup>pro/pro</sup>) leads to the creation of a prothrombotic state without a visible phenotype if unchallenged.

In humans, genetic mutations in the thrombomodulin gene were reported to be associated with an increased risk for myocardial infarction<sup>39,40</sup>. However, other reports could not confirm a clear association between arterial and venous thrombo-embolic diseases and the mutation. **Chapter 10** presents the results of a study investigating if a functional deficiency of TM influences thrombus formation in an acute experiment of arterial thrombosis of the carotid artery in TM<sup>pro/pro</sup> mice.

**Chapter 11** reports on E.coli peritonitis induced in TM<sup>pro/pro</sup> mice with the intention to test the hypothesis, whether an increase of systemic thrombin formation resulting from a reduced activation of the antiprotein C pathway would influence bacterial defense. In this experiment the importance of excluding genetic background differences between mouse strains in the susceptibility to bacteria is discussed as an additional topic. In a second project described in **Chapter 12** a different approach was used to find evidence for the hypothesis that a prethrombotic state might influence host defense in Gram-negative peritonitis. TM<sup>pro/pro</sup> mice infected intraperitoneally with E.coli were pretreated with a direct and an indirect thrombin inhibitor (low molecular weight heparin, hirudin, respectively) and compared to uncoagulated infected controls.
References

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