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

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Summary
Chapter 1 gives an outline of the thesis and describes briefly the various antiplatelet and antithrombotic agents used in the prevention of thrombosis in patients suffering from atherosclerotic disease such as myocardial infarction, stroke and peripheral vascular disease. In chapters two to four, meta-analytical studies are presented, that evaluate the optimal preventive treatment of patients with peripheral vascular disease, who had either undergone infrainguinal bypass surgery or balloon angioplasty.

In chapter 2 we evaluated whether antithrombotic treatment in patients with progressive atherosclerosis of the lower limbs undergoing infrainguinal bypass surgery improves graft patency, limb salvage and survival by performing a meta-analysis of randomized clinical trials. For each trial, the number of patients originally allocated to each treatment group and outcomes were extracted and pooled for an intention-to-treat analysis. An intention-to-treat analysis, which included three trials evaluating vitamin K antagonists (VKA) versus no VKA, suggests that oral anticoagulation favours venous, but not artificial, graft patency, as well as limb salvage and survival. Two other studies comparing VKA with aspirin (ASA), or aspirin/dipyridamole (ASA/DIP), supported a positive effect of VKA on the patency of venous but not artificial grafts. Randomized clinical trials with larger patient numbers comparing antithrombotic therapies with either placebo or antiplatelet therapies are needed in the future.

In the meta-analysis of chapter 3 we evaluated if antiplatelet treatment in patients with chronic peripheral arterial occlusive disease (PAOD) undergoing infrainguinal bypass surgery improves graft patency, limb salvage and survival. Data from all available randomized clinical trials comparing patients treated with one antiplatelet therapy to those treated with a placebo or another treatment were pooled for an intention-to-treat analysis. It was shown that the administration of platelet-inhibitors such as ASA, ASA/DIP, ticlopidine, or pentoxifylline, results in improved venous and artificial graft patency compared to no treatment. However, subgroup analysis for graft-type, i.e. venous versus artificial showed that patients receiving a prosthetic graft will profit more from ASA or ASA/DIP administration than those receiving a venous graft. Antiplatelet therapy with ASA has an inferior effect on venous graft patency compared with VKA. For further improvement of antiplatelet therapy in venous grafts, it might be worth, to combine aspirin and a thienopyridine, such as for example clopidogrel. In conclusion of chapter 2 and 3, patients operated on for an infrainguinal venous graft should be treated with VKA, whereas patients receiving artificial grafts might profit more from platelet inhibitors (aspirin).

In chapter 4 the efficacy of antiplatelet or antithrombotic agents on reocclusion was investigated in patients suffering from PAOD, who had undergone balloon angioplasty. In a meta-analysis including all randomized clinical trials meeting current methodological standards, treatment effect was compared of one antiplatelet agent versus a placebo or versus another antiplatelet agent or versus an antithrombotic agent or versus another therapy strategy. A 30 %
reduction of reocclusion was found with the administration of ASA/DIP compared to placebo at 6 months follow-up. Analysis of pooled data from four trials comparing high doses of ASA to low doses revealed that higher doses of ASA (300 to 1000 mg) do not improve patency rates following angioplasty at any timepoint of follow-up. One trial found that ASA/DIP improved patency after femoropopliteal angioplasty in contrast to VKA. The presented data are highly suggestive for antiplatelet agents to be the optimal therapy compared with no therapy or VKA in the described patient group.

The second part of the thesis concentrates on two new compounds applied in in vivo models of arterial and venous thrombosis in the rabbit. Chapter 5 is a systematic review of published studies containing experimental results obtained in venous thrombosis models in animals. The vast majority of publications concerned pharmacological studies. Most experimental animal models of venous thrombosis are based on similar principles, although animal species, methods of thrombus formation and outcome assessment may vary considerably. Animal models of venous thrombosis appear to play an important role in the study of venous thrombosis and the evaluation of antithrombotic properties of novel anticoagulant agents. However, dose-finding studies in experimental venous thrombosis models in animals and studies comparing the efficacy of different antithrombotic strategies should be interpreted with caution, since the outcome of these studies often inaccurately predicts the effect in clinical studies.

In chapter 6 a conjugate of low molecular weight heparin (LMW heparin) linked to autologous erythrocytes is described. This compound was tested in vitro with rabbit red blood cells and in vivo in an acute experiment of venous thrombosis in the jugular vein of New Zealand White rabbits. Evidence for its antithrombotic activity in vivo is presented in terms of reduction of thrombus growth and of thrombin generation. Radioactive labeling of LMW heparin-coated erythrocytes ('heparinocytes') and nuclear scanning showed presence of the cells in the circulation for at least 72 hours after intravenous administration. In conclusion, autologous heparinocytes maintain their antithrombotic properties in a rabbit in vivo model with a prolonged half-life compared to free LMW heparin.

In chapter 7 a cyclic RGD-peptide was conjugated to human albumin, in order to prolong its half-life. RGD-peptides are potential inhibitors of platelet aggregation. The conjugate did not lose its antiplatelet inhibition potency in vitro and exhibited a 30fold prolongation in half-life ex vivo compared with the free peptide. In chapter 8 the RGD-albumin conjugate was tested for its platelet inhibitory activity ex vivo in rabbit platelet rich plasma. In addition, the conjugate was evaluated in an arterial thrombosis model of the rabbit carotid artery using the ferric chloride method. Doses of 3.5 mg/kg and 7.5 mg/kg of the conjugate were administered intravenously before thrombus induction and compared to the effect of aspirin and to physiological saline. Ex vivo, platelet inhibition was still effective two hours following the acute experiment of thrombosis. Thrombus growth was prevented in 60% of the animals treated with the 7.5 mg/kg
of the conjugate, whereas a lower dose (3.5 mg/kg) failed to show an effect. In conclusion, RGD-albumin conjugate is still effective as a platelet inhibitor two hours after administration with a 20- to 30-fold prolongation of half-life compared to free peptide.

Part 3 of the thesis reports on experiments evaluating the influence of a functional thrombomodulin deficiency on coagulation and immunity in a genetically modified mouse model. These 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. After activation by the TM/thrombin complex, activated protein C proteolytically inactivates coagulation factors Va and VIIIa leading to impaired thrombin generation. A dysfunction in TM binding to thrombin, as achieved in the genetically modified 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. 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 chemically induced arterial thrombosis in the carotid artery of TM<sup>pro/pro</sup> mice. Complete occlusion as assessed in transonic flow measurements and histological analysis occurred in 76% of the TM<sup>pro/pro</sup> mice, while only in 19% of the wildtype controls.

Gram-negative sepsis is associated with acquired deficiencies of anticoagulant proteins, such as protein C, protein S, and antithrombin, and low levels of these proteins predict poor outcome of this disease. Administration of (activated) protein C improves survival in sepsis patients. These observations suggest that the activity of the coagulation system directly influences host defense. To investigate this, we performed the experiments described in chapter 11 where TM<sup>pro/pro</sup> mice and wildtype littersmates were inoculated intraperitoneally with E. coli. The outcome assessed were: signs of illness, bacterial counts, cytokine profiles in plasma, histological evidence for inflammation, and thrombosis. Coagulation activation was evaluated by the measurement of thrombin-antithrombin (TAT) complexes levels in plasma. The results showed comparable responses in mutant and wildtype littermates. The conclusion from this experiment is that in the present model of gram-negative peritonitis, impairment of the protein C pathway by dysfunctional thrombomodulin does not lead to impaired host defense.

In chapter 12, we further disturbed the coagulation system in the TM<sup>pro/pro</sup> mice by pretreatment with two thrombin inhibitors (hirudin and low molecular weight heparin, LMWH) just before intraperitoneal challenge with Escherichia coli. As controls we used inoculated TM<sup>pro/pro</sup> mice that did not receive antithrombotics. With one exception, a significant increase of bacterial outgrowth in peritoneal fluid, blood and liver of the anticoagulant treated mice was observed, in
parallel with an increase of IL-6 and TNFα levels in peritoneal fluid and blood. Female mice that were treated with LMWH formed the exception. The conclusion from this experiment is that thrombin inhibition in a prothrombotic mouse impairs host defense in a Gram-negative peritonitis, which further underlines a key role for thrombin in the crosstalk between the coagulation and the innate immune system.

Chapter 9 is a systematic review of published experiments performed in genetically modified mice used in thrombosis and hemostasis research. Over the last five years the interest into experimental research in thrombosis and hemostasis has considerably shifted from evaluating antithrombotic effects of new anticoagulant or antiplatelet compounds to questions of molecular functions of specific coagulation proteins. These models offer the opportunity not only to study the pathophysiological process of thrombosis and hemostasis but also open the way to interdisciplinary research. This is illustrated by demonstration of the crosstalk between coagulation and vascular development, coagulation and inflammation, coagulation and infection, and coagulation with underlying internal diseases such as diabetes mellitus, collagen disease, and sepsis, provided by transgene mouse models. However, this enthusiasm should not blind investigators to the fact, that explanations gained from successful transgenic mutations in mice mimicking human disease, usually cannot directly be translated to the human organism. The chance of obtaining phenotypes that are possibly due to gene interference or compensation rather than purely to the desired targeted mutation is an important pitfall. However, we will undoubtedly obtain clue information helping us to better understand a pathological process, or the network of relationships with other proteins into which a protein is imbedded. The more for example thrombus formation, has been investigated and compared in different mouse strains under comparable circulation conditions, the more will strain-related differences regarding susceptibilities to the applied model be ruled out. One way of avoiding such misinterpretation might be to obtain the same results in more than just one mutagen strain. In addition, an international definition on required background qualities, such as the number of back-crossings or of littermate preconditions needed, might enable clearer communication through published articles between research groups.