Lessons from snake venom: new insights into the structural and functional aspects of factor V and factor X

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Chapter 8

English summary
Nederlandse samenvatting

English summary

Coagulation of the blood, or simply blood clotting, is a vital process in which blood congeals to form a protective barrier to prevent the loss of blood. Formation of a blood clot is usually triggered upon damage to endothelial cells that line the wall of a blood vessel. Exposure of subendothelial collagen prompts the activation of blood platelets which aggregate into a plug that blocks off the damaged vessel wall. The damaged endothelium and activated platelets also recruit blood clotting factors to the site of injury. These clotting factors reinforce the platelet plug by promoting the formation of a protective mesh of fibrin polymers in and around the primary platelet plug. These polymers are produced by the blood clotting enzyme thrombin by cleaving fibrinogen into fibrin strands that can self-associate to form an intricate network of fibrin polymers. Controlling the enzymatic action of thrombin is key to maintaining hemostasis. For example, if the activity of thrombin is not correctly contained to the site of injury it could lead to indiscriminate fibrin formation and ultimately to a pathological and life-threatening condition known as thrombosis. On the other hand, insufficient thrombin activity could result in poor fibrin deposition and clot instability, ultimately leading to recurrent bleeding and debilitating physiological complications.

Thrombin is maintained in the blood in a quiescent pro-enzyme or zymogen form known as prothrombin. The conversion of prothrombin into thrombin is governed by an enzymatic complex known as prothrombinase. This complex is formed by two essential clotting factors: activated factor V (five) and activated factor X (ten); FVa and FXa in short. Both clotting factors are also maintained in a quiescent form and are activated upon vascular damage. After activation, FVa and FXa are only able to form a complex on surfaces of activated platelets and damaged endothelial cells, thereby limiting the activation of thrombin to the site of injury. In addition, several anticoagulant proteins circulate in the blood that can limit the action of thrombin, FVa and FXa, thereby controlling blood coagulation. Interestingly, the venom of the Australian common brown snake *Pseudonaja textilis* is known to contain a powerful prothrombinase-like enzyme complex that can convert prothrombin in the absence of activated platelets or damaged endothelium. This enzyme complex consists of 'weaponized' FVa and FXa homologs, which are evolutionarily adapted to derail the hemostatic system of its prey, leading to runaway coagulation. In this thesis, I have studied the structural and functional properties of the *P. textilis* venom FXa-FVa complex with the intention to harness its potent procoagulant potential for medical use.

The first part of this thesis is centered around the relationship between molecular modifications unique to the *P. textilis* venom FV molecule (ptFV) and their implications to FVa function. Under normal circumstances, FVa is stored in the

blood as an inactive molecule (referred to as FV) until coagulation is initiated. In humans, this inactive FV form consists of six subunits, three so-called A-domains, a large B-domain that regulates activity and two C-domains. Activation of FV requires removal of the B-domain segment through the enzymatic action of either thrombin or FXa. Previous studies into the ptFV molecule have shown that the regulatory B-domain segment is absent in the cDNA sequence of ptFV, leading to the expression of a consistently active FVa molecule. In **chapter 2** it was investigated whether this genetic feature is unique to the cDNA sequence of venom ptFV, or whether it is a general hallmark of FV in snakes. To investigate this, cDNA was isolated from the liver of 13 different snake species and analyzed using DNA sequencing. Two separate FV cDNA transcripts were discovered in each of the 13 snake species that were studied: a transcript with a full-length B domain sequence and a considerably shorter second transcript lacking the B domain sequence. In addition, a conserved pre-RNA splicing site was identified in the full-length B-domain sequence among most of the studied snake species. This discovery suggests that the B-domain segment may be removed during processing of the snake FV mRNA in the liver via alternative splicing. Thus, although the B domain has been preserved as a potential regulator of FV activity in snakes, current findings also indicate that in some snake species the function of the FV molecule may be altered by alternative splicing of the B-domain.

In **chapter 3** the function of the venom ptFV protein was studied at the molecular level by constructing new variants of ptFV and testing these for their ability to support prothrombin conversion. As mentioned, the *P. textilis* venom FXa-FVa complex can convert prothrombin in the absence of a procoagulant cellular lipid surface such as activated platelets. This ability is referred to as lipid-independent FVa function and it specifically allows the venom molecule to initiate blood coagulation throughout the body. It was investigated whether lipid-independent ptFV function stemmed from the presence of a unique covalent link between the A2- and A3-domains in ptFV. Such a covalent link could potentially stabilize the molecule and thereby facilitate FVa function in the absence of a lipid surface. Interestingly, the ptFV molecule that is expressed in the liver of the snake does not include this covalent link. However, functional analysis revealed that both the venom- and liver-derived ptFV molecules were able to support prothrombin conversion in the absence of lipids, irrespective of the presence or absence of the covalent link between the A2- and A3-domains in both ptFV species. Furthermore, a previous study had shown that the ptFV is functionally resistant to degradation by activated protein C (APC), which is an essential anticoagulant enzyme. In the current study it was also established that the covalent link was not essential to ptFV function after degradation by the anticoagulant enzyme APC.

In **chapter 4**, additional functional aspects of the venom ptFV molecule were examined by engineering hybrid variants (chimeras) of ptFV and human FV. To this end, two FV chimeras were created in which the A-domains of human FV were linked to the C-domains of ptFV, or vice versa. Functional analysis of these chimeras showed that the ptFV C-domains were of little relevance to its lipid-independent FVa function, as the chimera comprising the ptFV A-domains and human C-domains was also able to support prothrombin conversion in the absence of a lipid surface. In contrast, the chimera that comprised the human A-domains and ptFV C-domains was not able to support prothrombin conversion under similar conditions. Chapter 4 also investigated the relationship between the stability of the FV molecule and its ability to bind to lipid surfaces. It was established that the C-domain exchange adversely affected protein stability in ptFV, yet enhanced stability in human FV. Moreover, we found that lipid binding by the ptFV C-domains was enhanced when these were linked to the A-domains of human FV. On the other hand, lipid binding was reduced when the human C-domains were linked to the A-domains of ptFV. These findings indicate that the ability of the C-domains to bind lipids is coupled to the molecular stability of the entire FV molecule.

In the second part of this thesis, the structural and functional properties of the FXa molecule from snake venom (ptFXa) were studied. In **chapter 5** new findings are presented which show that the ptFXa enzyme is insensitive to inhibition of its catalytic activity by small molecular inhibitors of FXa, such as apixaban, rivaroxaban, and edoxaban. These synthetic FXa inhibitors are often prescribed as anticoagulant drugs to prevent or treat thrombosis. However, these drugs may also cause severe bleeding as serious side effect. Previous research has shown that the molecular structure of ptFXa differs from human FXa in several ways. Most importantly, the polypeptide chain constituting the “99-loop” is significantly longer in the serine protease domain of ptFXa compared to the homologous region in human FXa. The 99-loop is part of the so-called “catalytic site” of the enzyme and is important for the recognition of other molecules involved in blood clotting. In chapter 5, three hybrid variants of human FXa were generated in which the original 99-loop was replaced with the 99-loop from three different snake venom FXa species. These hybrid FXa variants were all found to be significantly less sensitive to inhibition by the synthetic FXa inhibitors, but otherwise functioned almost similarly to human FXa. In theory, these hybrid variants would thus make it possible to restore blood coagulation in patients using synthetic FXa inhibitors as anticoagulant drugs. One of these three hybrid FX molecules, FX-C, now known as VMX-C001, is currently in preclinical development as a drug against FXa inhibitors for patients who need restoration of blood coagulation, for example in event of an internal bleeding or before immediate surgery.

In **chapter 6** some of the preclinical research is described that was conducted for the development of VMX-C001. To identify a suitable animal model for the preclinical development of our new FX molecule, several animal plasmas have been tested using routine and special coagulation assays. It was discovered that inhibition of activated human FX was enhanced in the plasma of rabbits, rats, and especially mice. These plasmas are therefore less suitable for research with human FX. However, these new findings also highlight that there are species-specific differences that may inadvertently influence the interpretation of coagulation tests. Nevertheless, small animals are usually selected for at least part of the preclinical research. In general, rats may serve as a relevant animal model for the preclinical evaluation of VMX-C001 due to the sensitivity of its plasma in most coagulation assay.