Abstract

Blood delivers oxygen and nutrients to all tissues and removes their waste products, through a large and complicated system of vessels. Under normal circumstances, blood is separated from the surrounding tissues by a single layer of endothelial cells that form the innermost layer of all blood vessels (the endothelium). When the endothelium is damaged, for instance by trauma, blood comes in contact with the tissues surrounding the endothelium. Subsequently several systems are activated to minimize blood loss as much as possible. The blood forms a clot and the vessels contract so that less blood flows to the damaged area.

In this dissertation a part of the blood clotting system was studied. By quickly forming a clot, a leak in the vessel wall can be sealed. When blood comes in contact with the tissue outside of the endothelium, blood platelets are activated, which subsequently form an initial clot to plug the leak. This blood platelet clot has to be reinforced with fibrin strands that are the product of the coagulation reaction (also known as the ‘blood coagulation cascade’). Without the reinforcement of the fibrin strands the blood platelet clot will not be strong enough to keep the leak closed.

When the endothelium is disrupted blood is exposed to tissue factor, a cofactor for activated factor VII, which is an enzyme that is always present in blood in small quantities. This initiates the coagulation reaction and results in the formation of thrombin and fibrin strands. Figure 1 in Chapter 1 shows an abbreviated scheme of these reactions. Normally, the coagulation reaction is in balance. On one side the reaction can be started (procoagulant) and on the other side the reaction can also be stopped (anticoagulant). This prevents too much clotting taking place, which unchecked can lead to occlusion of the vessel (thrombosis).

The whole coagulation reaction is very sensitive to the activity of the two procoagulant cofactors, activated factor V (factor Va) and activated factor VIII (factor VIIIa). The enzyme activated protein C (APC) functions as an anticoagulant and inactivates factor Va and factor VIIIa by cleaving them (the cleaving of a protein is also called proteolysis), which reduces the formation of thrombin and fibrin strands significantly. APC has two cofactors that accelerate these anticoagulant reactions, protein S and factor V (see Figure 1B in Chapter 1). This means that factor V plays a double role: either it is activated to form factor Va and acts as a procoagulant cofactor for factor Xa, or it is not activated and acts as an anticoagulant cofactor for APC.

The importance of factor V, factor VIII, protein S and APC is showcased in patients that have reduced concentration, or defects of one or more of these clotting factors. This can result in excessive bleeding, or excessive blood clotting, either of which can be a life threatening situation for the patient.
Abstract

Factor VIIIa inactivation by APC
The factor VIIIa molecule is very unstable. Immediately after activation factor VIIIa quickly loses its activity because part of this molecule, the A2 domain, spontaneously dissociates from the rest of the molecule. Apart from this fast auto-inactivation, factor VIIIa is also inactivated through proteolysis by APC. Factor VIIIa is cleaved by APC in two spots, after amino acid arginine 336 and after amino acid arginine 562. To study the inactivation of factor VIIIa by APC, a factor VIIIa variant was created in which the spontaneous dissociation of the A2 domain is prevented by linking the A2 and the A3 domain together with a disulfide bridge (M662C/D1828C factor VIII). With this factor VIII variant we were able to measure how quickly factor VIIIa was inactivated by the cleavages after arginine 336 and arginine 562, without interference of additional inactivation due to spontaneous dissociation of the A2 domain.

Chapter 2 shows that the cleavage after arginine 336 was faster than the cleavage after arginine 562. Also, factor VIIIa was not completely inactivated by the cleavage after arginine 336. On the other hand, cleavage after arginine 562 did fully inactivate factor VIIIa. Both protein S and factor V stimulated factor VIIIa inactivation in these experiments, and both stimulated the cleavage after arginine 562 more than the cleavage after arginine 336. Protein S was able to do this alone, but for the effect of factor V the presence of protein S was also required.

Factor VIIIa interaction with APC
To cleave factor Va and factor VIIIa, APC will have to get in contact with these proteins. On the surface of APC a large area has been identified where factor Va and factor VIIIa can bind. Figure 1 in Chapter 6 shows the 3D structure of the protease domain of APC, where this area is shown in color. There are several differences in which exact amino acids on APC are important for inactivation of factor Va and factor VIIIa, but overall it is the same area on the surface of APC that is involved in the interaction with both factor Va and factor VIIIa. Most of these important amino acids are basic and therefore this area has a net positive charge.

In Chapter 3 the importance of the ‘autolysis loop’ of APC (amino acids 306-314) for the inactivation of factor VIIIa was investigated. The autolysis loop also contains a large number of basic amino acids. It has been previously shown that several of these basic amino acids are important for the inactivation of factor Va. In Chapter 3 we showed that the autolysis loop was also important for the inactivation of factor VIIIa. However, not every amino acid was equally important for these two substrates. For the inactivation of factor VIIIa amino acids arginine 306, lysine 311, and arginine 314 played a major role, and lysine 308 had a minor role. For the inactivation of factor Va these amino acids played the same roles, but also glutamic acid 307 (minor role) and arginine 312 (major role) were important. The work from Chapter 3, together with other published studies that investigated other loops of the basic area on APC, also showed that the anticoagulant cofactors for APC (protein S and factor V) do not affect the function of the basic surface area on APC. They probably stimulate the inactivation of factor Va and factor VIIIa by binding to APC elsewhere.
Factor V as cofactor for APC in the inactivation of factor Va
Because protein S and factor V are cofactors for APC in the inactivation of factor VIIIa, and protein S is also a cofactor for APC in the inactivation of factor Va, it sounds logical that factor V may also be able to stimulate the inactivation of factor Va. In Chapter 4 the first evidence is presented that this is indeed the case. To investigate this we measured the clotting time of human plasma, under specific conditions where the clotting time was sensitive to the activity of factor Va, but was not sensitive to the activity of factor VIIIa. The clotting time was increased by adding the anticoagulant APC to the plasma, which indicated that factor Va was inactivated.

The clotting time of factor V-deficient plasma in this experiment was very long, and a titration of factor V in this plasma showed a decrease in clotting time at higher concentrations of factor V. However, when APC was added to the plasma the clotting time increased as more factor V was present. This meant that when more factor V was present, APC was more able to increase the clotting time, which indicated an anticoagulant function of factor V. This suggested that the anticoagulant form of factor V stimulated the inactivation of the procoagulant factor Va by APC.

Because plasma is a very complex environment, our goal was to also show the anticoagulant effect of factor V on the inactivation of factor Va by APC in a purified system. This work is presented in Chapter 5. By visualizing the cleavages in factor Va by APC we were able to measure if cleaving factor Va by APC actually happened faster when factor V was also present. For this factor Va was labeled with a fluorescent compound and was then subjected to proteolysis by APC and protein S. After electrophoresis of samples the gels could be scanned for fluorescence directly, which visualized the fragments of the labeled factor Va. Quantification and analysis of the intensity of the factor Va fragments showed a small increase of factor Va proteolysis when factor V was present. This effect however, was fairly small and did not fully explain the clear anticoagulant effect that was observed in the plasma clotting time experiments. Further research will be required to investigate if one or more factors were not present in this purified system, that were present in the plasma and that further enhanced the anticoagulant function of factor V.

Conclusion
Defects in the coagulation system can have serious consequences for patients. This system is highly complex and is controlled by many different enzymes that under normal circumstances balance the procoagulant and anticoagulant reactions. Regulation of factor Va and factor VIIIa activity by the anticoagulant enzyme APC plays a pivotal role in managing proper formation of blood clots. The research as presented in this dissertation provides us with better understanding of the anticoagulant function of APC. This new knowledge can be used to improve currently available therapeutics and form a basis to design novel intervention strategies in the field of haemostasis.