Regulation of thrombin formation via the protein C pathway in normal and hypercoagulable states

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Regulation of Thrombin Formation via the Protein C Pathway in Normal and Hypercoagulable states

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ABSTRACT

Thrombin formation is a key event in the development of thrombotic disease. The protein C pathway is a major mechanism for regulating thrombin formation. In this review, we discuss the regulation of thrombin formation via the protein C pathway in normal and hypercoagulable states.

Key words: Thrombin, Protein C, Hypercoagulable states.

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Regulation of Thrombin Formation via the Protein C Pathway in Normal and Hypercoagulable states

PROEFSCHRIFT

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Studieknóbbelknee

Dao zag 'nen dokter "in spé":
"Studere? Dat hoof neet persé.
De loup vaan 't blood
Dee weit iech al good,
En 't hart hób iech oonder de knee!"

Fons Otten, 1988 in 'ABC in Mestreech'

Voor mijn moeder en vader,

Voor Silvie

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Abbreviations

α 2M	α 2 macroglobulin
α 2M-IIa	thrombin complexed to α 2 macroglobulin
Ala	Alanine
APC	activated protein C
APC-sr	APC sensitivity ratio
APTT	activated partial thromboplastin time
Arg	Arginine
ATIII	antithrombin III
BCA	bicinchoninic acid
BSA	bovine serum albumin
DNA	deoxyribonucleic acid
DOPC	1,2-dioleoyl- <i>sn</i> -glycero-3-phosphocholine
DOPE	1,2-dioleoyl- <i>sn</i> -glycero-3-phosphoethanolamine
DOPS	1,2-Dioleoyl- <i>sn</i> -glycero-3-phosphoserine
EDTA	ethylenediaminetetraacetic acid
ETP	endogenous thrombin potential
factor Va ^{R506Q}	abnormal factor Va in which Arg ⁵⁰⁶ is replaced by Gln
FPLC	fast protein liquid chromatography
FV	coagulation factor V
FVa	activated coagulation factor V
Fv _{int}	FVa cleaved at Arg ⁵⁰⁶
FVi	APC-catalyzed inactivated coagulation factor V
FX	coagulation factor X
FXa	activated coagulation factor X
Gln	Glutamine
Glu	Glutamic acid
HEPES	hydroxyethylpiperazineethanesulfonic acid
I2581	N-dansyl-(p-guanidino)-phenylalanine-piperidine hydrochloride
IIa	α -thrombin
K _{½Xa}	apparent K _d for the dissociation of the membrane-bound factor Xa-Va complex
k _{cat}	catalytic constant,
K _m	Michaelis constant
Mr	relative molecular weight
Mw	molecular weight
n-APC-sr	normalized APC sensitivity ratio

OC	oral contraceptives
PC	phosphatidylcholine
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
Phe	Phenylalanine
PL	phospholipid, phospholipid vesicles
pNA	<i>para</i> -nitroaniline
p-NPGB	p-nitrophenyl-p'-guanidino-benzoate hydrochloride
PPACK	D-Pro-Phe-Arg-CH ₂ Cl
Pro	Proline
PS	phosphatidylserine
PT	prothrombin
rAPC	recombinant human APC
RVV-X	purified factor X activator from Russel's viper venom
S2238	D-Phe-(pipecolyl)-Arg-pNA
S2366	L-pyroGlu-Pro-Arg-pNA
SDS	sodium dodecyl sulfate
SDS-PAGE	polyacrylamide gel electrophoresis in the presence of SDS
SEM	standard error of the mean
TF	tissue factor
V _{max}	maximal initial rate of substrate conversion expressed in terms of total amount of enzyme present

Chapter 1

Blood Coagulation, Factor V and Thrombosis

a general introduction to this thesis

1.1 Introduction

Haemostasis and Thrombosis

The vascular system in higher organisms constitutes the vital transport system between the various tissues and organs. This function is performed in cooperation with a pump and a carrier-medium, known to us as the heart and the blood, respectively. Poor maintenance of this system can be hazardous to the entire organism and vascular damage would cause fatal blood loss without a system that has the potency to repair the damaged blood vessels at the site of the injury: the haemostatic system.

Haemostasis is intimately associated with other host-defense systems such as the inflammatory response¹ and works in concert with physiological processes such as vasoconstriction in order to limit vascular damage and repair damaged bloodvessels.

Two prime events are essential to prevent blood loss after vascular injury: formation of an unstable primary platelet plug that is subsequently stabilized by the formation of a fibrin network²⁻³.

Haemostasis is capable of locally transforming the physical properties of the blood dramatically: an apparent fluid phase is changed into an apparent solid phase (clot). The haemostatic system thus also inherently possesses a dangerous capacity for clogging up the vessel system. Therefore, several mechanisms exist, that strictly control the process of clot formation and/or that can remove a clot once it has become redundant. It is generally believed that in healthy individuals in the absence of tissue injury, these pro- and antithrombotic processes, proceed simultaneously at a low, vigilant, level in a dynamic equilibrium that is shifted in favour of anticoagulation⁴.

In the case of thrombosis, a commonly observed pathological condition, it is hypothesized that loss of control over the haemostatic system induces a so-called hypercoagulable state that contributes at least in part to the pathogenesis of this disease⁵. As a result, undesirable formation of thrombi and their accumulation can lead to obstruction of the circulation. Due to obstruction ischemia occurs, which may cause necrosis or gangrene in underlying tissues and/or organs.

Both in normal haemostasis and in the pathological condition of thrombosis the generation of a proteolytic enzyme, thrombin, as well as the regulation of the functional activity of a cofactor protein, Factor V, play a pivotal role (see 1.2 and 1.3). Disproportionate activity of either protein can impose both a bleeding or a thrombotic tendency.

The following sections of this chapter contain information on some basic events in the blood coagulation process. The role of factor V (FV), especially its function in the control over and expression of procoagulant activities, will be discussed in more detail.

1.2 The Biochemistry of the Coagulation Cascade

As was stated in section 1.1 above, bleeding is put an halt to by the concerted actions of both platelets and plasma proteins. Platelet action and plasma protein activation are two inseparable processes, acting simultaneously both in temporal and spatial respects. Platelet action and function will be briefly discussed from a enzymological point of view (for excellent reviews on the histological/ cytological properties or signal transduction in platelets see refs.⁶⁻⁷).

Platelets

Platelets, or thrombocytes, are small cells that are abundantly present in human blood (3×10^{11} cells/l)⁸. Since most platelets circulate for 8-10 days in the blood, their number is constantly replenished by continuous production, presumably by the 'budding' of giant bone marrow precursor cells, the megakaryocytes⁹. Although platelets contain various cell organelles and granules, they are anucleate and are therefore sometimes considered as cell fragments, rather than entire cells.

Apart from their role in inflammation¹⁰, platelets play a major role in the arrest of bleeding. Upon exposure to subendothelial structures in damaged vessels (e.g. collagen or basement membrane), platelets are activated by a series of interactions, of which receptor-mediated interaction is most important. The result of this activation process is that platelets adhere to the subendothelium at the site of a vessel damage and simultaneously adhere to each other. By this mechanism they form a loose plug that prevents the loss of blood. In the course of platelet activation two further processes are important. Firstly, the phospholipid content of the platelet outer-membrane is changed by active and passive redistribution of phospholipids between the inner and outer leaflets of the platelet membrane. Most importantly, glycerophosphoryl-L-serine (PS) is exposed, which is essential for the binding of vitamin K dependent proteins of coagulation and the formation of enzymatically active membrane complexes involved in the haemostatic mechanism (see section 1.3 below)¹¹⁻¹².

Secondly, the contents of internal stores are released (α -granules, dense bodies) which contain coagulation factors and low molecular weight platelet activation/aggregation-agonists. The released molecules contribute to the initiation and maintenance of coagulation at the site of the injury. The physiological importance of this release is underscored by the fact that patients in which the release reaction is impaired or absent show a bleeding tendency (e.g. Gray platelet syndrome, Storage pool deficiency)⁸. Factor V_{Quebec}, a bleeding disorder which was for long ascribed to the qualitative deficiency in platelet FV, has recently been hypothesized to be associated with a general defect that leads to degradation (and therefore inactivation) of most proteins of the α -granules, including FV¹³. This again, stresses the importance of α -granule release for haemostasis.

General outline of reactions leading to thrombin formation

In 1964 it was recognized that the reactions of coagulation occur in an organized manner and are closely linked and sequential. Fibrin formation was hypothesized to be the result of a waterfall (cascade) of reactions¹⁴⁻¹⁵. Via a complex series of reactions, fibrin is formed from its soluble precursor protein, fibrinogen. For an accurate coagulation response the following properties of the cascade are required:

1. No fibrin deposition may take place under normal conditions
2. Response to injury must be fast
3. The response must be limited to the site of injury

Nature's elegant solution for these requirements embody the essential characteristics that make the clotting system so powerful. Clotting factors (indicated by Roman numbers I to XIII)¹ are plasma proteins circulating in the blood as *inactive precursor molecules*. Many of them are synthesized in the liver and in a number of cases vitamin K is required for the formation of functionally active proteins.

Initiation of coagulation is still a matter of debate. It is, however, generally accepted that exposure of tissue factor, a transmembrane glycoprotein that is located in many cell types including cells of the adventitia, to blood is essential for efficient triggering of coagulation¹⁶⁻²⁰. Since tissue factor is not expressed in intact endothelium, initiation of coagulation only occurs after vascular damage.

In most factors limited proteolysis converts the inactive profactor into an active form, which either is a non-enzymatic cofactor molecule or a serine

¹ By convention, the activated form of a coagulation factor is indicated by a small 'a' added to the roman number

protease. These activation reactions occur preferably on suitable phospholipid surfaces, i.e. membranes containing negatively charged phospholipids, such as PS. *In vivo* these surfaces are most likely presented by platelets or by small platelet particles, which expose negatively charged phospholipid molecules in their outer leaflet only *upon activation*. This mechanism ensures that activated platelet surfaces provide docking places for the assembly of enzyme complexes and that coagulation is *localized* to the site of injury (platelet). On these membranes, activated coagulation factors (serine proteases) form an active enzyme complex with a non-enzymatically cofactor molecule, that has an improved overall catalytic efficiency due to its organization at a surface (lowering of K_m). The importance of the cofactor presence needs to be stressed here, since their presence alone improves overall catalytic efficiency of the complex by 3 to 4 orders of magnitude. Newly formed proteases are on their turn capable of converting numerous other zymogen molecules into active clotting factors, thus resulting in an *amplification* of the clotting process.

Another way of amplification is the occurrence of positive feedback reactions in the coagulation cascade: many products promote their own formation by activation of other clotting factors acting 'earlier' in the coagulation process. The general outcome of all these processes is the obligatory massive formation of thrombin, the enzyme that is capable of transforming fibrinogen to its insoluble derivative fibrin. Since 1964 many researchers have shaped the cascade and a simplification of the current view on the blood coagulation cascade is given in Figure 1.1 below.

Fig 1.1 Schematic representation of the coagulation cascade. Solid arrows indicate proteolytic activations, clotting factors organized in ovals indicate membrane bound complexes of clotting factors. ⊕ indicates positive feedback reaction. 'TF' is tissue factor, 'F' stands for coagulation factor

Clinical relevance

The physiological relevance of the presence of the majority of the clotting factors is underscored by the association of a bleeding tendency with clotting factor deficiencies (it is only the FXII, prekallikrein- and high molecular weight kininogen-deficiencies that have not been related to excessive bleeding²¹). Historically, haemophilia A, a hereditary bleeding disorder, showed for the first time that deficiency of a coagulation factor (FVIII) can be associated with severe and sometimes lethal symptoms. Although partial deficiencies are mostly asymptomatic²², deficiencies of coagulation factors (<5-10% functional activities) are often accompanied by a variety of bleedings, including joint-, muscle- or retroperitoneal hemorrhages which can be life-threatening²³⁻²⁴.

Anticoagulant reactions counteracting thrombin formation

Two general mechanisms exist for the limitation of thrombin formation. These include 1) the actions of plasma inhibitors and 2) of proteases that convert active clotting factors into *inactive* products. These two systems are essentially different with the first being a constitutive and the second being a reactive inhibitory system. Inhibition by circulating plasma inhibitors results in the formation of enzyme-inhibitor complexes that have no biological activity. In contrast, each protease molecule catalyzes the inactivation of many clotting factor molecules. Moreover, the activity of the proteases is subject to control by their activation from inactive proenzymes and by their inhibition by plasma inhibitors. In both systems complex formation with cofactor molecules provides a mean of fine-tuning of activity and/or reactivity (e.g. heparin in case of plasma inhibitors or protein S for the activated form of the anticoagulant serine protease protein C).

Plasma inhibitors

Human plasma contains a number of inhibitors, fast- and slow acting, that are in a great surplus relative to the amount of active proteases they are to inhibit. Among these the serpins antithrombin III (ATIII), heparin cofactor II (HCII), protein C inhibitor (PCI) and α_1 -antitrypsin, as well as α_2 -macroglobulin (α_2 M), belonging to the α -macroglobulin family, are the most prominent. Inhibition by the serpins is fairly selective and reversible, although the dissociation rate of the complexes are so slow that for practical purposes inhibition is considered to be irreversible. The actions of ATIII and HCII are greatly potentiated by the presence of heparin. The inhibitory capacity of α_2 M is mechanistically different from the active-site directed serpins and shows

almost universal specificity. Binding of a protease to $\alpha 2M$ results in a conformational change following protease reaction, covalently anchoring the protease by transacylation, that appears to physically entrap the protease molecule and is therefore irreversible²⁵. The $\alpha 2M$ -protease complex retains activity towards small oligopeptide substrates (e.g. chromogenic substrates), but is almost completely inactive towards larger protein substrates ($>30kD$)²⁶. The major physiological inhibitors of thrombin, the central enzyme in haemostasis, are ATIII and $\alpha 2M$.

In recent years, interest in tissue factor pathway inhibitor (TFPI), a Kunitz-type protease inhibitor has been revived by many researchers. TFPI directly inhibits FXa and forms a complex with FXa that on its turn inhibits the FVIIa/tissue factor (TF) complex²⁷⁻²⁹.

The protein C pathway

Thrombin, when assembled after high affinity binding with the integral membrane protein thrombomodulin (TM), changes from a procoagulant into an anticoagulant protein. Binding to TM modulates the substrate specificity of thrombin. As a result, fibrinogen, platelets, factors V, VIII and XIII can no longer be activated³⁰⁻³². TM is present on the vascular surface of endothelial cells in almost all blood vessels and in a great variety of tissues (including the brain). Formation of the thrombin-TM complex not only diminishes the amount of available procoagulant thrombin but what is more important, this complex converts protein C, a vitamin K dependent zymogen with a normal plasma concentration of 48-81 nM, into activated protein C (APC)³³⁻³⁵. The activation rate of protein C by thrombin is increased by more than 4 orders of magnitude³⁶ upon binding of thrombin to TM.

Activated protein C is capable of proteolytically inactivating both the activated and non-activated forms of factors V and VIII, by cleavage of multiple peptide bonds. Another vitamin K dependent protein, protein S, serves as a cofactor for the inactivation of factors Va and VIIIa by APC. The cofactor activity of protein S is in its turn regulated by C4b-binding protein (C4BP), a multi-domain protein of the classical complement pathway. Protein S bound to C4BP (in 1:1 complex) lacks cofactor activity in the protein C pathway³⁶. Furthermore, APC is slowly neutralized *in vivo* by at least three protease inhibitors: the protein C inhibitor (PCI), $\alpha 1$ -antitrypsin and $\alpha 2$ -macroglobulin³⁷⁻⁴².

Recently, it has been shown by Shen et al. and by Varadi et al. that FV can act as cofactor for the inactivation of FVIIIa by APC in purified systems and in plasma respectively⁴³⁻⁴⁴.

APC-catalyzed inactivations of factors V(a) and VIII(a) occur in the absence of a phospholipid membrane. However, in the absence of phospholipids reaction rates are too slow to be considered important. The presence of a catalytic surface has an influence on both the order of consecutive peptide bond cleavages in factor V/Va and on the rate constants of these cleavages; stimulations of up to 1000-fold are reported by us (see chapter 3). Furthermore, it has been reported that incorporation of phosphatidylethanolamine into the lipid surface selectively enhances the FVa inactivation by APC⁴⁵.

Aside from their inactivation by APC, factors V and VIII have been reported to be cleaved by proteases such as plasmin and FXa. The physiological importance of these interactions have not yet been fully understood⁴⁶⁻⁴⁷.

Clinical relevance

The physiological relevance of the anticoagulant pathways is dramatically exemplified by the association of deficiencies of ATIII, protein C or protein S with thrombosis. In case of homozygous deficiencies, or combined heterozygous deficiencies, these thrombotic tendencies can be severe and the affected patients may develop *purpura fulminans* (a disease involving clotting of the microvasculature of the skin, and the uncontrolled coagulation generally leads to death in infancy unless treated)⁴⁸⁻⁴⁹. Recently, a hereditary disorder has been described, that is called APC resistance, that is associated with thrombosis and in which a DNA mutation renders FVa resistant to inactivation by APC (FV_{Leiden}⁵⁰, see section 1.5 below).

Since the formation of thrombin and the regulation of FV cofactor activity are topics of the research described in this thesis, the general concepts of thrombin generation will be described in more detail in the following sections.

1.3 The Prothrombinase Complex

Proteolytic activation of prothrombin is catalyzed by an enzyme complex (the prothrombinase complex, PTase) composed of the serine protease FXa that is noncovalently and reversibly associated with the cofactor FVa on membranes containing negatively charged phospholipids. The overall acceleration of prothrombin activation that is achieved by the assembled PTase, as compared to the activation by FXa alone, is >1,000,000 fold⁵¹.

Prothrombin

Human prothrombin (factor II, PT), the zymogen of the active products thrombin and meizothrombin, is a plasma glycoprotein that has a molecular weight of 72 kD and a plasma concentration of $1.48 \mu\text{M}$ ⁵². Prothrombin synthesis primarily occurs in the liver and the resulting protein shows several types of structural motifs or domains: fragment 1 (F1) and fragment 2 (F2) and the proteinase module (see fig 1.2 below). Synthesis of biologically active F1 domain is dependent upon the presence of vitamin K, which acts as a cofactor in the post-translational γ -carboxylation of 10 Glu residues in the F1 domain into Gla residues⁵³. The Gla-residues function in the Ca^{2+} -dependent binding of PT to negatively charged phospholipids.

Figure 1.2 **Schematic representation of prothrombin and several of its activation products.** Cleavage sites by FXa are indicated by solid arrows, the cleavages catalyzed by thrombin are indicated by the open arrows. Enzymatically active products are indicated in italics.

Recently it was shown that PT, apart from being the substrate for the PTase complex, actively contributes to the assembly of this complex⁵⁴.

Factor Xa

Factor Xa is a vitamin K dependent heterodimeric serine protease with a molecular mass of 45 kD that is formed after activation of its 59 kD plasma precursor factor X⁵⁵, which has a plasma concentration of $\sim 170 \text{ nM}$ ⁵⁵. FXa is generated from its inactive zymogen, FX, by proteolytic removal of a 52 amino acid activation peptide⁵⁵ by either an extrinsic (TF-FVIIa complex) or an intrinsic FX-activating complex (FIXa-FVIIIa complex). FXa consists of a 27 kD heavy chain and a 18 kD light chain that are linked via a disulfide bridge⁵⁶⁻⁵⁷. While lipid binding is considered to be mediated by the 11 γ -carboxyglutamic

acid residues in the Gla-domain⁵⁷, located in the N-terminal part of the light chain of FXa, interaction with FVa and substrate (PT) are thought to be mediated by regions located in the heavy chain domain of FXa, that also contain the His-Asp-Ser catalytic triad⁵⁸.

Phospholipid Surface

Although FVa and FXa can assemble in the absence of a phospholipid surface (PL) and can activate PT, assembly of a fully functional PTase requires the presence of a suitable membrane surface. The phospholipid membrane adds the protein-lipid interaction to the protein-protein and protein-metal ion interactions already present in the FVa-FXa complex in free solution. As an overall result the K_d for the FVa-FXa interaction drops from $\sim 10^{-5}$ - 10^{-6} mol/l to 10^{-9} mol/l in the presence of a membrane surface^{51,59-62}. However, not only the enzyme and its cofactor bind to the PL-surface, but also the substrate, PT which contains a Gla-domain (see above).

It is generally accepted that for efficient lipid activity the polar headgroup of a phospholipid molecule needs to contain a net negative electric charge. Factors, other than the polar headgroups that influence the activity of lipid membranes in prothrombin activation are the organization of the phospholipids (e.g. small unilamellar vesicles, or monolayers, macroscopic surfaces), the membrane fluidity (largely dependent upon the kind of fatty acyl side chains and temperature) and the stereochemical configuration of the phospholipid headgroup⁶³⁻⁶⁷.

In vivo, the procoagulant surface for PTase assembly and function is most likely provided by the PS that is exposed on the outer membranes of activated platelets⁶⁸. Endothelial cells⁶⁹, viruses⁷⁰ and monocytes/macrophages^{19,71-73} have however also been hypothesized to provide surfaces for haemostatic and thrombotic processes. Furthermore, *in vitro* experiments show that microvesicles, that are released from the platelet membrane upon activation and that also contain PS, might form a suitable surface for *in vivo* PT activation⁷⁴⁻⁷⁶. Reports on the physiological importance of procoagulant microvesicles are contradictory however (see ref. ⁷⁷).

Factors V and Va

Factor Va is the heterodimeric non-enzymatic cofactor that is formed after activation of its inactive pro-cofactor FV (activity < 0.27%, ⁶⁰) by limited proteolysis (see also section 1.4 below). FV is a 330 kD single chain

glycoprotein with a plasma concentration of $\sim 21 \text{ nM}$ ⁷⁸. It is synthesized mainly in the liver and in megakaryocytes where it is being transcribed from a 6672 base pair stretch of DNA located in the chromosome 1q21-25⁷⁹. The resulting procofactor consists of 2224 amino acids, which includes a 28-amino acid leader peptide, that after translation subsequently undergoes post-ribosomal modification. These include at least glycosilations⁸⁰⁻⁸¹, phosphorylations⁸² and sulphations⁸³⁻⁸⁴. The FV molecule shows a distinct multi-domain structure that resembles that of FVIII (see fig 1.3 below).

Figure 1.3 Schematic diagram of the multi domain structure of FV. Cleavage sites at Arg⁷⁰⁹, Arg¹⁰¹⁸ and Arg¹⁵⁴⁵ are indicated by open arrows^{refs.85-88}. Closed arrows indicate the reported cleavage sites for the inactivation of Factor V by activated protein C^{ref.89}. 'Y' indicates the presence of carbohydrate side chains in the A1-A2 domain, the fragment Thr¹⁰¹⁹-Arg¹⁵⁴⁵ of the B-domain and in the light chain^{refs.80-81}. Open circles indicate phosphorylation within the C-terminal part of the heavy chain^{ref.82}, filled circles indicate sulphations in the heavy chain region and in the Thr¹⁰¹⁹-Arg¹⁵⁴⁵ fragment.

The homology between the A1, A2, A3, C1 and C2 domains of FV and the corresponding domains in FVIII is $\sim 40\%$ on basis of the amino acid sequence. The B-domains of FV and FVIII show no homology⁹⁰. The A-domains of FV further show $\sim 40\%$ amino acid sequence homology with the three highly conserved domains in ceruloplasmin, a plasma copper-binding transport protein. It has been suggested that FV, FVIII and ceruloplasmin form a family of structurally related proteins that stem from a common ancestral gene⁹¹.

Human FVa consists of a 105 kD heavy chain (A1-A2, fig.3) and a 71-74 kD light chain (A3-C1-C2, fig. 3) that are noncovalently linked by a calcium ion⁸⁸. Since two different forms of the light chain region exist, FVa can occur in two forms in plasma, named FVa1 and FVa2. FVa1 and FVa2 differ in their binding properties to phospholipid membranes⁹², the basis for this

heterogeneity has been shown to be resulting from differential glycosylation of Asp²¹⁸¹ in the C2 domain (see fig. 1.3, ref.⁸¹). The heavy chain domain of the FVa molecule is largely responsible for the interactions with PT⁹³⁻⁹⁶, while several patches of the light chain, located near the C-terminal end, have been shown to be involved in phospholipid binding⁹⁷⁻¹⁰¹. It is noteworthy to mention that the C domains of FVa are approximately 70% identical to the duplicated C domains present in a murine breast epithelial cell protein of unknown function¹⁰². Both heavy and light chain contain regions that mediate interaction with FXa¹⁰³⁻¹⁰⁴.

The large B-domain that is released from the FV molecule on thrombin-catalyzed activation was considered to fulfill no more functional role than separating the heavy and light chains until recently. It has been shown by Kaufman and coworkers that efficient thrombin activation of FV requires the presence of an acidic amino acid rich regions within the B-domain¹⁰⁵. Furthermore, Lu et al. concluded that a portion of the B-domain is involved in the FV cofactor function for FVIII inactivation by APC¹⁰⁶.

Apart from circulating in the blood, ~25% of the FV contained in the whole blood is present in platelet α -granules and can be secreted along with other α -granule proteins upon activation of the platelet^{73,78}. A physiological necessity for the presence of platelet FV has not yet been shown, although it has been reported that platelet FV can support thrombin generation in patients with acquired immune inhibitors of plasma FV¹⁰⁷⁻¹⁰⁸. Platelet FV is stored as a complex with multimerin, a 155 kD specific binding protein of the light chain domain of FV(a). A possible role for multimerin as a carrier protein for FV in platelets has been suggested. Regulation of platelet FVa activity might differ from that of its plasma equivalent¹⁰⁹⁻¹¹⁰.

Integration of Interactions in the Prothrombinase Complex

What is it that makes the prothrombinase complex so efficient and the physiologically most relevant activator of PT? An insight into this can be effectively demonstrated by evaluating the influence of the individual components of the complex on the kinetic parameters of PT activation.

Activating Component	K _m (μ M)	V _{max} (mol IIa s ⁻¹ * (mol Xa) ⁻¹)	k _{cat} / K _m M ⁻¹ * s ⁻¹	Time to convert 1 PT molecule
Xa	84	0.011	131	1 hr 26 min 24 s
Xa-Va	34	6.22	1.83*10 ⁵	3.8 s
Xa-PL	0.06	0.038	6.33*10 ⁵	27.4 s
Xa-Va-PL	0.21	32.0	1.52*10 ⁸	0.036 s

Table 1.1 ***Kinetic properties of the prothrombinase complex.*** (Data adapted from references.^{51,111}).

In table 1.1 it is shown that FXa alone has only a limited potency to activate PT. Addition of FVa alters the maximum capacity with which FXa activates PT. Addition of phospholipid alone, lowers the K_m for PT activation some ~1400 times, whereas the combined addition of PL and FVa joins the beneficial effects brought about by each component alone and simultaneously lowers the K_m for prothrombin activation and increases the maximum turnover rate (k_{cat}) of the enzymatic complex. It is conceivable that each component contributes to the assembly, positioning and proteolytic activity of the PTase complex so that a more than million-fold increase in catalytic efficiency can be achieved.

The concept of the PTase, an enzyme-complex that is fully active only after the assembly of enzyme, cofactor and substrate at a phospholipid surface, further ensures a localization of thrombogenic response: complex assembly occurs only on suitable membranes and the catalyst is concentrated at the site of the injury. Also, through feedback reactions that influence the assembly and expression of activity in PTase, the complex can be modulated in various manners⁵⁹.

1.4 Control of Factor V Cofactor Activity

Since FV has an extremely low cofactor activity⁶⁰, activation to its active product is a prerequisite for the expression of prothrombinase activity. In fact, during in vitro coagulation, FV activation is the rate-limiting factor in prothrombin activation¹¹².

activation of the procofactor, Factor V

FV can be activated by thrombin, as well as by FXa via multiple cleavages at positions Arg⁷⁰⁹-Ser⁷¹⁰, Arg¹⁰¹⁸-Thr¹⁰¹⁹ and at Arg¹⁵⁴⁵-Ser¹⁵⁴⁶. Apart from these activations, a specific non-fysiological FV activator from Russels viper venom (RVV-V) can be used to activate FV (see fig 1.4 below^{86,88,113,114}).

Figure 1.4 Activation of factor V via limited proteolysis. Schematic representation of the thrombin, FXa and RVV-V mediated cleavages of FV during activation of the procofactor molecule. Small arrows mark the positions of the proteolytic cleavages at the indicated scissile bonds. Fragment molecular masses are given. Forms of FVa that contain the 72 kD or the 220 kD fragment express cofactor activity.

Whereas FXa catalyzed activation is lipid-dependent, activation by thrombin is not. From the k_{cat} / K_m values it is concluded that FXa is as efficient as thrombin in activating FV⁸⁶. Thrombin-activated FVa expresses the same cofactor activity as FXa-activated FVa. As can be deduced from the domain structures of FV, FXa and thrombin (FVa and FXa contain phospholipid binding domains, while thrombin does not), FXa-catalyzed activation of FV is greatly stimulated by the presence of a phospholipid surface whereas thrombin-catalyzed activation occurs both in the presence and the absence of a phospholipid surface⁸⁶.

Downregulation of Factor V cofactor activity

Although FVa is proteolysed effectively by plasmin⁴⁶ and FXa⁴⁷, it is generally thought that proteolytic inactivation of FVa (and FV) under physiological conditions is regulated by activated protein C (APC, see section 1.2). APC inactivates FVa via three cleavages in the heavy chain region of the cofactor at Arg³⁰⁶, Arg⁵⁰⁶ and Arg⁶⁷⁹ respectively, resulting in loss of cofactor activity^{89,115}. No cleavage of the light chain is observed⁸⁹. APC-catalyzed inactivation of FVa is stimulated by the presence of negatively charged membrane surfaces¹¹⁶⁻¹¹⁸ and by the vitamin K dependent cofactor Protein S¹¹⁸⁻¹²⁰. Recently, we have shown that in a purified protein system the

presence of FXa selectively inhibits the APC-catalyzed cleavage at Arg⁵⁰⁶, an inhibition that is independent of the presence of a catalytic triad in FXa¹²¹. In the same report it was shown that protein S selectively enhances cleavage at Arg³⁰⁶ some 20-fold.

1.5 APC resistance

original observations

In 1993, laboratory findings by Dahlbäck and coworkers indicated a poor anticoagulant response to APC in the plasma of a group of patients with a family history of familial thromboembolic disease¹²²⁻¹²³. The phenomenon was termed 'APC resistance' and it was recognized to be an important cause for familial thrombosis, since it is found in 20-60% of patients documented with idiopathic thromboembolism. It was proposed that patients that were APC resistant, were missing a cofactor of the APC anticoagulant system. Similar findings by Griffin et al.¹²⁴ and Koster et al.¹²⁵ in the same year 1993 also showed the diminished response to APC in plasma and established the abnormality in the anticoagulant protein C pathway. In February 1994 Dahlbäck and Hildebrand showed that *in vitro* APC resistance could be corrected by the addition of purified FV, thereby identifying FV as a cofactor for APC¹²⁶. The 'cofactor' theory could explain earlier observations from plasma experiments which showed that the impaired APC response in plasma of APC-resistant individuals could be corrected with normal plasma¹²⁷.

Hereditary Factors Causing APC Resistance

Shortly after the initial reports by Dahlbäck et al. and their suggestion of a hereditary nature for the defect¹²³, Bertina et al. showed⁵⁰, simultaneously with several other laboratories¹²⁸⁻¹³⁰, that APC resistance is linked to the occurrence of a G→A point mutation in the gene for FV (FV_{Leiden}). This causes the replacement of Arg⁵⁰⁶, located at an APC cleavage site, to Gln and as a result APC cannot cleave FVa at the 506-position. The mutation was estimated to occur in ~4% of the Dutch population^{50,125}, a percentage that was confirmed for several other western countries¹³¹. This occurrence rate is 10 times higher than that of the sum of all other known hereditary causes of familial thrombophilia. Epidemiological studies have shown that heterozygosity for the FV_{Leiden} mutation results in a ~7 fold increase in the risk of thrombosis, homozygosity in a 50-100 fold increase^{125,132}. In over 95% of APC-resistance cases, this molecular cause of resistance to APC is found¹³².

A general mechanism for the occurrence of thrombosis has not been reported so far. It is likely though that as well a) hampered downregulation of FVa activity, due to the R506Q mutation, as b) the reported anticoagulant cofactor activity of FV in the APC-catalyzed FVIIIa-inactivation^{43,133}, which is lower in case of FV_{Leiden}, are of major influence in the development of thrombosis in APC resistant individuals. Homozygous APC resistance appeared to be a mild risk factor for venous thrombosis compared with homozygous and heterozygous protein C and protein S deficiency. Therefore, it was deduced that multiple manifestations of heretic defects besides the FV_{Leiden} mutation ('double hits') play an important role in the manifestation of thrombosis related to the FV_{Leiden} mutation.

Thromboses that develop generally are venous by nature. Several investigators however, have associated APC resistance with the development of stroke and arterial thrombosis¹³⁴⁻¹³⁷, whether this holds true remains to be clarified.

Acquired APC Resistance

As detection of functional APC resistance requires plasma testing, reduction of clotting factors is likely to interfere with the outcome of an APC resistance assay and hence constitutes a problem to clinical laboratory testing. This is particularly true in the case of use of oral anticoagulant drugs or heparin which largely interfere with functional tests for APC resistance. When testing for APC resistance via functional assay systems, several different plasmas give assay outcomes which would qualify the plasmas tested as stemming from APC resistant individuals.

The presence of such 'acquired APC resistant' factors are generally observed in plasmas containing anti-phospholipid antibodies or lupus anticoagulants. Other known (hereditary) causes, resulting in the phenotype of APC resistance include protein S deficiency or protein C deficiency (depending on APC resistance assay).

Another acquired type of APC resistance is encountered in healthy women that are pregnant, or that are on contraceptive (OC) therapy. Assay outcomes for these groups of women have been reported to overlap with heterozygous FV_{Leiden} carriers. To some extent, both conditions (pregnancy, OC use) present hormonal and metabolic similarities, and both have reported influence on the haemostatic system which in general shifts in the direction of both an increased prothrombotic tendency and increased fibrinolytic activity¹³⁸⁻¹⁴². The net outcome of these changes in the haemostatic system are thought to balance each other. However, thromboembolic disease is a leading cause of

obstetric morbidity and mortality and accounts for a major percentage of maternal deaths¹⁴³. Recent (revived) concern about the association of OC use with thromboembolic disease (in particular those containing the third generation progestagens desogestrel, gestodene and norgestimate) has been a great impulse to retro- and prospective studies on this subject. The molecular etiology of the correlation between pregnancy/OC use and thrombosis remains, however, to be clarified.

For all of the above mentioned acquired causes of APC resistance modification of functional assays or use of DNA-based assay systems have so far overcome all of these false positive APC resistances. Whether a false-positive APC resistance test outcome reflects merely an interference with the APC resistance assay or gives information on the *in vivo* development of thrombosis in APC resistant patients is not known. Using this shortcoming of functional testing, several new assays have been developed that are intended to be sensitive for more than one component of the protein C pathway.

Shortly after the onset of the work that is described in this dissertation in 1993, Dahlbäck and coworkers firstly described the APC resistance. Since in our laboratory FV regulation in general and APC catalyzed inactivations in particular were subjects of investigation, research focused on the APC resistance using model systems with purified proteins, as well as in plasma-based systems. This thesis describes the research that was performed in this, for 'Factor V researchers', turbulent times in the period March 1993 to March 1997. It can roughly be divided into three parts with the chapters 2 and 3 describing the activation and inactivation mechanisms of human FV in purified protein systems and the chapters 5 and 6 describing downregulation of thrombin formation by APC in plasma-based systems, with chapter 4 being an intermediate between the model- and the plasma-based systems.

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Chapter 2

Activation of Human Factor V by Meizothrombin

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2.1 Summary

A recombinant human prothrombin was prepared in which Arg¹⁵⁵ was replaced by Ala. The recombinant prothrombin was converted into a meizothrombin derivative (R155A meizothrombin) that was resistant to autocatalytic removal of the fragment 1 domain. R155A meizothrombin appeared to be a potent factor V activator in reaction mixtures that contained negatively charged phospholipid vesicles. Factor V activation by R155A meizothrombin was characterized by a second order rate constant of $0.06 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ in the absence of phospholipid and $18 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ in the presence of $60 \text{ }\mu\text{M}$ phospholipid vesicles composed of a 10/90 (mol/mol) mixture of phosphatidylserine (PS) and phosphatidylcholine (PC). The rate constant for thrombin-catalyzed activation of factor V was hardly affected by the presence of phospholipid vesicles and was $4.0 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$. The initial rate of activation of 3 nM factor V by R155A meizothrombin was a function of the concentration of PS/PC vesicles present in the reaction mixture and the calculated rate constant reached a plateau value at $[\text{PS/PC}] \geq 50 \text{ }\mu\text{M}$. Gel electrophoretic analysis of factor V activation showed that R155A meizothrombin and thrombin cleaved the susceptible peptide bonds in factor V at different rates. However, both activators finally generated a factor Va molecule composed of a heavy chain with an $M_r = 104,000$ and a light chain doublet with M_r values of $74,000$ and $71,000$. Since meizothrombin is one of the major reaction products formed during the initial phase of prothrombin activation, these findings are indicative of a significant contribution of meizothrombin to *in vivo* factor V activation.

2.2 Introduction

Human blood coagulation factor V is a single chain glycoprotein^{1,2} that, during haemostasis, is converted into factor Va via limited proteolysis by enzymes (e.g. thrombin and factor Xa) generated after the initiation of blood coagulation¹⁻⁶. Factor Va is the non-enzymatic cofactor of the prothrombin-activating complex, which also comprises the serine protease factor Xa, calcium ions and a procoagulant membrane surface. Depending on the reaction conditions, factor Va accelerates prothrombin activation 10^3 - 10^5 -fold⁷⁻⁹.

The important function of factor Va in haemostatic plug formation is further underscored by the observation that, during *in vitro* coagulation, factor Va is the rate-limiting factor in prothrombin activation¹⁰ and by the fact that the activity of factor Va is efficiently regulated by activated protein C¹¹. Since

factor V is unable to act as a cofactor in the prothrombinase complex⁷, activation of factor V must be a key event in thrombin formation.

Here we report that meizothrombin, a reaction intermediate that accumulates during the initial phase of prothrombin activation¹²⁻¹⁵, may be an important factor V activator. Meizothrombin exhibits full activity on chromogenic substrates, but compared with thrombin it has a reduced activity on macromolecular protein substrates^{12,16}. Studies on the catalytic activities of meizothrombin are hampered, however, by the fact that it is autoproteolyzed to meizothrombin(desF1)^{12,17} or thrombin¹⁷. In this chapter the preparation of a recombinant human prothrombin is described in which Arg¹⁵⁵ is replaced by Ala. The recombinant prothrombin was converted into meizothrombin, which, due to the amino acid substitution, could not be converted to meizothrombin(desF1) and which was not measurably autolyzed to thrombin. This meizothrombin derivative is a powerful factor V activator in the presence of phospholipids. Since its activity can even exceed that of thrombin, activation of factor V by meizothrombin may be of prime importance for the initiation of prothrombin activation.

2.3 Materials and Methods

Materials

Ovalbumin, BSA, bovine brain PS and egg-yolk PC were purchased from Sigma. DOPC and DOPS were obtained from Avanti Polar Lipids (Pelham, AL). Small unilamellar phospholipid vesicles were prepared as described before¹⁸. S2238 (D-Phe-pipecolyl-Arg *p*-nitroanilide) and I2581 (N-dansyl-(*p*-guanidino)phenylalanine piperidide hydrochloride) were supplied by Chromogenix. Materials used for protein purification were purchased from Pharmacia Biotech Inc. The adenovirus transformed human kidney 293 cell line (ATCC CRL1573) was from Flow Laboratories, Inc. *Escherichia coli* 1061/P3 and plasmid pcDNA1 were from Invitrogen. Iscove's and Optimem1 medium were from Life Technologies, Inc. Vitamin K₁ was from Hoffman-La Roche. Human coagulation factors were prepared by earlier described procedures^{14,18}. The prothrombin activator from *Echis coloratus* was purified from the crude venom (Latoxan) by gel permeation chromatography on Superdex 200 and ion-exchange chromatography on Mono Q and Mono S. The venom prothrombin activator was applied to a Mono Q in 50 mM Tris (pH 7.9), 100 mM NaCl, 0.1 mM CaCl₂ and to a Mono S in 25 mM-Hepes (pH 7.7) 50 mM NaCl, 0.1 mM CaCl₂ and eluted from the columns at NaCl

concentrations between 150 and 200 mM. Details of the purification procedure will be described elsewhere.

Cloning of prothrombin and construction of R155A prothrombin

Full-length prothrombin cDNA was obtained as described before¹⁹. Mutagenesis was performed by the polymerase chain reaction overlap extension technique²⁰, using two overlapping oligonucleotides (5'-CGATGACTCCAGCGTCCGAAGGCTCC-3' and 5'-GGAGCCTTCG-GACGCTGAGATCATCG-3' (mutated sequence is underlined)), resulting in the replacement of Arg¹⁵⁵ with Ala. The mutated fragment was used to substitute the wild type *XhoI*-*Bst*EII fragment of prothrombin in pcDNA1 and the DNA sequence was confirmed for the entire fragment.

Expression of recombinant prothrombin

The prothrombin cDNAs obtained from the pcDNA1 plasmid by digestion with *Eco*RI and *Xba*I were inserted into the *Bcl*I site of expression vector p-GTh (a gift of Dr. B.W. Grinnell, Lilly Research Laboratories, Indianapolis, USA). This vector enables high expression levels in the adenovirus-transformed human kidney cell line 293 and selection of positive clones with hygromycin B since the vector contains the cistron for expression of hygromycin phosphotransferase.

Cells were grown in Optimem1 medium supplemented with 5% fetal calf serum and 10 µg/ml vitamin K₁. The cells were transfected and colonies were selected as described by Walls et al.²¹. The selected cell line was grown in 175-cm² culture dishes in the medium described above. After reaching confluency, the cells were kept serum-free in Optimem1 with 10 µg/ml vitamin K₁ and the medium was collected and refreshed each 72 hr during 8 weeks. The medium was centrifuged for 20 min at 14,000 x g and the supernatant was stored at - 80°C.

Purification and characterization of R155A prothrombin

In a typical purification, 0.5 L medium (to which 0.5 mg/ml ovalbumin was added to minimize protein losses during the first purification step) was loaded at 4°C on a 5-ml fast flow Q-Sepharose column. The column was washed with 100 ml 50 mM Tris (pH 7.5 at 4°C) and 150 mM NaCl, and R155A prothrombin was eluted with a 100-ml linear gradient of 150-1000 mM NaCl in the same buffer. R155A prothrombin-containing fractions were pooled and

diluted 5-fold in 50 mM Tris (pH 7.5 at 20°C) and applied to a Mono Q column (HR 5/5) connected to a Pharmacia FPLC system. R155A prothrombin was eluted with a 30-ml linear gradient of 100-1000 mM NaCl in the same buffer. R155A prothrombin containing fractions were pooled, dialyzed against 25 mM Hepes (pH 7.5) and 175 mM NaCl at 4°C, and stored at -80°C. R155A prothrombin appeared as a closely spaced doublet on non-reduced gels and as a single smeared band on reduced gels (Fig. 2.1). R155A prothrombin was fully carboxylated as determined by the method of Kuwada and Katayama²². Concentrations of R155A prothrombin were determined after complete activation with *Echis carinatus* venom and by enzyme-linked immunosorbent assay using a biotinylated rabbit polyclonal antibody to human prothrombin. Antigen/activity ratio's, initial rates of activation by the prothrombinase complex, and final levels of thrombin obtained were the same for R155A prothrombin and purified human plasma prothrombin.

Fig. 2.1 Gelelectrophoretic analysis of R155A prothrombin and R155A meizothrombin. R155A prothrombin (2.64 μ M) was incubated at 37 °C with 65 ng/ml purified prothrombin activator from the venom of *Echis coloratus* in a reaction medium containing 25 mM Hepes (pH 7.5), 175 mM NaCl, 5 mM CaCl_2 and 20 μ M I2581. After 4 min the activation mixture was applied to a Mono-S column (HR5/5) that was equilibrated with 25 mM Hepes (pH 7.7) and 50 mM NaCl. R155A meizothrombin passed through this column and I2581 was added to the meizothrombin-containing fractions to a final concentration of 20 μ M. The R155A meizothrombin preparation was stored at -80 °C. The gel represents SDS-PAGE analysis of: 1) R155A prothrombin, 2) R155A meizothrombin, 3) molecular weight standards, 4) R155A prothrombin + 5% β -mercaptoethanol and 5) R155A meizothrombin + 5% β -mercaptoethanol.

Protein concentrations

R155A meizothrombin concentrations were determined with S2238, considering that the amidolytic activity/mole of R155A meizothrombin is the same as that of thrombin^{12,16}.

Factor Va was quantitated by determining the rate of factor Xa-catalyzed prothrombin activation in reaction mixtures that contained a limiting amount of factor Va and saturating concentrations phospholipid vesicles (50 μ M PS/PC, 10:90, M/M), factor Xa (5 nM) and prothrombin (0.5 μ M)¹⁸. The inhibitor I2581 (20 μ M) was present to prevent further activation of factor V by thrombin generated in the assay mixture. I2581 did not interfere with the subsequent thrombin determination since it is a reversible inhibitor whose inhibitory action in the thrombin assay was essentially eliminated by dilution and by the presence of the S2238. The molar factor Va concentration was calculated from the rate of prothrombin activation using a turnover number of 6000 mol prothrombin activated per min/mol of factor Xa-Va complex¹⁴.

2.4 Results

Preparation of R155A Meizothrombin

R155A prothrombin was converted into R155A meizothrombin with the purified prothrombin activator from *Echis coloratus* venom. R155A meizothrombin was separated from the venom activator on a Mono S column. This column material did not bind R155A meizothrombin, but retained the venom activator. Immediately after elution of R155A meizothrombin, the thrombin inhibitor I2581 (final concentration 20 μM) was added to prevent autocatalytic peptide bond cleavages. SDS-PAGE analysis of R155A prothrombin and of the R155A meizothrombin that eluted from the Mono S column showed that R155A prothrombin was completely converted into R155A meizothrombin and that no further peptide bond cleavages had occurred. R155A meizothrombin was stored at -80°C . Before use, I2581 was removed by passage through a prepacked Sephadex G-25 (PD-10) column. The meizothrombin preparation thus obtained was diluted to 50 nM and was stable for at least 12 hrs at 0°C .

R155A meizothrombin-, meizothrombin(desF1)- and thrombin-catalyzed activation of factor V

Time courses of factor V activation by R155A meizothrombin, meizothrombin(desF1) and thrombin are presented in Fig. 2.2. In free solution, R155A meizothrombin and meizothrombin(desF1) were poor factor V activators, with activities that were less than 5% of that of thrombin (Fig. 2.2A).

Fig. 2.2 Time courses of factor V activation by thrombin, R155A meizothrombin and meizothrombin(desF1). Human factor V (3 nM) was activated at 37°C with 0.15 nM thrombin (●), R155A meizothrombin (□) or meizothrombin(desF1) (▲) in the absence (A) or presence (B) of 60 μM DOPS/DOPC (10/90, mol/mol) vesicles in a reaction mixture containing 25 mM Hepes (pH 7.5), 175 mM NaCl, 5 mM CaCl_2 and 5 mg/ml BSA. After the time intervals indicated in the figure aliquots from the activation mixture were assayed for the presence of factor Va as described under "Materials and Methods".

However, when negatively charged phospholipid vesicles were included in the activation mixture, there was a drastic increase of the rate of R155A meizothrombin-catalyzed activation of factor V, while there was hardly any effect on factor V activation by thrombin or meizothrombin(desF1) (Fig. 2.2B). We verified that factor V activation in reaction mixtures containing phospholipids and R155A meizothrombin was not due to α -thrombin formed by autoproteolysis of R155A meizothrombin. This is concluded from the fact that R155A meizothrombin present in activation mixtures retained full amidolytic activity when incubated with antithrombin III + heparin, which shows that it is not converted into α -thrombin since this would be readily inhibited by these inhibitors¹².

Initial rates of factor Va generation were linear in time between 0 and 3 min and directly proportional to the concentrations of factor V (0.5 nM) and activator (0-0.3 nM) present in the reaction mixture (data not shown). This shows that factor V activation under these conditions is first-order in both factor V and activator (R155A meizothrombin, meizothrombin(desF1) or thrombin), which allows the calculation of second-order rate constants for factor V activation from the initial rates of factor Va formation. These rate constants are summarized in Table 2.1.

Table 2.1***Second order rate constants of factor V activation***

Rate constants of factor V activation by thrombin, meizothrombin(desF1) or R155A meizothrombin were calculated from initial rates of factor Va formation obtained from the time courses of factor V activation presented in Fig. 2.2.

Factor V activator	$k \text{ (M}^{-1}\text{s}^{-1}\text{)}$	
	no lipid	+ 60 μM DOPS/DOPC
Thrombin	4.0×10^6	3.6×10^6
Meizothrombin(desF1)	0.17×10^6	0.22×10^6
R155A meizothrombin	0.06×10^6	18×10^6

Thrombin catalyzed factor V activation with a second-order rate constant of $4.0 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ in the absence of phospholipid vesicles and $3.6 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ in their presence. The rate constant for meizothrombin(desF1)-catalyzed factor V activation was ~20-fold lower ($k = 0.17 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$) and was also hardly affected by the presence of phospholipid. In free solution, R155A meizothrombin was the weakest factor V activator ($k = 0.06 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$). However, the presence of 60 μM PS/PC (10:90, mol/mol) vesicles caused a

300-fold increase in the initial rate of R155A meizothrombin-catalyzed factor V activation, yielding a rate constant ($k = 18 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$) that even exceeds that of thrombin.

The initial rate of factor V activation by R155A meizothrombin was a function of the phospholipid concentration in the reaction mixture. The rate constant calculated from the initial phase of time courses of activation of 3 nM factor V by R155A meizothrombin increased from $0.06 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ in the absence of phospholipid vesicles to an average plateau value of $19 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ at concentrations PS/PC (10:90, mol/mol) vesicles concentrations of $\geq 50 \mu\text{M}$ (Fig. 2.3). Half-maximal rates of factor V activation were observed at $\sim 10 \mu\text{M}$ PS/PC.

Fig. 2.3 Phospholipid dependence of R155A meizothrombin-catalyzed activation of factor V. Human factor V (3 nM) was activated at 37°C with 0.03 nM R155A meizothrombin in a reaction mixture containing 25 mM Hepes (pH 7.5), 175 mM NaCl, 5 mM CaCl_2 , 5 mg/ml BSA and concentrations of DOPS/DOPC (10/90, mol/mol) vesicles indicated in the figure. After appropriate time intervals aliquots were taken from the activation mixture and assayed for the presence of factor Va as described under "Materials and Methods". Initial rates of factor V activation were obtained from the time courses of factor Va formation by linear regression.

A remark is due here regarding the equation that describes time courses of factor V activation such as those presented in Fig. 2.2. Theoretically, the time course of product generation of an enzyme-catalyzed reaction determined under pseudo first-order conditions is described by the equation $P_t = S_0(1 - e^{-k \cdot t})$, in which P_t is the product concentration at time t , S_0 is the substrate concentration at $t=0$ and k is the pseudo first-order rate constant of the reaction. The second-order rate constant of the reaction is obtained after dividing the pseudo first-order rate constant by the enzyme concentration. The time courses of thrombin-catalyzed factor V activation could be fitted with this equation and yielded a rate constant equal to that calculated from the initial rate of factor Va formation. However, the time course of R155A

meizothrombin-catalyzed activation of factor V (Fig. 2.2B) did not adhere to the equation for a pseudo first-order reaction. The experimental curve was biphasic, with a rapid initial phase (60% of the reaction) followed by a slower second phase that completes factor V activation. Possible explanations for this biphasic behaviour are given in the discussion.

Fig. 2.4 Gelelectrophoretic analysis of factor V activation by thrombin and R155A meizothrombin. Human factor V (365 nM) was activated at 37°C with 2.5 nM thrombin (A) or 2.5 nM R155A meizothrombin in a reaction mixture containing 25 mM Hepes (pH 7.5), 175 mM NaCl, 5 mM CaCl₂ and 60 μM DOPS/DOPC (10/90, mol/mol) vesicles. After different time intervals aliquots from the activation mixture were assayed for the presence of factor Va as described under "Materials and Methods". At the same times 10 μl aliquots from the activation mixture were mixed with 10 μl SDS- and mercaptoethanol-containing gelbuffer. To allow correlation of activation with product formation the gel represents SDS-PAGE analysis of samples which are matched as close as possible with respect to the factor Va activities generated in the reaction mixtures.

SDS-PAGE analysis of factor V activation by thrombin and R155A meizothrombin

Generation of reaction intermediates and products during factor V activation by thrombin and R155A meizothrombin was analysed by SDS-PAGE (Fig. 2.4A,B). In agreement with the literature¹⁻⁶ thrombin-catalyzed activation of factor V proceeds via the characteristic high molecular weight intermediates with $M_r = 280,000$ and $220,000$. The $280,000$ intermediate was, however, hardly formed when factor V was activated by R155A meizothrombin (Fig. 2.4B). This indicates differences in either the order or the

rate of peptide bond cleavage during factor V activation by thrombin or R155A meizothrombin. Further processing of the high molecular weight intermediates resulted in both cases in the generation of activation fragments, which were hardly visible on the gel due to poor staining with Coomassie Blue⁵ and in the formation of a factor Va molecule that consisted of a heavy chain with an M_r of 104,000 and a light chain doublet with M_r values of 74,000 and 71,000.

2.5 Discussion

Factor V activation is an important reaction in hemostatic plug formation. It results in the formation of factor Va, the protein cofactor that greatly accelerates prothrombin activation and that regulates the rate of thrombin formation. Factor V is rapidly activated by thrombin¹⁻⁶ and by factor Xa⁶, which are the proteases that are generally considered to be the physiological activators of factor V.

In this chapter, it is reported that factor V is also readily activated by meizothrombin, provided that negatively charged membranes are present as a catalytic surface. Meizothrombin is a reaction intermediate that accumulates in the early phase of prothrombin activation at concentrations that exceed those of thrombin¹²⁻¹⁵. Meizothrombin is a serine protease that, compared with thrombin, was reported to exhibit greatly reduced activity towards macromolecular thrombin substrates such as fibrinogen^{12,16}, platelets¹⁶ and factor V¹⁶. Evaluation of the catalytic activity of meizothrombin is, however, hampered by the fact that it is further processed into meizothrombin-desF1 (fast) or α -thrombin (slow)^{12,17} via an autocatalytic reaction. Removal of the fragment 1 domain, which contains the γ -carboxyglutamic acid residues that function in the binding of meizothrombin to procoagulant membranes, may especially influence those catalytic activities of meizothrombin that may occur on a phospholipid surface *e.g.* protein C and/or factor V activation. To enable activity studies of meizothrombin, we have prepared a recombinant human prothrombin in which Arg¹⁵⁵ was replaced by Ala. The recombinant prothrombin was converted into a meizothrombin derivative (R155A meizothrombin) that is resistant to autocatalytic removal of the fragment 1 domain. In free solution, R155A meizothrombin is a poor activator of factor V, and activates factor V at a rate that is < 2% of that of thrombin ($k = 0.06 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ *versus.* $4.0 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$). However, when negatively charged phospholipid vesicles are included in the reaction mixture, there is a phospholipid-dependent increase of the rate of R155A meizothrombin-catalyzed factor V activation, reaching a plateau value with an average activation rate constant of $k = 19 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ at $\geq 50 \mu\text{M}$ PS/PC. Since the

activation of factor V by thrombin or meizothrombin(desF1) is hardly affected by the presence of phospholipid, we conclude that the stimulation in the case of R155A meizothrombin is due to the fact that both the enzyme (R155A meizothrombin) and the substrate (factor V) bind to the membrane surface. This simultaneous binding promotes the interaction between two poorly interacting components (R155A meizothrombin and factor V), which results in an increased rate of factor V activation. Such a mechanism of action of membranes has also been proposed for other phospholipid-dependent coagulation factor activations²³.

The final product of both thrombin- and R155A meizothrombin-catalyzed activation of factor V is a factor Va molecule that consists of the characteristic heavy- and light chains. There are, however, some differences in the pattern of high molecular weight intermediate generation in the early phase of the activation reaction. Gel electrophoretic analysis (Fig. 2.4) shows that thrombin activates factor V via M_r 280,000 and 220,000 intermediates (designated A₁ and B in Ref. 1) by successive cleavage of peptide bonds 1, 2 and 3 (Fig. 2.5). The M_r 280,000 intermediate is, however, hardly formed during factor V activation by R155A meizothrombin. This indicates that, under these conditions, there is a change in either the rate or the order of peptide bond cleavages during factor V activation.

Fig. 2.5 **Peptide bonds cleaved by thrombin during factor V activation.** The heavy and light chain of factor Va are indicated by HC and LC, respectively.

Such an effect may be specific for situations in which membrane-bound factor V is activated by a membrane-bound enzyme since a similar activation pattern (*i.e.* reduced M_r 280,000 intermediate formation) was also observed in the case of phospholipid-dependent activation of factor V by factor Xa⁶. A phenomenon like this may be caused by a steric or juxtaposing effect that results from coordinate binding of substrate and enzyme to the membrane surface.

The observations that meizothrombin-catalyzed factor V activation occurs at a membrane surface via a peptide bond cleavage pattern that differs from that of factor V activation by thrombin may explain why time courses of factor Va formation are biphasic in case of meizothrombin and monophasic in the case of thrombin. Recently, we reported that factor V preparations contain two forms of factor V with different affinities for negatively charged membranes¹⁸. Although there may be several explanations for biphasic time courses of product generation during pseudo first-order reactions, we would like to mention two possibilities that relate to the observations mentioned above: 1) fast generation of an activation intermediate with a cofactor activity that is lower than that of a slowly formed end product (factor Va) or 2) fast activation of the factor V that binds with high affinity to phospholipid, followed by slow activation of the factor V molecules that have a low affinity for phospholipid.

The data presented in this chapter show that meizothrombin is a potent factor V activator in the presence of phospholipids. The rate constant of R155A meizothrombin-catalyzed factor V activation ($18 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$) even exceeds those determined for activation by thrombin ($4 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$, this chapter and ref. 6) or factor Xa ($3.26 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$, ref. 6). Since the first prothrombin molecules that are activated during coagulation in whole blood are likely converted into meizothrombin¹⁵, meizothrombin should be considered as a physiologically important factor V activator with a major function during the phase of coagulation in which the prothrombinase complex is generated.

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Chapter 3

Molecular Events in the Cleavage of FVa and FVa_{Leiden} by Activated Protein C

*Based on: Peptide Bond Cleavages and Loss of Functional Activity During Inactivation of Factor Va and Factor Va^{R506Q} by Activated Protein C, Journal of Biological Chemistry 1995, 270 (36), 21158-21166
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3.1 Summary

Factor V was purified from the plasma of an APC-resistant patient who is homozygous for the mutation Arg⁵⁰⁶→Gln (factor VR^{506Q}). Factor VR^{506Q} was converted by thrombin into factor Va which was further purified yielding a factor Va preparation that had the same cofactor activity in prothrombin activation as normal factor Va. Inactivation of low concentrations of normal factor Va (<5 nM) by 0.15 nM activated protein C (APC) in the presence of phospholipid vesicles proceeded via a biphasic reaction that consisted of a rapid phase ($k = 4.3 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$), yielding a reaction intermediate with reduced cofactor activity that was fully inactivated during the subsequent slow phase ($k = 2.3 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$). Inactivation of factor Va^{R506Q} proceeded via a monophasic reaction ($k = 1.7 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$). Immunoblot analysis showed that APC-catalyzed inactivation of factor Va occurred via peptide bond cleavages in the heavy chain. The rapid phase of inactivation of normal factor Va was associated with cleavage at Arg⁵⁰⁶ and full inactivation of factor Va required subsequent cleavage at Arg³⁰⁶. The slow monophasic inactivation of factor Va^{R506Q} correlated with cleavage at Arg³⁰⁶. Cleavage at Arg⁵⁰⁶ in normal factor Va resulted in accumulation of a reaction intermediate that exhibited 40 % cofactor activity in prothrombin activation mixtures that contained a high factor Xa concentration (5 nM). Compared with native factor Va, the reaction intermediate retained virtually no cofactor activity at low factor Xa concentrations (0.3 nM). This demonstrates that factor Va that is cleaved at Arg⁵⁰⁶ is impaired in its ability to interact with factor Xa. Michaelis-Menten kinetic analysis showed that cleavage at Arg⁵⁰⁶ in membrane-bound factor Va was characterized by a low K_m for factor Va (20 nM) and $k_{\text{cat}} = 0.96 \text{ s}^{-1}$. For cleavage at Arg³⁰⁶ in factor Va^{R506Q} the kinetic parameters were $K_m = 196 \text{ nM}$ and $k_{\text{cat}} = 0.37 \text{ s}^{-1}$. This means that differences between APC-catalyzed inactivation of factors Va and Va^{R506Q} will become much less pronounced at high factor Va concentrations. When factor Va^{R506Q} was inactivated by APC in the absence of phospholipids, cleavage at Arg⁶⁷⁹ of the heavy chain also contributed to factor Va inactivation. Comparison of rate constants for APC-catalyzed cleavage at Arg³⁰⁶, Arg⁵⁰⁶ and Arg⁶⁷⁹ in the absence and presence of phospholipids indicated that phospholipids accelerated these cleavages to a different extent. This indicates that the binding of factor Va to phospholipids changes the accessibility of the cleavage sites and/or the sequence of peptide bond cleavage by APC.

3.2 Introduction

Human blood coagulation factor Va is a heterodimeric glycoprotein¹ that consists of a heavy chain ($M_r = 105,000$) associated via a single Ca^{2+} ion with a light chain ($M_r = 74,000$ or $72,000$, cf. ref²). Factor Va is formed during hemostasis from the inactive procofactor V after limited proteolysis. Factor Va is an essential nonenzymatic cofactor of the prothrombin-activating complex, which further comprises the serine protease factor Xa, calcium ions and a procoagulant membrane surface. Depending on the reaction conditions factor Va accelerates prothrombin activation 10^3 - 10^5 fold³⁻⁵.

Proteolytic inactivation of factor Va by activated protein C (APC), is one of the key reactions in the regulation of thrombin formation. APC-catalyzed cleavage of factor Va is stimulated by the presence of negatively charged membrane surfaces⁶⁻⁸ and by the protein cofactor, protein S⁸⁻¹⁰. The loss of cofactor activity of factor Va is associated with peptide bond cleavages in the heavy chain of factor Va at Arg³⁰⁶, Arg⁵⁰⁶ and Arg⁶⁷⁹^{11,12}. The physiologic importance of the down-regulation of factor Va activity by APC is underscored by the observation of recurrent thromboembolic events in individuals that are deficient in either protein C or protein S¹³⁻¹⁵.

Since a first publication by Dahlbäck et al.¹⁶, several laboratories have associated the occurrence of familial thrombophilia in a large group of patients with a poor anticoagulant response to APC (APC resistance). APC resistance is at least 10 times more common than all other known genetic thrombosis risk factors together and has an allelic frequency of ~2% in the Dutch population¹⁷. The molecular defect in APC-resistant patients was recently demonstrated¹⁸⁻²¹ to be linked to a single point mutation in the factor V gene that causes an amino acid substitution (Arg⁵⁰⁶ → Gln) at a site that is cleaved during APC-catalyzed inactivation of factor Va¹¹.

In this chapter we present a kinetic analysis of APC-catalyzed inactivation of human factor Va that was either obtained from normal plasma or from the plasma of a patient who is homozygous for the mutation (Arg⁵⁰⁶ → Gln). Parallel experiments were performed in which changes of functional activity of factor Va during inactivation by APC were correlated with the cleavage of peptide bonds in the factor Va molecule. This study was undertaken in order to elucidate the consequences of the molecular defect in factor V for the regulation of *in vivo* prothrombin activation.

3.3 Materials and Methods

Materials

Hepes, Tris, bovine serum albumin and Russel's viper venom were purchased from Sigma Chemical Co., USA. DOPC and DOPS were obtained from Avanti Polar Lipids, Pelham, AL., USA. The chromogenic substrates S2238 and S2366 were supplied by Chromogenix, Mölndal, Sweden. PPACK was obtained from Calbiochem and p-NPGB was from Nutritional Biochemicals. Micro BCA protein assay kits were from Pierce, Rockford, USA. FPLC equipment and column materials used for protein purification were purchased from Pharmacia, Uppsala, Sweden.

Proteins

Human coagulation factors used in this study were isolated from fresh frozen plasma. Human prothrombin and factor X were purified according to DiScipio et al.²². Human thrombin was prepared from prothrombin activation mixtures as described by Pletcher and Nelsestuen²³. Human factor Xa was obtained from purified factor X after activation with RVV-X and isolation from the activation mixture by affinity chromatography on soybean trypsin inhibitor-Sepharose²⁴. RVV-X was purified from Russel's viper venom according to Schiffman et al.²⁵. Protein C was purified and activated as described by Gruber et al.²⁶. The first steps of the preparation of human factor V and factor V^{R506Q} were essentially as described by Dahlbäck²⁷ with minor modifications²⁸. The factor V (in 25 mM Hepes pH 7.5, 50 mM NaCl, 5 mM CaCl₂) thus obtained was applied to a Resource S column and eluted with 20 ml of a linear gradient (50 mM - 1000 mM NH₄Cl) in 25 mM Hepes (pH 6.5), 5 mM CaCl₂. Aliquots from column fractions were incubated for 15 min with 5 nM thrombin and factor Va-containing fractions were pooled. Factor Va was prepared by incubating factor V (33 µg/ml) for 20 min with 5 nM thrombin in 25 mM Hepes (pH 7.5), 175 mM NaCl and 5 mM CaCl₂. After activation 15 nM PPACK was added to inhibit thrombin. Factor Va was further subjected to fast protein liquid chromatography (FPLC) on a Mono S column (HR 5/5) at room temperature². The factor Va bound to the Mono S column was eluted with 15 ml of a linear gradient (50 mM - 1000 mM NH₄Cl) in 25 mM Hepes (pH 7.5), 5 mM CaCl₂. Factor Va activity eluted from the column in two well-separated protein peaks containing factor Va with light chains of $M_r = 74,000$ and $M_r = 71,000$, respectively². The two factor Va containing peaks were pooled and brought into 25 mM Hepes (pH 7.5), 175 mM NaCl, 5 mM CaCl₂.

by a buffer exchange on a PD-10 column. Factor V and factor Va from an APC-resistant patient were purified by the same methods. The patient was a female with a history of deep vein thrombosis and pulmonary embolism. Plasma from the patient was obtained by plasmapheresis. The APC-resistance ratio was 1.31 (Coatest APC-resistance kit, Chromogenix, Mölndal, Sweden). The patient was identified as homozygous for the substitution G1691→A in the factor V gene (amino acid substitution Arg509→Gln in factor V) by genetic analysis in genomic DNA samples isolated from peripheral blood²⁹.

Protein Concentrations

Protein concentrations were determined with the micro BCA protein assay³⁰. Molar thrombin and factor Xa concentrations were determined by active site titration with p-NPGB^{31,32}. Prothrombin concentrations were determined after complete activation of prothrombin with *Echis carinatus* venom and quantitation of thrombin with p-NPGB. APC concentrations were determined with S2366 using kinetic parameters reported by Sala et al.³³ Factor V and factor Va were quantitated as described below.

Phospholipid Vesicle Preparations

Small unilamellar phospholipid vesicles were prepared as described earlier². Phospholipid concentrations were determined by phosphate analysis³⁴.

Gel Electrophoretic and Immunoblot Analysis

Purity of protein preparations was analyzed by SDS-PAGE on 8% acrylamide slabgels³⁵ and staining with Coomassie Brilliant Blue R-250. The inactivation patterns of factor Va were analyzed after SDS-PAGE, electrophoretic transfer of proteins from the gel to Immobilon-P membranes and visualization with a polyclonal rabbit-antibody directed against the heavy chain of factor Va and goat-anti-rabbit IgG conjugated with alkaline phosphatase as described by Blake et al³⁶.

Factor Va Assay

Factor Va cofactor activity was quantitated by determining the rate of factor Xa-catalyzed prothrombin activation in reaction mixtures that contained a limiting amount of factor Va, a saturating concentration factor Xa (5 nM), 50 μ M phospholipid vesicles (DOPS/DOPC, 10/90, mol/mol) and 0.5 μ M prothrombin²⁸. Rates of prothrombin activation were determined with the chromogenic substrate S2238⁴. The molar factor Va concentration in the assay mixture was calculated from the rate of prothrombin activation using a turnover number of 6000 moles prothrombin activated per min per mole of factor XaVa complex³⁷. Factor V was quantitated by the same method after activation with 5 nM thrombin.

Kinetic Data Analysis

The formation of a membrane-bound factor XaVa complex was determined by measuring rates of prothrombin activation in the presence of phospholipid vesicles at a fixed (limiting) concentration of factor Va and varying amounts of factor Xa. Factor Xa, factor Va and phospholipid vesicles were preincubated for 5 min at 37°C in 25 mM Hepes (pH 7.5), 175 mM NaCl, 2 mM CaCl₂ and 5 mg/ml BSA. Prothrombin activation was started by the addition of prothrombin (preincubated at 37°C in the same buffer) and rates of prothrombin activation were determined with the chromogenic substrate S2238⁴. The apparent K_d for dissociation of the membrane-bound factor Xa-Va complex ($K_{1/2}X_a$) was obtained from plots of the rate of prothrombin activation as function of the factor Xa concentration that were fitted to the equation for a single site binding isotherm (*hyperbola*) via non-linear least squares regression analysis.

The kinetic parameters (K_m and V_{max}) for factor Xa-catalyzed prothrombin activation were determined by measuring the rate of thrombin formation at varying prothrombin concentrations in the presence of a fixed phospholipid concentration, a limiting amount of factor Xa and a saturating concentration of factor Va as described in the previous paragraph. The kinetic parameters were obtained by fitting the data to the Michaelis-Menten equation using non-linear least squares regression analysis.

Curve Fitting of Pseudo-first Order Time Courses of Factor Va Inactivation

Time courses of factor Va or factor Va^{R506Q} inactivation by APC were determined by following the loss of cofactor activity of factor Va as a function of time. Data reported in literature^{11,12} and in this chapter demonstrate that the loss of factor Va cofactor activity can proceed via 2 pathways:

in which factor Va_{int} is a reaction intermediate with partial cofactor activity, factor Va_i is a reaction product that has no cofactor activity, k_1 is the second order rate constant for cleavage of peptide bond 1, k_2 is the second order rate constant for cleavage of peptide bond 2 in factor Va_{int} and k_3 is the second order rate constant for cleavage of peptide bond 2 in native factor Va.

Under first order conditions, i.e. conditions at which the inactivation rate is directly proportional to the factor Va and APC concentrations, the loss of cofactor activity is described by equation 3³⁸:

$$\text{Va}_t = \text{Va}_0 \cdot e^{-(k_1+k_3) \cdot t} + B \cdot \text{Va}_0 \cdot \frac{k_1 \cdot e^{(-k_2 \cdot t)}}{(k_1 + k_3 - k_2)} \cdot (1 - e^{\{-(k_1+k_3-k_2) \cdot t\}}) \quad (\text{Eq. 3})$$

in which Va_t is the cofactor activity determined at time t, Va₀ is the cofactor activity determined before APC is added, B is the cofactor activity of factor Va_{int} (expressed as fraction of the cofactor activity of native factor Va) and k_1 , k_2 and k_3 are the rate constants defined above. The rate constants and the cofactor activity of factor Va_{int} were obtained by fitting the data to equation 3 using non-linear least squares regression analysis.

In the case of normal factor Va inactivation, k_3 appeared to be associated with cleavage of the peptide bond at Arg³⁰⁶. Therefore, time courses of normal factor Va inactivation were fitted with a fixed k_3 , obtained from kinetic analysis of factor Va^{R506Q} inactivation.

In some experiments (factor Va inactivation in free solution) there was a slow loss of factor Va cofactor activity in the absence of APC. In these cases the spontaneous loss of cofactor activity (0.4 %/min) was added to k_3 .

3.4 Results

Peptide Bonds in Factor Va Susceptible to Proteolysis by APC

Recently it was reported¹¹ that APC can cleave 3 peptide bonds in the heavy chain of human factor Va that are located at Arg³⁰⁶, Arg⁵⁰⁶ and Arg⁶⁷⁹. Fig. 3.1 represents a schematic diagram of the heavy chain of factor Va in which we have depicted the sites that are susceptible to APC and the molecular weights of possible cleavage products. In the present chapter we will refer to the rate constants associated with the indicated peptide bond cleavages as k_{306} , k_{506} and k_{679} , respectively.

FIG. 3.1 *Schematic representation of APC cleavage sites in the heavy chain of factor Va.* The heavy chain of human factor Va contains three peptide bonds that can be cleaved by APC and that are located at Arg³⁰⁶, Arg⁵⁰⁶ and Arg⁶⁷⁹. The molecular weights of possible cleavage products correspond to those given in other publications^{11,12}.

Structural and Functional Properties of Factor Va Purified from Normal and from APC-Resistant Plasma

Factor V and thrombin-activated factor Va were purified from normal and homozygous APC-resistant plasma as described in the Materials and Methods. The thrombin-activated factor Va and factor Va^{R506Q} preparations had identical gel electrophoretic properties with the characteristic heavy chain

with $M_r = 105,000$ and a light chain doublet at $M_r = 74,000$ and $71,000$ (data not shown).

Factor Va and factor Va^{R506Q} had similar cofactor activities in prothrombin activation. Kinetic analysis showed that prothrombin was activated in the presence of 25 μM phospholipid vesicles (DOPS/DOPC, 10/90, mol/mol) with the following kinetic parameters: K_m for prothrombin = 0.13 μM , $k_{\text{cat}} = 8570 \text{ min}^{-1}$ and $K_{1/2}\text{Xa}$ of XaVa complex formation = 0.083 nM for prothrombinase complexes with normal factor Va and K_m for prothrombin = 0.12 μM , $k_{\text{cat}} = 8450 \text{ min}^{-1}$ and $K_{1/2}\text{Xa}$ of XaVa complex formation = 0.105 nM for prothrombinase complexes with factor Va^{R506Q} (data not shown).

This means that the factor Va assay that we used, which measures the cofactor activity of factor Va in prothrombin activation, allows quantitative comparison of factor Va and factor Va^{R506Q}.

Kinetic Analysis of APC-Catalyzed Inactivation of Membrane-Bound Factor Va and Factor Va^{R506Q}

Time courses of APC-catalyzed inactivation of factor Va and factor Va^{R506Q} in the presence of phospholipid vesicles are shown in Fig. 3.2.

Fig. 3.2 Inactivation of factor Va and factor Va^{R506Q} by APC in the presence of phospholipids. 0.3 nM purified human factor Va (▲) or factor Va^{R506Q} (■) was incubated with (A) 0.15 nM APC or (B) 1.5 nM APC and 25 μM phospholipid vesicles (DOPS/DOPC, 10/90, mol/mol) in 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl₂ and 5 mg/ml BSA at 37 °C. At the indicated time points factor Va activity was determined as described in the "Materials and Methods". The solid lines represent exponential curves obtained after fitting the data using equation 3 given in the "Materials and Methods". The rate constants and activity of the reaction intermediate were determined by nonlinear least-squares regression of the data.

At a low APC concentration (0.15 nM) normal factor Va was inactivated via a biphasic reaction during which 50 % of the factor Va activity was lost within 3 min. Factor Va^{R506Q} is inactivated at a considerably lower rate requiring more than 45 min for 50% loss of cofactor activity (Fig. 3.2A). When a similar experiment was performed at a high APC concentration (1.5 nM) inactivation of factor Va^{R506Q} appeared to occur via a monophasic reaction (the data points could be fitted with a single exponential), whereas the inactivation of normal factor Va still proceeded via a biphasic reaction (fig. 3.2B).

The time courses of factor Va and Va^{R506Q} inactivation (% factor Va activity vs. time) were not affected by varying the concentrations of factor Va or Va^{R506Q} between 0 and 5 nM. Since the rates of factor Va inactivation were also directly proportional to the amount of APC present (0.05 - 5 nM) we concluded that factor Va and Va^{R506Q} inactivation were first-order in both factor Va (Va^{R506Q}) and APC. This allows calculation of apparent second-order rate constants for factor Va inactivation from time courses such as shown in Fig. 3.2.

On the basis of literature data^{11,12} and experiments presented below it can be postulated that the biphasic time courses of inactivation of normal factor Va presented in Fig. 3.2 result from rapid cleavage at Arg⁵⁰⁶ yielding a reaction intermediate that possesses partial cofactor activity in prothrombin activation. This reaction intermediate is fully inactivated by subsequent slow cleavage at Arg³⁰⁶. Inactivation of factor Va^{R506Q} likely proceeds via a monophasic reaction that is the result of slow cleavage at Arg³⁰⁶.

The time courses of inactivation could indeed be fitted (Fig. 3.2, *solid lines*) using an equation that describes the time-dependent loss of cofactor activity when factor Va is inactivated via the random ordered two-step reaction model described in the "Materials and Methods". The fits of the particular experiment presented in Fig. 3.2 yielded the following second-order rate constants: $k_{506} = 4.7 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$, $k_{306} = 1.7 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ and 40 % cofactor activity for the reaction intermediate (factor Va cleaved at Arg⁵⁰⁶) in the case of normal factor Va inactivation and $k_{306} = 1.4 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ and a reaction product that has virtually lost its cofactor activity in the case of factor Va^{R506Q} inactivation.

Immunoblot Analysis of APC-Catalyzed Inactivation of Membrane-Bound Factor Va and Factor Va^{R506Q}

Generation of heavy chain fragments during APC-catalyzed factor Va and factor Va^{R506Q} inactivation was analyzed by SDS-PAGE, transfer to Immobilon-P membranes and visualization of the reaction products with a

polyclonal antibody directed against the heavy chain domain of human factor Va (Fig. 3.3).

FIG. 3.3 Immunoblot analysis of factor Va and factor Va^{R506Q} inactivation by APC in the presence of phospholipids. (A) 6 nM purified human factor Va and 1 nM APC or (B) 15 nM factor Va^{R506Q} and 5 nM APC were incubated with 25 μ M phospholipid vesicles (DOPS/DOPC, 10/90, mol/mol) in 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl₂ and 0.1 mg/ml BSA at 37°C. In the case of normal factor Va (A) the APC concentration was increased to 110 nM after 5 min. At the indicated time intervals samples from the inactivation mixture were subjected to SDS-PAGE. After transfer to Immobilon-P membranes, the heavy chain and derived fragments were visualized with a polyclonal antibody directed against the heavy chain of factor Va. The positions of the molecular weight markers are indicated at left of Panels A and B. The percentages cofactor activity at the indicated time points were (A) 100, 63, 46, 40, 25, 19, 17, and 0 % and (B) 100, 80, 67, 43, 12, 3, 2, and 1 %. Further experimental details are described under "Materials and Methods".

In the description of the gel patterns presented below, the formation of reaction products was associated with peptide bond cleavages at Arg³⁰⁶, Arg⁵⁰⁶ or Arg⁶⁷⁹ on the basis of molecular weights of heavy chain fragments indicated in Fig. 3.1.

During the initial phase of inactivation of normal factor Va, reaction products with M_r of 75,000 and 26,000/28,000 were formed, which is indicative of cleavage at Arg⁵⁰⁶ (Fig. 3.3A). Prolonged incubation of factor Va with a high APC concentration resulted in the disappearance of the M_r = 75,000 reaction intermediate (cleavage at Arg³⁰⁶) and formation of reaction products with M_r of 45,000 and 30,000, respectively. The fragment with M_r = 30,000 stains poorly with our polyclonal antibody and was hardly visible on

the gel. During the final stage of the inactivation reaction the peptide bond at Arg⁶⁷⁹ was also cleaved, as is indicated by the disappearance of the $M_r = 26,000/28,000$ doublet and the formation of low molecular weight products migrating with the dye front in the gel.

The immunoblot of the time course of APC-catalyzed inactivation of membrane-bound factor Va^{R506Q} (Fig. 3.3B) differs from that of normal factor Va. Inactivation of factor Va^{R506Q} correlated with the appearance of only two reaction products with $M_r = 60,000/62,000$ and $M_r = 45,000$, which corresponds with cleavage at Arg³⁰⁶. The immunoblot further demonstrates that cleavage at Arg⁶⁷⁹ did not significantly contribute to factor Va^{R506Q} inactivation since no additional bands became visible in the heavy chain or $M_r = 54,000/56,000$ kDa region of the immunoblot. This would have been expected if cleavage at Arg⁶⁷⁹ had occurred.

The immunoblot analysis supports the model for APC-catalyzed proteolysis of factor Va presented in the previous section. The rapid phase of the time course of inactivation of normal factor Va is associated with cleavage at Arg⁵⁰⁶ and the subsequent slow phase is due to cleavage at Arg³⁰⁶. Inactivation of factor Va^{R506Q} proceeds via a monophasic reaction which is explained by cleavage at Arg³⁰⁶.

APC-Catalyzed Inactivation of Factor Va and Factor Va^{R506Q} in the Absence of Phospholipids

In order to quantify the effect of the membrane on the peptide bond cleavages during factor Va and factor Va^{R506Q} inactivation by APC we have also determined time courses of factor Va inactivation in the absence of phospholipids under pseudo-first order conditions (Fig. 3.4).

FIG. 3.4 *Inactivation of factor Va and factor Va^{R506Q} by APC in the absence of phospholipids.* 19 nM purified human factor Va (▲) or factor Va^{R506Q} (■) was incubated with 390 nM APC in 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl₂ and 5 mg/ml BSA at 37°C. At the indicated time points factor Va activity was determined as described in the "Materials and Methods". The solid lines represent exponential curves obtained after fitting the data using equation 3 given in the "Materials and Methods". The rate constants and activity of the reaction intermediate were determined by nonlinear least-squares regression analysis of the data.

It was not possible to fit these time courses of inactivation with a single exponential. For both factor Va and factor Va^{R506Q} it was necessary to postulate that inactivation proceeded via a biphasic reaction with a partially active reaction intermediate.

To correlate the loss of cofactor activity with peptide bond cleavages in the heavy chain we analyzed the product generation pattern by immunoblot analysis of samples taken from the inactivation mixture at selected time intervals (Fig. 3.5).

A

B

FIG. 3.5 Immunoblot analysis of factor Va and factor Va^{R506Q} inactivation by APC in the absence of phospholipids. (A) 40 nM purified human factor Va and 15 nM APC or (B) 40 nM factor Va^{R506Q} and 500 nM APC were incubated in 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl₂ and 0.2 mg/ml BSA at 37°C. In the case of normal factor Va (A) the APC concentration was increased to 500 nM APC after 35 min. At the indicated time intervals samples from the inactivation mixture were subjected to SDS-PAGE. After transfer to Immobilon-P membranes, the heavy chain and derived fragments were visualized with a polyclonal antibody directed against the heavy chain of factor Va. The positions of the molecular weight markers are indicated at left of Panels A and B. The percentages cofactor activity at the indicated time points were (A) 100, 88, 71, 59, 39, 24 and 1 % and (B) 100, 95, 85, 69, 46, 34, 18, and 6 %. Further experimental details are described under "Materials and Methods".

Compared with the peptide bond cleavage pattern of membrane-bound factor Va a striking difference was observed. In the absence of phospholipids cleavage at Arg⁶⁷⁹ occurred at appreciable rates in factor Va^{R506Q} (Fig. 3.5B). This is concluded from time-dependent appearance of an additional band in the heavy chain region of the gel.

Thus, it appears that in the case of factor Va^{R506Q} inactivation proteolysis at Arg⁶⁷⁹ and Arg³⁰⁶ contributed to factor Va inactivation and that cleavage at Arg⁶⁷⁹ preceded cleavage at Arg³⁰⁶. Fitting the time course of APC-catalyzed factor Va^{R506Q} inactivation (Fig. 3.4, *solid lines*) to the equation for a biphasic reaction with a partially active intermediate yields rate constants of $8.0 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ and $1.0 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ for k₆₇₉ and k₃₀₆.

respectively and 70 % cofactor activity for the reaction intermediate that has been cleaved at Arg⁶⁷⁹.

Immunoblot analysis (Fig. 3.5A) indicates that during the time courses of normal factor Va inactivation (Fig. 3.4) only peptide bond cleavages at Arg⁵⁰⁶ and Arg³⁰⁶ significantly contributed to the loss of factor Va cofactor activity. Inspection of the immunoblot that represents inactivation of normal factor Va reveals that cleavage at Arg⁶⁷⁹ had a minor contribution to the loss of factor Va cofactor activity. As a first approximation we have, therefore, also fitted this time course with a biphasic exponential. This fit (Fig. 3.4, *solid line*) yielded rate constants of $1.3 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ and $4.0 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ for cleavage at Arg⁵⁰⁶ and Arg³⁰⁶, respectively and a reaction intermediate with 40 % cofactor activity. It should be emphasized that the values of the latter rate constants may be slightly overestimated due to the assumption that cleavage at Arg⁶⁷⁹ did not contribute to inactivation.

Rate Constants for APC-Catalyzed Cleavage of Peptide Bonds During Inactivation of Free and Membrane-Bound Factor Va and Factor Va^{R506Q}

The rate constants for APC-catalyzed cleavage at Arg³⁰⁶, Arg⁵⁰⁶ and Arg⁶⁷⁹ in factor Va and Va^{R506Q} are summarized in Table 3.1. It was not possible to calculate rate constants for cleavage at Arg⁶⁷⁹ in normal factor Va from the time courses of inactivation presented in Figs. 3.2 and 3.4. However, from the disappearance of the $M_r = 26,000/28,000$ doublet on immunoblots (time points between 10 and 60 min, data not shown) it could be estimated that cleavage at Arg⁶⁷⁹ in free factor Va occurred with $k_{679} \approx 5.9 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ ($t_{1/2} \approx 16 \text{ min}$ at 156 nM APC) and that the same peptide bond in membrane-bound factor Va was cleaved with $k_{679} \approx 7.6 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ ($t_{1/2} \approx 30 \text{ min}$ at 50 nM APC).

Table 3.1

**Rate constants of APC-catalyzed peptide bond cleavages
in factor Va and factor Va^{R506Q}**

Rate constants of APC-catalyzed inactivation of factor Va and factor Va^{R506Q} were obtained by fitting time courses of factor Va or Va^{R506Q} inactivation such as presented in Figs. 3.2 and 3.4 to equation 3 given in the "Materials and Methods". k_{679} for normal factor Va was calculated from the $t_{1/2}$ of disappearance of the $M_r = 26,000/28,000$ doublet ($k_{679} = \ln 2 / t_{1/2}$) obtained by scanning immunoblots of time courses of APC-catalyzed factor Va inactivation. The rate constants given are averages calculated from at least 10 experiments. The standard error of mean (SE) was < 20% in all cases.

	normal factor Va		factor Va ^{R506Q}	
	- PL ^a	+PL	- PL	+PL
	$(M^{-1}s^{-1})$		$(M^{-1}s^{-1})$	
k ₅₀₆	1.4×10^5	4.3×10^7		
k ₃₀₆	3.7×10^3	2.3×10^6	9.4×10^2	1.7×10^6
k ₆₇₉	$\approx 5.9 \times 10^3$	$\approx 7.6 \times 10^3$	7.5×10^3	ND ^b

^aPL, 25 μ M phospholipid vesicles composed of 10% DOPS and 90% DOPC

^bND, not determined

The data presented in Table 3.1 show that phospholipids stimulated the APC-catalyzed peptide bond cleavages in factor Va to a different extent. Phospholipids appeared to have no effect on k_{679} . Furthermore it is clear that cleavage at Arg³⁰⁶ in free factor Va^{R506Q} occurred at a lower rate than in normal factor Va. For membrane-bound factor Va^{R506Q} and factor Va this difference was annulated. These observations are indicative for specific effects of phospholipids and/or prior cleavage at Arg⁵⁰⁶ on the cleavages at Arg³⁰⁶ and Arg⁶⁷⁹ (see discussion).

**Kinetic Parameters for APC-Catalyzed Cleavage at Arg⁵⁰⁶ and
Arg³⁰⁶ in Membrane-Bound Factor Va and Factor Va^{R506Q}**

The differences in rate constants for cleavage at Arg⁵⁰⁶ and Arg³⁰⁶ in membrane-bound factor Va must result from differences between the Michaelis-Menten kinetic parameters (k_{cat} and/or K_m) for these peptide bond cleavages. In order to obtain information on the kinetic basis of the differences we have determined the kinetic parameters, K_m and k_{cat} , by measuring the initial rates of factor Va inactivation (between 0 and 20% loss of cofactor activity) at varying factor Va or factor Va^{R506Q} concentrations.

The experiment with normal factor Va yields the k_{cat} and K_{m} for cleavage at Arg⁵⁰⁶ since this peptide bond is preferentially cleaved during the initial phase of inactivation. Michaelis-Menten kinetic analysis of factor Va^{R506Q} inactivation will give kinetic parameters for cleavage at Arg³⁰⁶.

The Lineweaver-Burk plots presented in Fig. 3.6 show that cleavage at Arg⁵⁰⁶ was characterized by a K_{m} of 20 nM and a k_{cat} of 0.96 s⁻¹. Inactivation of factor Va^{R506Q}, which corresponds with cleavage at Arg³⁰⁶, had a high K_{m} (196 nM) and a k_{cat} of 0.37 s⁻¹.

Fig. 3.6 Lineweaver-Burk plots for inactivation of factor Va and factor Va^{R506Q} by APC in the presence of phospholipids. Initial rates of factor Va inactivation were determined at varying concentrations of factor Va (▲) or factor Va^{R506Q} (■) that were incubated with 0.28 nM APC (▲) or 1 nM APC (■) and 25 μ M phospholipid vesicles (DOPS/DOPC, 10/90, mol/mol) in 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl₂ and 5 mg/ml BSA at 37°C. After different time intervals the factor Va activity was determined as described in the "Materials and Methods" and initial rates of factor Va inactivation were expressed as nM factor Va inactivated/min/nM APC (assuming that normal factor Va is converted into an intermediate with 40 % cofactor activity). The solid lines represent a fit of the data according the Michaelis-Menten equation with $K_{\text{m}} = 20$ nM and $V_{\text{max}} = 57.6$ nM factor Va inactivated/min/nM APC ($k_{\text{cat}} = 0.96$ s⁻¹) for factor Va (▲) and $K_{\text{m}} = 196$ nM and $V_{\text{max}} = 22.2$ nM factor Va^{R506Q} inactivated/min/nM APC ($k_{\text{cat}} = 0.37$ s⁻¹) for factor Va^{R506Q} (■).

Theoretically the $k_{\text{cat}}/K_{\text{m}}$ obtained by Michaelis-Menten analysis should equal the second order rate constant calculated from a time course of inactivation determined at low factor Va concentrations (Fig. 3.2). Indeed the $k_{\text{cat}}/K_{\text{m}}$ values determined for cleavage at Arg⁵⁰⁶ (4.8×10^7 M⁻¹s⁻¹) and Arg³⁰⁶ (1.9×10^6 M⁻¹s⁻¹) closely resemble the rate constants obtained at pseudo first-order reaction conditions (Table 3.1). This shows that the kinetic parameters, K_{m} and k_{cat} , are indeed associated with the indicated peptide

bond cleavages. The data further demonstrate that the differences between the rate constants of cleavage at Arg⁵⁰⁶ and Arg³⁰⁶ are mainly due to differences between the K_m values for these peptide bond cleavages.

Cofactor Activity of Factor Va after Cleavage at Arg⁵⁰⁶ by APC

Pseudo first-order time courses of inactivation of normal factor Va (Fig. 3.2 and 3.4) could only be fitted in a reaction model that consisted of two steps with the formation of a reaction intermediate (factor Va cleaved at Arg⁵⁰⁶) that retained partial cofactor activity.

The actual cofactor activity of factor Va is dependent on its interaction with procoagulant membranes, factor Xa, prothrombin and on its ability to increase the catalytic activity (k_{cat}) of factor Xa^{2-5, 40}. To examine which of these functions was impaired in factor Va that is cleaved at Arg⁵⁰⁶, we have characterized the functional properties of the inactivation intermediate. To this end normal factor Va was inactivated by APC in the absence of phospholipids (*cf.* Fig. 3.4) and after completion of the first phase of inactivation (Arg⁵⁰⁶ cleavage) the reaction mixture was diluted and chromatographed on a Mono S column. The reaction intermediate bound to Mono S and eluted in a NH_4Cl gradient as an overlapping doublet between 700 mM and 800 mM NH_4Cl . These column fractions contained almost pure inactivation intermediate (Fig. 3.7B).

To compare the abilities of factor Va and the purified inactivation intermediate to assemble with factor Xa into a membrane-bound prothrombinase complex we determined initial steady state rates of prothrombin activation at limiting amounts of factor Va or factor Va cleaved at Arg⁵⁰⁶ as a function of the factor Xa concentration (Fig. 3.7A,B). Low amounts of factor Xa were required for full expression of prothrombinase activity in the case of normal factor Va ($K_{1/2}X_a = 0.088$ nM). With factor Va that was cleaved at Arg⁵⁰⁶ much higher factor Xa concentrations ($K_{1/2}X_a = 3.9$ nM) were needed to obtain maximal prothrombinase activity.

A

B

FIG. 3.7A,B. Cofactor activities of factor Va and factor Va cleaved at Arg⁵⁰⁶. Purified human factor Va (40 nM) was incubated with 15 nM APC in 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl₂ and 5 mg/ml BSA at 37°C. After 45 min the inactivation mixture was diluted 10-fold and applied to a Mono S column. Bound protein was eluted with 20 ml of a linear NH₄Cl gradient (50 - 1000 mM) in 25 mM Hepes (pH 7.5). Immunoblot analysis of factor Va (A) and the fraction eluting between 700 and 800 mM NH₄Cl (B) is shown in the inset. Aliquots taken from the inactivation mixture after 0 min (native factor Va) and from the column fraction were used to determine rates of prothrombin activation at (A) 1.1 ng/ml factor Va (▲) or (B) 1.2 ng/ml factor Va cleaved at Arg⁵⁰⁶ (■) in a reaction mixture containing

25 μ M phospholipid vesicles (DOPS/DOPC, 10/90, mol/mol), 0.5 μ M prothrombin and varying concentrations of factor Xa in 25 mM Hepes (pH 7.5), 175 mM NaCl, 2 mM CaCl₂ and 5 mg/ml BSA at 37°C. Rates of prothrombin activation were determined as described under "Materials and Methods" and were corrected for thrombin formed in the absence of factor Va. The solid lines represent hyperbolas obtained after fitting the data with $K_{1/2}Xa = 0.088$ nM and $V_{max} = 50.2$ nmoles Ila/min/ μ g factor Va ($k_{cat} = 151$ s⁻¹) for factor Va (▲) and $K_{1/2}Xa = 3.9$ nM and $V_{max} = 28.7$ nmoles Ila/min/ μ g protein ($k_{cat} = 72$ s⁻¹) for the column fraction [factor Va cleaved at Arg⁵⁰⁶] (■).

(Inset) Double reciprocal plots of the same data.

The V_{max} of prothrombin activation obtained with the purified inactivation intermediate was lower than that determined for native factor Va. The V_{max} values could be converted into k_{cat} values on basis of the protein contents of

the titrated fractions. This yielded k_{cat} values of 153 s^{-1} and 96 s^{-1} for native and cleaved factor Va, respectively.

The interaction of the membrane-bound factor Xa-Va complex with prothrombin was not affected by cleavage at Arg⁵⁰⁶ since the K_{m} values for prothrombin were virtually the same for prothrombinase complexes with normal and cleaved factor Va (data not shown).

From these data we conclude that APC-catalyzed cleavage at Arg⁵⁰⁶ of the heavy chain of factor Va results in the formation of a reaction product that has lost part of its cofactor activity mainly because of a reduction of its affinity for factor Xa.

The Effect of the Factor Va Concentration and the Assay Conditions on Observed Time Courses of Inactivation of Membrane-Bound Factor Va and Factor Va^{R506Q} by APC

The data presented in this chapter demonstrate that differences in rate constants of cleavage at Arg⁵⁰⁶ and Arg³⁰⁶ are primarily due to differences in the K_{m} for these peptide bond cleavages. This means that at high factor Va concentrations, where the rates of cleavage approach V_{max} , differences in time courses of inactivation of factor Va and factor Va^{R506Q} should become less pronounced.

We have also shown (Fig. 3.7) that the relative cofactor activities of native factor Va and factor Va cleaved at Arg⁵⁰⁶ strongly depend on the factor Xa concentration present in the factor Va assay. It was demonstrated that factor Va that is cleaved at Arg⁵⁰⁶ will exhibit much lower cofactor activity than native factor Va when assayed at factor Xa concentrations below $K_{1/2}\text{Xa}$ (3.9 nM).

These observations predict that the observed time courses of factor Va or factor Va^{R506Q} inactivation by APC will depend 1) on the factor Va concentration in the inactivation mixture and 2) on the assay conditions at which the loss of cofactor activity of factor Va is followed. Compared with factor Va^{R506Q}, normal factor Va will be more rapidly inactivated at low than at high factor Va concentrations. The apparent loss of cofactor activity of normal factor Va will be even more rapid and approach complete loss of activity much faster when samples from the inactivation mixture are assayed at a factor Xa concentration at which the inactivation intermediate has no activity i.e. $[\text{factor Xa}] \ll 3.9 \text{ nM}$ ($K_{1/2}\text{Xa}$).

These predictions were confirmed by an experiment in which time courses of factor Va inactivation were determined at low (Fig. 3.8A) or high concentrations of factor Va or Va^{R506Q} (Fig. 3.8B) and in which samples

from the same inactivation mixture were assayed for the loss of cofactor activity in assay mixtures that contained a low (Fig. 3.8A) or a high factor Xa concentration (Fig. 3.8B).

As predicted, the differences in APC-catalyzed loss of cofactor activity of normal factor Va and factor Va^{R506Q} were most pronounced when factor Va was inactivated at a low concentration and assayed in a prothrombin activation mixture that contained a low factor Xa concentration (Fig. 3.8A). Differences in time courses of inactivation of factor Va and factor Va^{R506Q} were indeed greatly reduced when high factor Va concentrations were inactivated by APC and the loss of cofactor activity was monitored in prothrombin activation mixtures that contained a high factor Xa concentration (Fig. 3.8B).

Fig. 3.8 The effect of assay conditions and factor Va concentration on the loss of cofactor activity during factor Va inactivation by APC. Purified human factor Va (▲) or factor Va^{R506Q} (■) was incubated with APC and phospholipid vesicles in 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl₂ and 5 mg/ml BSA at 37°C. At the indicated time points factor Va activity was determined as described in the "Materials and Methods" in assay mixtures that contained 10 nM or 0.4 nM factor Xa. (A) Inactivation mixture: 0.8 nM factor Va (▲) or 0.8 nM factor Va^{R506Q} (■), 0.15 nM APC, 25 μM phospholipid vesicles (DOPS/DOPC, 10/90, mol/mol); cofactor assay mixture: 0.4 nM factor Xa (B) Inactivation mixture: 210 nM factor Va (▲) or 210 nM factor Va^{R506Q} (■), 2.7 nM APC, 25 μM phospholipid vesicles (DOPS/DOPC, 10/90, mol/mol); cofactor assay mixture: 10 nM factor Xa.

3.5 Discussion

Proteolytic inactivation of membrane-bound factor Va by APC requires cleavage of two peptide bonds located at Arg⁵⁰⁶ and Arg³⁰⁶ in the heavy chain domain of factor Va (Fig. 3.1). At low factor Va concentrations cleavage at Arg⁵⁰⁶ occurs at a rate that is approximately 20-fold higher than the rate of cleavage at Arg³⁰⁶. This results in the accumulation of a reaction intermediate that is cleaved at Arg⁵⁰⁶ and that exhibits partial cofactor activity in prothrombin activation (Fig. 3.2). Cleavage at Arg³⁰⁶, which may occur both in the reaction intermediate and in native factor Va (see below), results in a complete loss of cofactor activity.

The cofactor activity of the factor Va derivative cleaved at Arg⁵⁰⁶ strongly depends on the factor Xa concentration in the prothrombin activation mixture in which factor Va is assayed. Cofactor activity of the reaction intermediate in prothrombin activation is only observed at high factor Xa concentrations. Compared with native factor Va, full expression of cofactor activity of factor Va that is cleaved at Arg⁵⁰⁶ requires a 45-fold higher factor Xa concentration. This indicates that cleavage at Arg⁵⁰⁶ in the heavy chain of factor Va affects its interaction with factor Xa and results in a considerable loss of affinity for factor Xa.

Factor Va^{R506Q} obtained from factor V purified from the plasma of a homozygous APC-resistant patient is slowly inactivated by APC (*cf.* ref¹²). This factor Va molecule lacks the cleavage site at Arg⁵⁰⁶, due to replacement of Arg by Gln¹⁸⁻²¹. Time courses of inactivation of low concentrations of membrane-bound factor Va^{R506Q} could be fitted with an equation for a single exponential curve. Immunoblotting experiments (fig. 3.3B) show preferential cleavage at Arg³⁰⁶ during the inactivation of membrane-bound factor Va^{R506Q}. These data indicate that APC inactivates factor Va^{R506Q} by a single cleavage at position Arg³⁰⁶ which results in the formation of a reaction product that has virtually lost its cofactor activity in prothrombin activation.

Under the conditions of our experiments (low factor Va concentrations) cleavage at Arg⁶⁷⁹ has a negligible contribution to the loss of cofactor activity in the case of APC-catalyzed inactivation of membrane-bound factor Va or factor Va^{R506Q}. This is concluded from the fact that cleavage at Arg⁶⁷⁹ (*i.e.* disappearance of the $M_r = 26,000/28,000$ fragment or appearance of doublet bands in the heavy chain or the $M_r = 54,000/56,000$ region of immunoblots) is only observed after time intervals at which more than 90% of the cofactor activity of factor Va is already lost.

These data suggest that inactivation of membrane-bound factor Va and factor Va^{R506Q} by APC proceeds via the pathways depicted below.

Scheme 3.1

In this scheme 3.1 factor Va_{int} exhibits partial cofactor activity and factor Va_i is a reaction product that has no detectable cofactor activity. We propose that APC-catalyzed cleavage at Arg⁵⁰⁶ and Arg³⁰⁶ in membrane-bound factor Va occurs in a random fashion and that cleavage at Arg³⁰⁶ is not affected by prior cleavage at Arg⁵⁰⁶. This is inferred from the fact that the rate constants for cleavage at Arg³⁰⁶ calculated from time courses of inactivation of normal and APC-resistant factor Va are approximately the same (k_{306} is $2.3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and $1.7 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ for normal Va and factor Va^{R506Q}, respectively).

Michaelis-Menten analysis provided additional information regarding the mechanism that accounts for the observed difference between k_{506} and k_{306} . The kinetic parameters determined for cleavage at Arg⁵⁰⁶ in normal factor Va were: K_m for factor Va = 20 nM and $k_{\text{cat}} = 0.96 \text{ s}^{-1}$. The peptide bond at Arg³⁰⁶ in factor Va^{R506Q} was cleaved with $K_m = 196 \text{ nM}$ and $k_{\text{cat}} = 0.37 \text{ s}^{-1}$. The fact that the calculated catalytic efficiencies (k_{cat}/K_m) have the same values as the second order rate constants calculated for cleavage at Arg⁵⁰⁶ in normal factor Va and for cleavage at Arg³⁰⁶ in factor Va^{R506Q} verifies that the Michaelis-Menten kinetic parameters are indeed associated with the indicated peptide bond cleavages. These data suggest that the differences in rate constants for cleavage at Arg⁵⁰⁶ and Arg³⁰⁶ mainly result from differences in the K_m and explain why at high factor Va concentrations ($> K_m$) differences between time courses of factor Va and factor Va^{R506Q} inactivation are much less pronounced (Fig. 3.8B).

APC can also fully inactivate factor Va and factor Va^{R506Q} in the absence of a membrane surface (Fig. 3.4). Under these conditions cleavage at Arg⁶⁷⁹ also contributes to the loss of factor Va cofactor activity. This is particularly observed for factor Va^{R506Q}, in which the peptide bond at Arg⁶⁷⁹ is even cleaved at a 8-fold higher rate than the site at Arg³⁰⁶. Cleavage at Arg⁶⁷⁹ does not require prior cleavage at Arg⁵⁰⁶ or Arg³⁰⁶ since immunoblot analysis shows the appearance of a doublet band in the M_r = 105,000 region of the gel, which indicates that cleavage at position Arg⁶⁷⁹ can already occur in the intact heavy chain.

Comparison of rate constants obtained for free and membrane-bound factor Va shows that phospholipids greatly accelerate APC-catalyzed factor Va inactivation. Part of this stimulation will presumably be due to the fact that both the substrate (factor Va) and the enzyme (APC) bind to the membrane surface, a condition that promotes their interaction and reaction in a way similar to that of phospholipid-dependent coagulation factor activations^{4,5,8,40-42}. However, in the case of APC-catalyzed factor Va inactivation, phospholipids do not accelerate the peptide bond cleavages to the same extent (Table I). Cleavage at Arg⁵⁰⁶ and Arg³⁰⁶ in normal factor Va is stimulated 300-600 fold, whereas cleavage at Arg³⁰⁶ in factor Va^{R506Q} is enhanced 1800-fold by phospholipids. This is indicative of a specific effect of phospholipids on APC-catalyzed cleavage at Arg³⁰⁶ (cf. ref.^{11, 12}). We observed that k_{679} is not significantly increased in membrane-bound factor Va. This demonstrates that after binding of factor Va to phospholipids the peptide bond at position Arg⁶⁷⁹ is hardly accessible to cleavage by membrane-bound APC.

Taken together our conclusions seem to contradict earlier observations of Kalafatis et al.^{11,12} who reported that APC-catalyzed cleavages in the heavy chain of membrane-bound factor Va occur in an ordered, sequential fashion in which cleavage at Arg⁵⁰⁶ promotes cleavage at Arg³⁰⁶ and Arg⁶⁷⁹. They also suggested that cleavage at Arg⁵⁰⁶ does not affect the cofactor activity of normal factor Va and that cleavage at position Arg⁶⁷⁹ is required for full inactivation of factor Va. These apparent discrepancies may be explained by our observations that 1) the relative rates of peptide bond cleavages in the heavy chain of factor Va depend on the factor Va concentration present in the inactivation mixture (Fig. 3.6) and 2) that the residual cofactor activity of partially active reaction intermediates strongly depends on the reaction conditions at which the functional activity of factor Va and its degradation products is assayed (Figs. 3.7 and 3.8). In this respect it is essential to mention that Kalafatis et al.^{11,12} collected their data at high factor Va concentrations (> 200 nM) and used a factor Va assay system with high

concentrations of coagulation factors, while our conclusions are based on experiments at low factor Va concentrations (<10 nM) and assessment of cofactor activity of factor Va in prothrombin activation mixtures that contained varying concentrations of coagulation factors.

To emphasize the importance of reaction and assay conditions, we demonstrated that the apparent loss of factor Va cofactor activity during inactivation by APC indeed strongly depends on the concentration at which factor Va is inactivated and on the prothrombinase conditions at which the loss of cofactor activity is monitored (Fig. 3.8). Time courses of factor Va and factor Va^{R506Q} inactivation at a high factor Va concentration, in which the loss of cofactor activity of factor Va is followed in prothrombin activation mixtures that contain a high factor Xa concentration, show relatively little difference (Fig. 3.8A and ref.¹²). However, when factor Va is inactivated at a low concentration and when its cofactor activity is assayed in a prothrombin activation mixture that contains a low amount of factor Xa, the differences between factor Va and factor Va^{R506Q} are profound (Fig. 3.8B). This observation may have significant physiological consequences. If thrombotic events result from ongoing coagulation at low concentrations of factor Xa and factor Va (the plasma factor V concentration is ~25 nM), down-regulation of the cofactor activity of factor Va by APC will be most efficient in the case of normal factor Va and will be maximally impaired in case of factor Va^{R506Q}. This underscores the increased risk for venous thromboembolism in individuals whose plasmas are APC-resistant as a consequence of a factor V phenotype in which Arg⁵⁰⁶ is substituted by Gln.

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Chapter 4

A Prothrombinase-Based Assay for Detection of Resistance to Activated Protein C

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4.1 Summary

In this chapter we present a new method for the detection of resistance to activated protein C (APC) that is based on direct measurement of the effect of APC on the cofactor activity of plasma factor Va. The factor V present in a diluted plasma sample was activated with thrombin and its sensitivity towards APC was subsequently determined by incubation with phospholipids and APC. The loss of factor Va cofactor activity was quantified in a prothrombinase system containing purified prothrombin, factor Xa and phospholipid vesicles and using a chromogenic assay for quantitation of thrombin formation. The reaction conditions were optimized in order to distinguish normal, heterozygous- and homozygous APC-resistant plasmas. Maximal differences in the response of these plasmas towards APC were observed when factor Va was inactivated by APC in the absence of protein S and when the cofactor activity of factor Va was determined at a low factor Xa concentration (0.3 nM).

Addition of 0.2 nM APC and 20 μ M phospholipid vesicles to a 1000-fold diluted sample of thrombin-activated normal plasma resulted in loss of more than 85 % of the cofactor activity factor Va within 6 min. Under the same conditions, APC inactivated ~ 60 % and ~20 % of the factor Va present in plasma samples from APC-resistant individuals that were heterozygous or homozygous for the mutation Arg⁵⁰⁶→Gln in factor V, respectively. Discrimination between the plasma samples from normal and heterozygous and homozygous APC-resistant individuals was facilitated by introduction of the so-called APC-sensitivity ratio (APC-sr). The APC-sr was defined as the ratio of the factor Va cofactor activities determined in thrombin-activated plasma samples after 6 min incubation with or without 0.2 nM APC and was multiplied by 100 to obtain integers ($\text{APC-sr} = \{\text{factor Va}_{+\text{APC}}/\text{factor Va}_{-\text{APC}}\} \times 100$). Clear differences were observed between the APC-sr of plasmas from normal healthy volunteers (APC-sr: 8-20, n=33) and from individuals that were heterozygous (APC-sr: 35-50, n=17) or homozygous APC resistant (APC-sr: 82-88, n=7). There was no mutual overlap between the APC-sr of normal plasmas and plasmas from heterozygous- or homozygous APC resistant individuals ($p < 0.0001$). In all cases our test gave the same result as the DNA-based assay. Since the test is performed on a highly diluted plasma sample there is no interference by conditions that affect APC resistance tests that are based on clotting time determinations (e.g. coagulation factor deficiencies, oral anticoagulation, heparin treatment, the presence of lupus anticoagulants, pregnancy or the use of oral contraceptives). Furthermore, we show that part

of the factor Va assay can be performed on an autoanalyzer which increases the number of plasma samples that can be handled simultaneously.

4.2 Introduction

In 1993, Dahlbäck et al.¹ described a hereditary defect in the anticoagulant response to activated protein C (APC) that is associated with an increased risk for venous thrombosis. This abnormality, called APC resistance, was subsequently identified as the most common defect in thromboembolic patients²⁻⁶ and was found in 20-50% of patients with venous thrombosis. APC resistance is associated with familial thrombophilia and is inherited as an autosomal dominant trait. The molecular defect responsible for APC resistance is a single point mutation in the gene encoding for coagulation factor V. This mutation results in the substitution of Arg⁵⁰⁶ by Gln in the heavy chain domain of factor V(a)⁷⁻¹¹. Since Arg⁵⁰⁶ constitutes one of three APC cleavage sites in factor Va¹², the mutated factor V(a), also described as factor V^{Leiden} or factor V^{R506Q}, is much less effectively inactivated by APC than normal factor Va^{13,14}. Since APC-catalyzed inactivation of factor Va is a crucial step in the down-regulation of thrombin formation this likely explains the increased risk for venous thromboembolism in individuals with APC-resistant plasma.

The requirement for reliable detection of APC resistance became important when it was discovered that at least 2 to 4 % of the Caucasian population is APC-resistant^{2,3,6,7}. Several methods for the diagnosis of APC resistance have been described so far¹⁵. Most of these methods are based on sequence analysis of genomic DNA^{7,16} or on measurement of the effect of APC on the activated partial thromboplastin time (APTT)^{1,17,18} or on thrombin generation in plasma¹⁹. APTT-based APC-resistance assays appear to be less reliable in the case of other coagulation disorders^{17,20-22}, anticoagulant therapy^{2,4,6,23}, pregnancy²¹ or the use of oral contraceptives^{24,25}.

In this chapter, we report a different method for the identification of APC-resistant plasmas. The method is based on direct measurement of the effect of APC on the activity of factor Va in diluted thrombin-activated plasma samples and subsequent determination of factor Va cofactor activity in prothrombin activation.

4.3 Materials and Methods

Materials and Buffers

The phospholipids 1,2-dioleoyl-sn-glycero-3-phosphoserine (DOPS) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) were obtained from Avanti Polar Lipids, Alabaster, Alabama, USA. The chromogenic substrates D-Phe-(pipercolyl)-Arg-pNA (S2238) and L-pyroGlu-Pro-Arg-pNA (S2366) were supplied by Chromogenix, Mölndal, Sweden. Recombinant human APC (rAPC) was a kind gift of Immuno A.G., Vienna, Austria. Human protein S was purchased from Enzyme Research Laboratories, Swansea, United Kingdom. Human prothrombin and human factor X were purified as described by DiScipio et al.²⁶. Human factor X was converted into factor Xa by incubation with the purified factor X activator from Russel's viper venom and factor Xa was isolated from the activation mixture by chromatography on soybean trypsin inhibitor-Sepharose²⁷.

All proteins used in the APC resistance test were diluted in buffer I (25 mM Hepes [pH 7.5], 175 mM NaCl, 3 mM CaCl₂ and 5 mg/ml BSA).

Phospholipid Vesicle Preparations

Small unilamellar phospholipid vesicles composed of a mixture of DOPS/DOPC (10/90, M/M) were prepared as described earlier²⁸. Phospholipid concentrations were determined by phosphate analysis²⁹.

Protein Concentrations

APC concentrations were determined with S2366 using kinetic parameters reported by Sala et al.³⁰. Thrombin concentrations were determined with the chromogenic substrate S2238³¹. Protein S concentrations were calculated from the A₂₈₀ using an A^{1%}₂₈₀ of 9.5 and M_r = 70,000 for protein S³². Factor V and factor Va concentrations were determined by measuring their activity in prothrombin activation^{14,33} under conditions described in the legends to the figures.

Collection and Handling of Plasma Samples

Nine parts of blood from normal healthy volunteers or from individuals (both men and women) that were shown to be heterozygous or homozygous for the Arg⁵⁰⁶→Gln mutation in factor V by DNA analysis³⁴ were collected in one part of 0.13 M trisodium citrate (pH 7.8). The blood was centrifuged twice for 15 minutes at 3,000 x g at room temperature. The platelet poor plasma thus obtained was stored at -80°C. The healthy volunteers had a normal APTT and prothrombin time, a normal response to APC and DNA analysis showed that they were not carrying the Arg⁵⁰⁶→Gln mutation. A normal plasma pool was obtained by mixing the plasma of 84 different healthy volunteers (both men and women). Coagulation factor concentrations (including protein S and C) of all volunteers were within the normal range.

Chromogenic APC-resistance test

The procedure that we have developed basically consists of three steps that are performed at 37°C:

- 1) activation of plasma factor V with thrombin
- 2) incubation of factor Va with or without APC
- 3) quantification of factor Va cofactor activity

Step 1: to two tubes (sample A en B) with 215 µl of 1/1000 dilutions of the same plasma sample in buffer I, 5 µl of a mixture of 175 nM thrombin and 1 mM phospholipid vesicles (10/90 M/M DOPS/DOPC) was added to activate the factor V present in the plasma sample.

Step 2: After 10 min 10 µl buffer I was added to sample A and 10 µl purified rAPC (4.6 nM) was added to sample B and the incubation at 37°C was continued.

Step 3: After 6 min incubation with APC, 10 µl prothrombin (25 µM) and 10 µl factor Xa (7.5 nM) were added to the samples A en B in order to determine the factor Va cofactor activity present. After 1 min, the amount of thrombin generated in the reaction aliquots was quantitated by transferring a 10 µl aliquot into a disposable cuvette with 990 µl of a buffer containing 235 µM S2238 in 50 mM Tris (pH 7.5 at 37°C), 175 mM NaCl, 0.5 mg/ml ovalbumin and 20 mM EDTA. The factor Va concentration in the plasma sample was calculated from the thrombin formed using a calibration curve made with known amounts of purified factor Va³⁴.

The final concentrations of essential reaction components in the different steps were: *step 1*) 1/1000 dilution of plasma sample, 4 nM thrombin and 22.7 µM DOPS/DOPC (10/90, M/M) vesicles; *step 2*), 0.2 nM APC and

21. 7 μM DOPS/DOPC (10/90) vesicles; step 3) 0.25 μM prothrombin, 0.3 nM factor Xa and 20 μM DOPS/DOPC (10/90) vesicles. The factor Va assay procedure (step 3) is essentially the same as reported earlier³³. However, in this particular application factor Va was assayed at 0.3 nM factor Xa since at this factor Xa concentration the residual cofactor activity of the reaction intermediate cleaved at Arg⁵⁰⁶ is negligible compared to native factor Va¹⁴. This increased the difference in response between normal and factor Va^{R506Q} in the test (see Results). Plasma samples were assayed within 1 hour of thawing, although samples, kept at room temperature for 20 hours, could be assayed without loss of assay accuracy. The intra-assay variation of the test was 3% (n=17), the inter-assay variation is 4% (n=20).

Semi-automated method for the detection of APC resistance:

Plasma samples were diluted 750 times in a 25 mM Hepes buffer (pH 7.5), containing 175 mM NaCl, 5 mM CaCl₂ and 5 mg/ml BSA, (buffer II). After prewarming two aliquots of 400 μl of the same plasma sample at 37 °C, 16 μl of a mixture of 67 nM thrombin and 667 μM phospholipids (10/90 DOPS/DOPC M/M) without (sample A) or with 10 nM APC (sample B) was added yielding final concentrations of 4 nM thrombin, 20 μM phospholipid vesicles with or without 0.39 nM APC, respectively. After 20 min incubation, i.e. activation of plasma factor V (sample A) or activation and inactivation of plasma factor V (sample B) the reaction was stopped by the addition of 4500 μl cold buffer I (4°C). After dilution of the APC-treated plasma sample APC-catalyzed factor Va inactivation at 37°C is slowed down ~100-fold and becomes considerably slower than the rate of spontaneous loss of factor Va cofactor activity (< 0.3 %/min at 37°C). Since spontaneous loss of factor Va activity occurs both in non-treated and APC-treated plasma samples any loss of factor Va activity during the preincubation/incubation procedure in the ACL-300 will not affect the APC-sr.

The cofactor activity of factor Va present in the plasma samples was determined on an ACL-300 Research Automated Coagulation apparatus (Instrumentation Laboratory, Milan, Italy). Prothrombin activation was started by mixing 50 μl aliquots of the plasma dilutions with 50 μl 2 μM prothrombin, 0.6 nM factor Xa and 40 μM phospholipids (10/90 DOPS/DOPC M/M) in 25 mM Hepes (pH 7.5), 175 mM NaCl. After 2 min prothrombin activation was stopped by mixing with 50 μl of 60 mM EDTA in 25 mM Hepes (pH 7.5), 175 mM NaCl, 0.5 mg/ml ovalbumin and 2.35 mM S2238 and the thrombin present was assayed by monitoring S2238 conversion for 1 minute at 405 nm. Under the conditions described above S2238 conversion appeared to be proportional with the amount of factor Va present in the plasma sample.

Statistics

Data are presented as ranges of values observed, median values or as mean $\pm$ S.E.M.. The Student's t-test was used to determine the significance of differences between test results of groups of plasmas.

4.4 Results

APC-mediated inactivation of factor Va generated in normal-pooled plasma and in plasmas from heterozygous and homozygous APC-resistant individuals.

The chromogenic method to screen plasmas for APC resistance is actually based on earlier publications from our group concerning a sensitive assay for factor Va³³, kinetic analysis of APC-catalyzed inactivation of factor Va and factor Va^{R506Q} ref¹⁴ and the effects of protein S and factor Xa thereon³⁵.

Recently, we have shown¹⁴ that APC inactivates normal factor Va 20 times faster than factor Va^{R506Q}. The difference between the observed rates of inactivation of factor Va and factor Va^{R506Q} was maximal when the loss of cofactor activity of factor Va was monitored in prothrombin activation mixtures that contained a low factor Xa concentration (e.g. 0.3 nM, cf. ref. 14). Furthermore, we reported that differences in the rates of inactivation of factor Va and factor Va^{R506Q} were less pronounced or even nullified when factor Va was inactivated in the presence of protein S and/or factor Xa³⁵.

Since the plasma of normal healthy volunteers contains ~ 25 nM factor V³⁶ and since our factor Va assay allows quantitation of factor Va as low as 0.2 pM it is possible to determine the sensitivity of plasma factor Va towards APC at high plasma dilutions (>1000-fold), independent from and unaffected by other plasma components. The procedure that we have developed for distinguishing normal and APC-resistant plasmas basically consists of three steps:

- 1) activation of the factor V present in a highly diluted plasma sample with thrombin
- 2) incubation of factor Va in the presence of phospholipids with or without APC
- 3) quantification of factor Va cofactor activity.

4) In order to obtain optimal differentiation between plasmas from normal healthy individuals, heterozygous and homozygous APC individuals the inactivation of factor Va should theoretically be performed in the absence of factor Xa and protein S³⁵ and the cofactor activity of factor Va should be assayed at a low factor Xa concentration¹⁴.

Fig. 4.1A shows that under these conditions the factor Va formed by thrombin in a normal plasma sample was indeed readily inactivated by 0.2 nM APC whereas the factor Va present in the plasma sample from a heterozygous APC-resistant patient showed intermediate sensitivity and that in plasma from a homozygous APC-resistant patient was very slowly inactivated by APC. The differences in response

towards APC were much less pronounced when protein S was present during factor Va inactivation (Fig. 4.1B) or when the loss of cofactor activity of factor Va was assessed in a prothrombinase mixture that contained a high concentration of factor Xa (Fig. 4.1C).

For practical reasons it is preferable that an APC-resistance test is based on measurement of sensitivity of plasma factor Va towards APC at a single time point. Quantitative comparison of the plasma samples was facilitated by expressing their response towards APC in terms of the so-called APC sensitivity ratio (APC-sr, *cf.* ref. 2, 11, 17-18). The APC-sr is defined as the ratio of the factor Va activities determined after incubation of the plasma factor Va in the presence and absence of APC and was multiplied by 100 to obtain integers ($\text{APC-sr} = \{\text{factor Va}_{+\text{APC}}/\text{factor Va}_{-\text{APC}}\} \times 100$). It appeared that under the reaction conditions of the experiment presented in Fig. 4.1A, clear differences between the APC-sr of the various plasmas were obtained for incubation times between 2 and 15 min. Therefore, in the case of a single time point assay, we have arbitrarily chosen for determination of the APC-sr after 6 min incubation with APC. Under these conditions the APC-sr was 13 for normal pooled plasma and 50 and 81 for heterozygous- and homozygous APC-resistant plasma, respectively.

FIG. 4.1 **Effect of APC on the activity of factor Va generated in normal plasma and in plasma from heterozygote- and homozygote APC-resistant individuals.** Normal-pooled plasma (■), plasma from heterozygous (○) or homozygous (▲) APC-resistant individual was diluted 1000 times in buffer I and factor V was activated at 37 °C with 4 nM thrombin in the presence of phospholipid vesicles (10/90 DOPS/ DOPC M/M). After 10 min APC with or without protein S was added to

inactivate factor Va. The formation and loss of factor Va cofactor activity was followed as described in "Materials and Methods". For each plasma sample the factor Va activity is given as % of its plateau value. Time courses of factor V activation in the different plasmas and the spontaneous loss of factor Va activity were indistinguishable and are therefore, given in the figure as a single averaged curve (●) for the three different plasmas. The final concentrations of components during the inactivation and assay procedures were: (A) 0.2 nM APC and 21.7 μ M phospholipid vesicles during inactivation and 0.3 nM factor Xa, 1 μ M prothrombin and 20 μ M phospholipid vesicles in the factor Va assay mixture; (B) 0.2 nM APC, 200 nM protein S and 21.7 μ M phospholipid vesicles during inactivation and 0.3 nM factor Xa, 1 μ M prothrombin and 20 μ M phospholipid vesicles in the factor Va assay mixture and (C) 0.2 nM APC and 21.7 μ M phospholipid vesicles during inactivation and 10 nM factor Xa, 1 μ M prothrombin and 20 μ M phospholipid vesicles in the factor Va assay mixture.

Influence of plasma dilution on the APC-sr

To establish the range of plasma dilutions at which the APC resistance test can be performed, we have determined the APC-sr as a function of the plasma dilution (Fig. 4.2).

FIG. 4.2 *The influence of the plasma dilution on the response of the various plasmas to APC. The factor V present in varying dilutions of normal-pooled plasma (■) or plasma from a heterozygous (○) or a homozygous APC-resistant individual (▲) was activated by incubating the plasma dilution during 10 min with 4 nM thrombin at 37°C. Factor Va was further incubated for 6 min with or without 0.2 nM APC in the presence of 21.7 μM phospholipid vesicles (10/90*

DOPS/DOPC M/M). The remaining cofactor activity of factor Va was determined in a prothrombinase mixture containing 0.3 nM factor Xa, 1 μM prothrombin and 20 μM phospholipid vesicles (10/90 DOPS/DOPC M/M). The APC-sr (factor Va_{+APC} /factor Va_{-APC}) was corrected for an assay blank, determined with a reaction mixture that did not contain plasma. For practical reasons, the APC-sr was multiplied by 100 in order to obtain integers. Further experimental details are given under 'Materials and Methods'.

At low plasma dilutions the APC-sr of the different plasmas approached the same value (~50). This may be caused by effects of other plasma proteins (e.g. protein S, *cf. ref. 35*) on the inactivation of factor Va by APC or by underestimation of the amount of factor Va present in the plasma sample. When too much factor Va is introduced in the factor Va assay mixture, factor Xa and prothrombin become limiting, which will cause an underestimation of the factor Va concentration especially in the plasma sample that was not treated with APC.

Reliable differences between the APC-sr of normal and APC-resistant plasmas were obtained when the test was performed on plasma samples that were diluted 500 to 5000 times. At higher dilutions of the plasma samples, the thrombin used to activate factor V in the plasma sample significantly contributed to the thrombin determined in the factor Va assay. Correction for this contribution (typically <5% in the absence of APC, and 5-25% in the presence of APC) required subtraction of an assay blank in which plasma was substituted with buffer.

Test results for a population of normal plasmas and plasmas from heterozygous or homozygous APC-resistant individuals.

Plasmas from a number of normal (n=33), heterozygous (n=17) and homozygous individuals (n=7), both men and women, were screened using the method described in the previous paragraphs. For all plasmas the presence or absence of the factor V^{R506Q} mutation was verified by genetic analysis of genomic DNA samples. In agreement with the results presented in previous paragraphs for the single plasmas, clear differences were observed for the three different groups of plasmas (Fig. 4.3).

FIG. 4.3 **Range of APC-sr of plasmas from healthy volunteers and heterozygous- and homozygous APC-resistant individuals.** The effect of APC on the cofactor activity of factor Va generated in a 1/1000 dilution of the plasma samples was determined as described in the Materials and Methods (cf. Fig. 4.2). The plasma samples were classified as belonging to normal healthy individuals (○), heterozygous APC-resistant individuals (◐) or homozygous APC-resistant individuals (●) by DNA analysis. The data obtained were plotted as

APC-sr = factor Va_{+APC}/factor Va_{-APC} multiplied by 100. Median values are indicated per group. P-values are given. The position of the theoretical APC-sr (see Discussion) is indicated per group of plasma samples (→).

The APC-sr values of the plasmas actually divided into three populations: 12.3 ± 0.38 (mean $\pm$ S.E.M.), for normal plasmas, 42.3 ± 1.0 for the plasmas of heterozygous APC-resistant individuals and 85.6 ± 0.63 for the plasmas of homozygous APC-resistant individuals. The ranges of APC-sr observed for these three populations were 8-20, 35-50 and 82-88 respectively. There was no overlap between the three groups of plasmas, i.e. the APC-sr of homozygous APC resistant individuals was significantly higher than the APC-sr of heterozygous APC-resistant individuals ($P<0.0001$). A similar difference was observed between the APC-sr of the plasma of heterozygous APC-

resistant individuals and the plasma of normal individuals. The outcome of the chromogenic APC resistance test was in full agreement with the results obtained by DNA analysis.

The chromogenic APC resistance test and plasma with coagulation abnormalities

APC resistance tests that are based on measurement of the effect of APC on the APTT give less reliable results in plasmas with abnormal APTT values e.g. in the case of coagulation factor deficiencies^{17, 20,21}, anticoagulant treatment with heparin or oral anticoagulants^{2,4,6,17,23}, the presence of Lupus anticoagulant²², pregnancy²¹ or the use of oral contraceptives^{24,25}. Since our test is performed on highly diluted plasma samples it is only dependent on plasma factor V and is not dependent on other plasma components. Hence, it is not surprising that for all "abnormal plasmas" tested the APC-sr values obtained by our assay were within the range of normal plasmas (Table 4.1).

Table 4.1

**APC-sr for normal plasmas and plasmas
with abnormal coagulation properties**

Plasma	APC-sr [#]	range	n
Plasma from normal individuals	12.3 ± 0.38	8-20	33
Heterozygous APC resistant plasma	41.9 ± 1.0	35-50	17
Homozygous APC resistant plasma	86.0 ± 0.63	82-88	7
Protein S deficient plasma*	12.8 ± 0.70	12-14	2
Plasma (oral anticoagulation)	15.4 ± 1.20	12-20	8
Plasma (Heparin treatment)	15.9 ± 0.74	14-18	4
Plasma (oral contraception)	13.1 ± 0.49	10-18	15
Plasma (pregnancy)	12.6 ± 0.61	12-14	3
Plasma (Lupus anticoagulans)	12.0 ± 0.96	10-14	3

[#]The APC-sr for the different plasmas was determined as described in the legend of Fig. 4.2.

Mean ± S.E.M. are given, n: number of different plasma samples tested.

* immunodepleted and plasma from a heterozygous protein S deficient patient (<5% free protein S antigen)

Modification of the chromogenic APC resistance test for use on an autoanalyzer.

Although the test described above is rather easy to perform we have adapted the assay for use on an ACL-300 Research Automated Coagulation Apparatus. In this application the plasma dilution, the activation of factor V and inactivation of factor Va were performed outside the apparatus but the factor Va was determined on the autoanalyzer. It appeared possible to activate plasma factor V and inactivate the factor Va with APC in a single step by incubating the plasma dilution with 20 μ M phospholipids, 4 nM thrombin in the absence or presence of 0.4 nM APC during 20 min. After this incubation the sample was diluted 12-fold with cold Hepes buffer and stored on ice in order to block further factor Va inactivation. The factor Va present in the plasma samples thus obtained appeared to be stable for at least one hour, which allowed a factor Va determination on the autoanalyzer. In the autoanalyzer the treated plasma sample was mixed with a solution containing prothrombin, factor Xa and phospholipid vesicles ($t = 0$ min) and subsequently with a S2338/EDTA mixture ($t = 2$ min) to stop prothrombin activation and to determine thrombin. Under the chosen assay conditions the thrombin formed was proportional to the amount of factor Va present in the plasma sample. The results obtained with the semi-automated method are shown in fig. 4.4 and are in good agreement with the data shown in Fig. 4.3, they also gave a distinct APC-sr for the normal plasmas and the heterozygous and homozygous APC-resistant plasmas. The differences between the three groups of plasmas tested were highly significant ($p < 0.0001$). The APC-sr (mean $\pm$ S.E.M.) for the three groups of plasmas tested were 12.2 ± 0.24 , 45.3 ± 0.72 and 77.3 ± 0.3 for the plasmas of normal, heterozygous APC-resistant and homozygous APC-resistant individuals respectively.

4. 5 Discussion

In this chapter we present a new functional test for the screening of plasmas for APC resistance that is based on probing the effect of APC on the cofactor activity of factor Va that is present in a thrombin-activated plasma sample. APC-catalyzed loss of cofactor activity of normal factor Va is due to proteolytic cleavage of peptide bonds located at Arg⁵⁰⁶ and Arg³⁰⁶ of the heavy chain of factor Va (ref 12-14). Factor Va from APC-resistant individuals (factor Va^{R506Q}) is much slower inactivated by APC¹⁴ since it lacks the cleavage site at Arg⁵⁰⁶.

In this chapter we show that maximal differences in APC-mediated loss of factor Va cofactor activities, present in thrombin-activated plasma samples from normal individuals and heterozygous and homozygous APC-resistant individuals, were obtained when 1) factor Va was inactivated by APC in a high plasma dilution in the absence of factor Xa and protein S and when 2) the factor Va cofactor activity was determined in a prothrombinase mixture containing a low factor Xa concentration (Fig. 4.1).

This led to a rapid and simple method for the screening of plasmas for APC resistance. Our procedure is based on comparison of the amounts of factor Va cofactor activity present in thrombin-activated plasma samples that were incubated during 6 minutes with phospholipid vesicles either in the absence or presence of 0.2 nM APC. The anticoagulant response of a given plasma sample to APC can be easily quantitated from the so-called APC sensitivity ratio (APC-sr) which is defined as the ratio of amounts of factor Va present in the APC-treated and the non-treated plasma sample. The chosen reaction conditions allow full distinction between the normal plasmas and the plasmas from individuals that were shown to be heterozygous or homozygous APC resistant by DNA analysis (Fig. 4.3). This is in contrast to results obtained with APTT-based APC-resistance assays, in which borderline zones for the APC response are observed in which it is difficult to distinguish normal, heterozygous and homozygous APC-resistant individuals^{11,15,37} unless the plasma sample is diluted in factor V-deficient plasma^{38,39} or buffer⁴⁰. The functional method for screening of APC resistance described in this chapter gives results that are fully compatible with those obtained by DNA analysis. This is not surprising since the determination is performed on highly diluted plasma samples which makes the assay independent of other plasma components that may affect inactivation or quantitation of the factor Va present in the plasma sample. This has the additional advantage that our assay is not affected by conditions that may interfere with coagulation-based tests such as coagulation factor abnormalities^{20,21}, the presence of lupus

anticoagulants²², anticoagulant treatment^{2,4,6,17,23}, pregnancy²¹ or the use of oral contraceptives^{24,25} (Table 4.1). A number of the heterozygote and homozygote APC-resistant individuals had been suffering from episodes of venous thromboembolism. In the prothrombinase-based assay they were indistinguishable from healthy APC-resistant individuals.

It should be emphasized that the actual value of the APC-sr is dependent on reaction conditions such as pH, temperature, the concentrations of APC and phospholipid vesicles and the time of incubation with APC. When the reaction conditions are not strictly controlled, different APC-sr may be obtained. Day to day variation of the APC-sr can, however, be minimized by predetermining the APC-sr of a normal pooled plasma as a function of the incubation time with APC and taking in the actual test an incubation time that yields an APC-sr of 10-15 in the pilot experiment.

Actually, the APC-sr obtained in the experiment presented in Fig.4.3 should equal a theoretical value that can be calculated using rate constants for APC-catalyzed cleavage of the peptide bonds located at Arg⁵⁰⁶ and Arg³⁰⁶ reported in chapter 3¹⁴. Using rate constants of $6.7 \cdot 10^{-5} \text{ s}^{-1}$ for the spontaneous loss of factor Va cofactor activity and $4.3 \cdot 10^7 \text{ M}^{-1} \text{ s}^{-1}$ for APC-catalyzed cleavages at Arg⁵⁰⁶ and $2.3 \cdot 10^6 \text{ M}^{-1} \text{ s}^{-1}$ for cleavage at Arg³⁰⁶ (ref¹⁴), theoretical APC-sr of 10, 46 and 85 are calculated for the plasma of normal individuals, heterozygous (assuming 50% normal factor V and 50% factor V^{R506Q}) and homozygous APC-resistant individuals, respectively. The theoretical values for the APC-sr closely approach the values experimentally obtained (Fig. 4.3).

We have shown that it is possible to use an autoanalyzer in order to increase the number of samples that can be tested simultaneously. In the semi-automated method the activation of plasma factor V by thrombin and the inactivation of factor Va by APC occur in a single step in a 37°C waterbath and the amount of factor Va present in the plasma sample is determined on an autoanalyzer by subsequently mixing the plasma sample with prothrombinase components (phospholipids, Ca²⁺ions, prothrombin and factor Xa) and with a solution containing the thrombin-specific chromogenic substrate S2238 in EDTA (to block further prothrombin activation). When the reaction conditions and the incubation times were chosen properly, the rate of S2238 conversion appeared to be proportional with the amount of factor Va present in the plasma sample and values for APC-sr allowed discrimination between normal, heterozygous and homozygous APC-resistant plasmas (Fig. 4.4).

The combination of reliability, reproducibility and easy performance, together with the fact that the assay is not disturbed in plasmas with abnormal

coagulation characteristics, make the APC-resistance test that is presented in this chapter an excellent method to screen plasma samples for APC resistance. The test actually gives direct information on the presence of Arg506→Gln mutation and gives distinct results with normal, homozygous and heterozygous APC-resistant plasmas that are fully compatible with those obtained with DNA-based assays. It is to be expected that the test will also recognize other abnormalities related to the factor V gene that can cause APC resistance (e.g. mutations or variable expression of factor V alleles in R506Q heterozygotes).

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Chapter 5

Effect of activated protein C on thrombin generation and on the thrombin potential in plasma of normal and APC-resistant Individuals

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5.1 Summary

This chapter describes the effect of activated protein C (APC) on thrombin generation initiated in platelet poor plasma via the extrinsic or the intrinsic pathway. Thrombin was determined with a specific chromogenic substrate and quantitated by calculating the time integral of the thrombin generation curve, *i.e.* the endogenous thrombin potential (ETP). Addition of APC to normal plasma after both extrinsic and intrinsic initiation of coagulation resulted in a dose-dependent inhibition of thrombin generation as reflected by the decrease of the ETP. Data obtained in intrinsically-triggered plasma of normal individuals were subject to rather large variation. Therefore, the effect of APC on thrombin generation in APC-resistant plasmas was only studied in extrinsically-stimulated reaction systems. APC had much less effect on the ETP of plasma from individuals that were heterozygous or homozygous for the mutation Arg⁵⁰⁶ → Gln⁵⁰⁶ in factor V (APC resistance). There appears to be a linear relationship between the ETP and the amount of α_2 -macroglobulin-thrombin complex (α_2 M-IIa) that accumulates in plasma during thrombin formation. Since the α_2 M-IIa complex possesses amidolytic activity, we measured the effect of APC on thrombin generation via the so-called normalized APC sensitivity ratio (APC-sr). The latter was defined as the ratio of the end levels of amidolytic activity of the α_2 M-IIa complex determined in the presence and absence of 50 nM APC (α_2 M-IIa_{+APC}/ α_2 M-IIa_{-APC}) divided by the ratio of a normal plasma pool. Significant differences ($p < 0.001$) were observed between APC-sr of plasmas from normal individuals (APC-sr: 0.5-1.9, $n=25$) and of plasmas from individuals that were heterozygous (APC-sr: 2.1-6.7, $n=17$) or homozygous APC resistant (APC-sr: 3.9-5.9, $n=5$). There was no overlap between APC-sr of normal plasmas and plasma from individuals, bearing the factor V mutation. Abnormal APC-sr in certain plasmas (pregnancy, use of oral contraceptives, anticoagulant therapy, protein S deficiency or Lupus anticoagulant) were corrected by performing the assay on a plasma sample that was diluted 10-fold in factor V-deficient plasma. Our data show that measurement of the effect of APC on the ETP yields valuable information about the (pro)thrombotic status of plasma (e.g. APC resistance, pregnancy, use of oral contraceptives).

5.2 Introduction

APC-resistance¹ is a hereditary disorder which appears to be associated with an increased risk for venous thrombosis.²⁻⁶ APC-resistance is caused by a single point mutation (G1691→A) in the gene encoding for coagulation factor V which results in the replacement of Arg⁵⁰⁶ by Gln in the heavy chain domain of factor V(a).⁷⁻¹¹ Since Arg⁵⁰⁶ is located at a predominant APC cleavage site¹², the mutated factor V molecule (also called factor V_{Leiden} or factor V^{R506Q}) is less efficiently down-regulated by APC^{13,14} which probably explains the increased risk for venous thromboembolism in APC resistant individuals. Since the prevalence of APC resistance is relatively high (3-5% in Europe)^{2,3,6,7} screening for the presence of the mutation is useful and advisable in several clinical situations.^{15,16}

The methods that are currently used for the diagnosis of APC resistance are either variations of APTT assays^{1,17} or are based on sequence analysis of genomic DNA.^{7,18} This chapter, describes an essentially different method. The technique is based on measurement of the effect of APC on the time course of thrombin generation in plasma that is triggered either via the extrinsic pathway or via the intrinsic pathway. As a characteristic parameter we have determined the time integral of thrombin formation, i.e. the endogenous thrombin potential (ETP).¹⁹⁻²³ The ETP appears to be a good indicator for the pro- or anti-thrombotic condition of a plasma sample and can be used to screen individuals for the presence of congenital and acquired APC resistance.

5.3 Materials and Methods

Materials

The phospholipids 1,2-dioleoyl-*sn*-glycero-3-phosphoserine (DOPS), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) were obtained from Avanti Polar Lipids, Alabaster, Alabama, USA. D-Phe-(pipecolyl)-Arg-pNA (S2238) and L-pyroGlu-Pro-Arg-pNA (S2366) and Coatest® APCTM Resistance APTT reagent (used to initiate the intrinsic coagulation pathway) were supplied by Chromogenix, Mölndal, Sweden. The extrinsic pathway of coagulation was initiated in plasma samples with relipidated recombinant tissue factor (Innovin® without additives), which was a kind gift of Dr. H. Pelzer, Dade International, Florida, USA. Recombinant human APC (rAPC) was a kind gift

of Immuno A.G., Vienna, Austria. Ancrod[®] was obtained from the WHO International Laboratory for Biological Standards, Hertfordshire, England. The fibrin polymerization inhibitor Pefabloc Fg[®] was purchased from Pentapharm, Basel, Switzerland. Immuno-depleted factor V-deficient plasma was obtained from Organon Teknika, Durham, NC, USA.

Phospholipid vesicle preparations

Small unilamellar phospholipid vesicles composed of a mixture of DOPS/DOPE/DOPC (20/20/60, M/M/M) were prepared as described earlier.²⁴ Phospholipid concentrations were determined by phosphate analysis.²⁵

Protein concentrations

APC concentrations were determined with S2366 using kinetic parameters reported by Sala *et al.*²⁶ Thrombin concentrations were determined with the chromogenic substrate S2238.²⁷

Collection and handling of plasma samples

Nine parts of blood from normal healthy volunteers or from individuals heterozygous or homozygous for the mutation Arg⁵⁰⁶→Gln in factor V was collected in one part of 0.13 M trisodium citrate (pH 7.8). The blood was centrifuged twice for 15 min at 3,000 g at room temperature. The platelet poor plasma thus obtained was stored at -80°C until it was used. Plasma defibrination was achieved by adding 1 U/ml Ancrod[®] to a plasma sample and allowing the fibrin formed to polymerize during 10 min at 37°C. The resulting clot was removed from the plasma by winding it out on a plastic spatula.

The normal donors were healthy individuals with a normal APTT and prothrombin time, a normal response to APC and which after DNA analysis were identified as not carrying the mutation Arg⁵⁰⁶→Gln. The APC-resistant donors were also healthy individuals but they were characterized as heterozygous or homozygous APC resistant by PCR analysis of genomic DNA.²⁸ Coagulation factor concentrations (including protein S and C) were all within the normal range. A pool of plasma from 10 different healthy donors was used in the experiments presented in Figures 5.1 and 5.2.

Determination of thrombin generation in plasma

Extrinsically initiated thrombin generation curves¹⁹ were determined as follows: 240 μ l of defibrinated plasma was mixed with 60 μ l of a solution containing 9.0 μ M of phospholipid vesicles (DOPS/DOPE/DOPC, 20/20/60, M/M/M) in 25 mM Hepes (pH 7.5), 175 mM NaCl, 5 mg/ml BSA. The mixture was incubated for 3 min at 37°C and thrombin generation was subsequently started by the addition of 60 μ l of a prewarmed mixture containing 0.6 ng/ml relipidated recombinant tissue factor in 25 mM Hepes buffer (pH 7.5), 175 mM NaCl, 5 mg/ml BSA and 100 mM CaCl_2 with or without 200 nM rAPC. This resulted in final concentrations of 1.5 μ M phospholipid, 0.1 ng/ml relipidated tissue factor, 50 nM rAPC and 8 mM CaCl_2 . At regular time intervals, 10 μ l samples were withdrawn and the amidolytic activity towards S2238 (thrombin plus α_2 M-thrombin) was determined as described before.²³

Intrinsically initiated thrombin generation curves were obtained in a similar way: 320 μ l of defibrinated plasma was mixed with 160 μ l of the Coatest® APC™ Resistance APTT reagent (containing purified phospholipids and colloidal silica) and the mixture was incubated for 4 min at 37°C. Thrombin generation was started by the addition of 20 μ l of a prewarmed mixture containing 400 mM CaCl_2 in 25 mM Hepes (pH 7.5), 175 mM NaCl, 5 mg/ml BSA with or without 1250 nM rAPC. Final concentrations of reaction components were 8 mM CaCl_2 , 50 nM rAPC and 160 μ l APTT reagent per 500 μ l final volume. Sampling and stopping methods, as well as chromogenic determination of thrombin formation were performed as described for the extrinsically initiated thrombin generation tests (TGT).²³

The thrombin generation curves thus obtained were corrected for the contribution of the α_2 -macroglobulin-thrombin (α_2 M-IIa) complex to the total amidolytic activity as described earlier.¹⁹ The resulting curve, representing the free thrombin concentration as a function of time, was used to calculate the ETP,¹⁹ which was defined as the time-integral of free thrombin concentration in a TGT.¹⁹⁻²³

Determination of the effect of APC on the ETP of plasmas by measurement of end levels of the α_2 M-IIa complex

Thrombin generation curves generally reach a residual level of amidolytic activity that can be attributed to the α_2 M-IIa complex.^{19,22,29-31} The end level of α_2 M-IIa is constant in time and is a direct indicator for the ETP in plasmas with the same α_2 M levels.²² This is particularly the case when the same plasma is tested with and without APC. The relationship between the end

level of $\alpha_2\text{M-IIa}$ and the ETP of a given plasma sample can be calculated from the rate of formation of the $\alpha_2\text{M-IIa}$ complex *i.e.*:

$$d[\alpha_2\text{M-IIa}]/dt = k \cdot [\text{IIa}]_t \cdot [\alpha_2\text{M}]_t \quad \text{eq. 1}$$

in which k is the second order rate constant for $\alpha_2\text{M-IIa}$ complex formation and $[\text{IIa}]_t$ and $[\alpha_2\text{M}]_t$ are the free thrombin (IIa) and $\alpha_2\text{M}$ concentrations at time t , respectively. Equation (1) yields:

$$\int (d[\alpha_2\text{M-IIa}]/dt) dt = \int (k \cdot [\text{IIa}]_t \cdot [\alpha_2\text{M}]_t) dt \quad \text{eq. 2}$$

Since $[\alpha_2\text{M}]_0 \gg [\alpha_2\text{M-IIa}]_t$, $[\alpha_2\text{M}]_0 \approx [\alpha_2\text{M}]_t$ equation (2) can be rearranged into:

$$[\alpha_2\text{M-IIa}]_t = k \cdot [\alpha_2\text{M}]_0 \cdot \int [\text{IIa}]_t dt = k \cdot [\alpha_2\text{M}]_0 \cdot [\text{ETP} + c] \quad \text{eq. 3}$$

in which ETP is the time integral of the free thrombin concentration in the TGT. Since at $t=0$, $[\alpha_2\text{M-IIa}]=0$, it follows that $c=0$ and that

$$[\alpha_2\text{M-IIa}]_t = k \cdot [\alpha_2\text{M}]_0 \cdot \text{ETP} \quad \text{eq. 4}$$

Equation (4) verifies that variations of the ETP in plasma with identical $[\alpha_2\text{M}]_0$ will result in a proportional variation of the end level of the $\alpha_2\text{M-IIa}$ complex. This implies that the effect of APC on the ETP of a given plasma sample can be simply quantitated on the basis of single amidolytic assays of the end levels of $\alpha_2\text{M-IIa}$ in plasma determined in the presence and absence of APC (see below).

APC sensitivity ratio: APC-sr

Similar to APTT-based diagnostic methods for APC resistance^{1,2,6,32} an APC sensitivity ratio (APC-sr) has been defined, to screen plasmas for the effect of APC on thrombin generation. We have used the so-called normalized APC sensitivity ratio,³² defined as the ratio of the endlevels of $\alpha_2\text{M-IIa}$ determined in the presence and absence of APC divided by the ratio determined in the normal plasma pool *i.e.*:

$$\text{APC-sr} = (\alpha_2\text{M-IIa}_{+\text{APC}}/\alpha_2\text{M-IIa}_{-\text{APC}})/(\alpha_2\text{M-IIa}_{+\text{APC}}/\alpha_2\text{M-IIa}_{-\text{APC}})_{\text{normal plasma}}$$

The APC-sr obtained appears to be independent of the plasma level of antithrombin III due to the fact that the fraction of thrombin that is inhibited by antithrombin III in an individual plasma sample is the same with and without APC.

Since constant end levels of amidolytic activity ($\alpha_2\text{M-IIa}$) were in almost all cases reached within 10 min (see figures 5.1 and 5.3), APC-sr were routinely calculated from two amidolytic assays (plasma samples with and without

APC) performed 15 min after initiating thrombin formation according the procedure described above ("determination of thrombin generation in plasma").

Statistics

Data are presented as ranges of values observed, median values or as mean $\pm$ S.E.M.. The Student's t-test was used to determine the significance of differences between test results of different groups of plasmas.

5.4 Results

Effect of APC on thrombin generation in normal plasma

In a first series of experiments, the effect of various amounts of added APC (0-75 nM) on thrombin generation in normal-pooled plasma was determined. Figure 5.1A shows typical thrombin generation curves that were obtained in a thrombin generation test initiated via the extrinsic pathway.^{19,20} During a short interval, thrombin is formed at an increasing rate. After about 2 min the thrombin concentration in plasma reaches a maximum. The thrombin that has been formed is subsequently irreversibly inhibited, mainly by the action of the plasma inhibitors antithrombin III and α_2 M.^{19,20} The plateau in amidolytic activity that is finally reached represents the amount of α_2 M-IIa complex that is formed. This complex lacks activity on macromolecular substrates but is still capable of cleaving small synthetic substrates.²⁹⁻³¹

FIG 5.1. Effect of APC on thrombin generation in normal-pooled plasma. Thrombin formation was initiated in plasma with a mixture of varying amounts of APC and recombinant tissue factor, phospholipids, Ca^{2+} -ions, phospholipids, Ca^{2+} -ions. The final APC concentrations in plasma were: ($\diamond$) no APC added; ($\bullet$) 2.5 nM; ($\times$) 5 nM; ($\blacksquare$) 10 nM; (+) 25 nM; ($\circ$) 37.5 nM, ($\square$) 50 nM and ($\blacktriangle$) 75 nM. Formation of thrombin was measured as described in 'Materials and Methods'. 1 arbitrary unit equals 1 mA / min, determined as described in 'Materials and Methods'.

The thrombin generation curve obtained after initiation via the intrinsic pathway was somewhat different (Figure 5.1B) and was characterized by a pronounced lag-phase in thrombin generation.

FIG 5.1B Effect of APC on thrombin generation in normal-pooled plasma.

Thrombin formation was initiated in plasma with a mixture of varying amounts of APC and colloidal silica, phospholipids, Ca^{2+} -ions. The final APC concentrations in plasma were: (◆) no APC added; (●) 2.5 nM; (x) 5 nM; (■) 10 nM; (+) 25 nM; (○) 37.5 nM, (○) 50 nM and (▲) 75 nM. Formation of thrombin was measured as described in 'Materials and Methods'.

1 arbitrary unit equals 1 mAU/min, determined as described in 'Materials and Methods'.

When APC was present in the TGT experiments, thrombin formation was inhibited. At 50 nM APC, inhibition in normal plasma appeared to be more than 90%. From Figure 5.1A,B it is clear that the presence of APC lowers both the formation of free thrombin and the end level of the $\alpha_2\text{M-IIa}$ complexes in a dose-dependent manner. In intrinsically triggered plasma, APC not only affected the amount of thrombin formed but also prolonged the lag-phase that preceded thrombin generation.

The thrombin generation curves were used to calculate the effect of APC on the ETP^{19,23} and on the end levels of $\alpha_2\text{M-IIa}$. At increasing APC concentrations the ETP, calculated from the area under the thrombin generation curve, decreased parallel with the end level of the $\alpha_2\text{M-IIa}$ complex (Figure 5.2A,B), which confirms the close relation between ETP and the $\alpha_2\text{M-IIa}$ plateau ($r=0.986$ and $r=0.992$ for the extrinsic and intrinsic TGT experiments, respectively) and justifies the approach to quantitate the effect of APC on the ETP via measurement of $\alpha_2\text{M-IIa}$ endlevels (cf. ref 22 and Methods). There appeared to be a marked difference in the APC sensitivity in the two systems: 50% inhibition of thrombin generation was obtained at ~ 8 nM APC after extrinsic initiation and ~ 25 nM APC after intrinsic initiation.

FIG 5.2 Effect of APC on the ETP and the end level of α_2 M-IIa generated in normal plasma. ETP values (■, normal error bars) were calculated from thrombin generation curves (cf. Figure 5.1 and ref.²³) and data are given as average $\pm$ 2SD ($n=3$). The α_2 M-IIa levels (▲, bold error bars) were determined 15 min (panel A) or 20 min (panel B) after initiation of thrombin formation ($n=10$). Values are expressed as percentage of the ETP or α_2 M-IIa level obtained in the absence of APC. (A) Thrombin formation was initiated with a mixture of recombinant tissue factor, phospholipids, Ca^{2+} -ions containing varying amounts of APC. (ETP, 100% = 490 ± 31 nM IIa·min (mean $\pm$ SD); α_2 M-IIa, 100% = 57 ± 6 mA/min). (B) Thrombin formation was initiated in plasma with a mixture of colloidal silica, phospholipids, Ca^{2+} -ions containing varying amounts of APC. (ETP, 100% = 486 ± 51 nM IIa·min; α_2 M-IIa, 100% = 65 ± 7 mA/min). The insets show the relation between the ETP and α_2 M-IIa levels calculated from the extrinsically ($r=0.986$) and intrinsically triggered thrombin generation curves ($r=0.992$) ($n=3$).

Effect of assay conditions on ETP measurements with and without APC

Optimal reaction conditions for APC-induced inhibition of thrombin generation initiated via the extrinsic pathway have been determined. At 1.5 μM phospholipid, the ETP hardly varied at free Ca^{2+} concentrations between 5 and 25 mM and relipidated tissue factor concentrations between 0.025 and 0.15 ng/ml (data not shown). At high phospholipid concentrations less APC and at high tissue factor concentrations more APC was required to inhibit thrombin formation. The activity of recombinant APC used in this study was 30% lower than that of an APC preparation that was purified from human plasma. In our further experiments we have chosen for reaction conditions (50 nM APC and 1.5 μM phospholipid) that approach those of conventional APTT-based clotting assays.^{1,17,32}

Inter-individual variation of sensitivity to APC

The experiments in which the ETP of intrinsically initiated thrombin formation is measured as a function of APC in a plasma pool of healthy volunteers consistently showed an as yet unexplainable scattering (compare Figure 5.2A and Figure 5.2B). Moreover, the inter-individual variation of the sensitivity of thrombin formation for APC in plasmas from normal individuals ($n=10$) appeared to be much larger in the intrinsic system than in the extrinsic system. The residual amidolytic activity determined at 75 nM APC ranged from 2.5 - 40% after intrinsic initiation while the same individuals gave a much narrower range (5 - 18% at 50 nM APC) after extrinsic initiation of coagulation. In order to compare normal individuals with carriers of factor V_{Leiden} we have therefore studied APC-resistant plasma only in extrinsically-stimulated reaction systems.

Effect of APC on thrombin generation in plasma of different factor V^{R506Q} genotypes

In the absence of APC there were no differences in thrombin generation in the plasmas from normal healthy volunteers (Figure 5.3A) and from individuals that were heterozygous (Figure 5.3B) or homozygous (Figure 5.3C) for the factor V^{R506Q} mutation. The values calculated for the ETP of these plasmas determined in the absence of APC were virtually the same (Table 5.1).

Clear differences between normal and APC-resistant plasmas were observed when 50 nM APC was present during thrombin formation. In agreement with Figure 5.1A, 50 nM APC strongly inhibited thrombin generation in plasma of the healthy volunteer (Figure 5.3A, thrombin generation curve corrected for $\alpha_2\text{M-IIa}$) and caused ~90% reduction of the ETP (Table 5.1). APC had much less effect on thrombin generation in plasma of the individual, who is heterozygous for the factor V^{R506Q} mutation (Figure 5.3B) and caused ~65% reduction of the ETP (Table 5.1). The effect of APC on thrombin formation in the plasma of the individual who is homozygous for factor V^{R506Q} mutation (Figure 5.3C) was even less as can be concluded from ~40 % reduction of the ETP (Table 5.1).

In agreement with the data presented in Figure 5.2, the effect of APC on the thrombin formation and on the ETP closely correlated with its effect on the final levels of the $\alpha_2\text{M-IIa}$ complex (Table 5.1).

FIG 5.3 Effect of APC on thrombin generation in single normal and APC-resistant plasmas. Thrombin formation was initiated in normal plasma (A), heterozygous APC-resistant plasma (B) or homozygous APC-resistant plasma (C) with a mixture of recombinant tissue factor, phospholipids, Ca^{2+} -ions, without APC ($\blacktriangle$) or with an amount of APC ($\bullet$) yielding a final APC concentration of 50 nM in plasma. Further experimental details are described in 'Materials and Methods'.

Table 5.1

Effect of APC on the ETP and the levels of α_2 M-IIa of plasmas from a normal, a heterozygous and a homozygous APC-resistant individual

	ETP _{-APC} [*] nM·min	ETP _{+APC} [*] nM·min	ETP _{+APC} ^{**} %	α_2 M-IIa-APC arb. Units	α_2 M-IIa _{+APC} ^{***} arb. Units	α_2 M-IIa _{+APC} ^{**} %
normal plasma	533	58	11	44	3.6	8.2
heterozygote	482	170	35	37	11	29
homozygote	523	312	60	67	38	57

^{*} ETP = time integral of thrombin formation calculated from Figures 5.3 A,B and C respectively.

^{**} % of value determined in the absence of APC

^{***} Corrected for the amidolytic activity of APC on the chromogenic substrate for thrombin

Test results for a population of plasmas from normal, heterozygous and homozygous APC-resistant individuals

In order to verify whether the different effects of APC on thrombin generation in plasmas from a normal, a heterozygous and a homozygous APC-resistant individual could be used as a diagnostic tool, we have screened groups of healthy individuals ($n=25$) and individuals that were identified as heterozygous ($n=17$) and homozygous ($n=5$) for the factor V^{R506Q} mutation by genetic analysis of genomic DNA. In order to provide a method that can be easily carried out in any laboratory we assessed the effect of APC on thrombin generation by measuring its influence on end levels of the α_2 M-IIa complex (cf. Figure 5.1A and Figure 5.2A).

The APC sensitivity ratios obtained are summarised in Figure 5.4.

FIG 5.4 APC sensitivity ratios calculated from α_2 M-IIa endlevels in plasmas from normal, heterozygous and homozygous APC-resistant individuals. Thrombin formation was initiated in plasma with a mixture of recombinant tissue factor, phospholipids, Ca^{2+} -ions with or without APC. In the incubations with APC the final plasma APC concentration was 50 nM. After 15 min the α_2 M-thrombin end-levels were determined with S2238. Further experimental details are given in 'Materials and Methods'.

The APC sensitivity ratio is defined as $([\alpha_2\text{M-IIa}]_{+APC} / [\alpha_2\text{M-IIa}]_{-APC})_{\text{divided by } ([\alpha_2\text{M-IIa}]_{+APC} / [\alpha_2\text{M-IIa}]_{-APC})_{\text{normal plasma}}}$. The absence or presence of the Factor V^{R506Q} mutation was determined by genetic analysis of genomic DNA samples isolated from peripheral blood.²⁸ The solid lines indicate the mean values observed.

As expected there was a clear difference in APC sensitivity ratios for the three groups tested. The APC sensitivity ratios roughly divided into three populations: APC-sr < 1.9 for normal plasmas, between 2.1 and 3.6 for

heterozygotes and ≥ 3.9 for homozygotes, with median values of 1.1, 2.5 and 4.1, respectively. There was no overlap between the normal and APC-resistant populations ($p < 0.001$ for the difference between the normal population and either of the APC-resistant populations) and some overlap between the homozygous and heterozygous APC-resistant populations since two of the seventeen heterozygotes had an APC-sr > 3.9 ($p > 0.01$).

We have also determined APC-sr for part of our population after intrinsic initiation of coagulation. Due to the earlier described large inter-individual variation of APC response in the intrinsic system there was a large overlap between APC-sr of normal individuals (range 0.3-3.3) and heterozygous carriers of factor V_{Leiden}. Approximately 30% of the non-carriers of factor V_{Leiden} fell in the range of heterozygous APC-resistant individuals. This percentage is similar to that observed for the APTT-based assay in non-diluted plasma.³⁶

Conditions that affect APC-sr measurements via ETP or α_2 M-IIa plateau determination

Reliable diagnosis of APC-resistance with APTT-based assays in undiluted plasma appears difficult for pregnant women,¹⁶ women using oral contraceptives^{16,33} or for patients that are on oral anticoagulation,² treated with heparin,³² diagnosed as protein S deficient³² or positive for lupus anticoagulant,³⁴ In such plasmas thrombin generation was also affected which hampered the screening for APC resistance via the method presented in this chapter (Table 5.2).

Table 5.2

APC-sr of various plasma samples after dilution in FV-deficient plasma.

Plasma	Normal assay			Modified assay		
	APC-sr	SEM	n	APC-sr	SEM	n
normal	1.04	0.08	25	1.09	0.16	4
Heterozygous APC resistant	3.03	0.27	17	2.24	0.13	3
Homozygous APC resistant	4.75	0.44	5	3.35	0.43	4
Protein S deficient	1.87	-	1	1.32	0.04	2
Oral anticoagulation	n.d.	-	3	1.41	0.12	4
Heparin treatment	n.d.	-	3	1.34	0.12	5
Lupus anticoagulans	n.d.	-	2	0.94	0.24	2
Oral contraception	2.40	0.85	8	1.63	0.20	3
Pregnancy	2.81	1.10	3	1.68	-	1

Note: mean values of APC-sr were calculated from final levels of α_2 M-IIa in undiluted plasma samples (normal assay) or in plasma samples diluted 10-fold in factor V-deficient plasma (modified assay).

n.d. Not determined. Strongly inhibited thrombin formation in the absence of APC

Assays performed in the presence of 40 ng/ml polybrene.³⁷

It has been investigated whether these interferences can be overcome by modifying the test *e.g.* by performing the assays on plasma samples diluted in factor-V deficient plasma.^{35,36} Preliminary data, for a limited number of plasma samples are given in Table 5.2. It is clear that dilution in factor V-deficient plasma normalized the sensitivity of these plasmas towards APC since APC-sr approached the values obtained for normal-pooled plasma.

In all experiments presented thus far the plasma samples were defibrinated by pre-treatment with Ancrod®, a thrombin-like enzyme purified from the venom of *Agkistrodon rhodostoma*. It is not necessary, however, to defibrinate the plasma samples when thrombin is formed in the presence of Pefabloc Fg®, an inhibitor of fibrin polymerisation.³⁸ At a final concentration of 2 mg/ml plasma, Pefabloc Fg® prevented clot formation and hardly affected the ETP or the α_2 M-IIa endlevel (data not shown).

It is well documented that chromogenic substrates do not show a unique selectivity towards the specific protease for which they were designed. The thrombin specific substrate used in the experiments presented in this chapter (S2238) is for instance also converted by APC albeit much less efficient.

Therefore, especially in the case of a normal plasma sample (low α_2 M-IIa levels in the presence of APC) the APC-related amidolytic activity can potentially contribute to the final amidolytic end-level determined. At the APC concentrations used in our experiments (~ 50 nM) the contribution of APC to the final amidolytic activity in individual normal plasmas ($n=10$) was ~30% (range 20 - 50%). For APC-resistant plasmas the contribution of APC to the final amidolytic activity becomes negligible (< 5%) due to the much higher level of α_2 M-IIa_{APC}. To obtain a correct APC-sr for normal plasma the APC-sr needs to be corrected for the contribution of APC to the amidolytic activity. It should be emphasised, however, that it is not necessary to determine the contribution of APC for each individual plasma. Application of a standard correction (amidolytic activity of APC in non-triggered normal plasma) yields APC-sr close to the value obtained after individual correction and will not interfere with the distinction between normal and heterozygous APC-resistant individuals.

5.5 Discussion

Unlike a clotting time, analysis of thrombin generation in plasma provides direct information on the entire blood coagulation process.^{20-23,39-41} The results presented in this chapter show that determination of the effect of APC on the ETP of a plasma sample allows detection of an abnormal anticoagulant response of plasma to APC (APC resistance).

In normal plasma, the presence of 50 nM APC almost completely inhibited thrombin formation initiated via the extrinsic pathway (Figure 5.1A) and reduced the ETP to values that were ~ 10 % of that observed in the absence of APC, whereas in heterozygous and homozygous APC-resistant plasmas the residual ETP's were 35% and 60%, respectively. These observations seem to be easily explained by the fact that APC inactivates factor Va^{R506Q}, isolated from APC-resistant plasma, some 10-20 times slower than normal factor Va.¹³ However, recently we reported¹⁴ that, dependent on their actual concentrations, factor Xa and protein S diminish the difference between the sensitivities of factor Va and factor Va^{R506Q} for APC. This was concluded from studies in model systems with purified proteins. The data reported in the present chapter show that care should be taken to simply apply theories developed on the basis of model experiments to plasma systems.

In order to simplify a TGT-based screening method for APC-resistant plasmas, we verified (Figure 5.2A,B) the linear relationship between the ETP and the end level of α_2 M-IIa present in plasma after completion of thrombin generation.²² This allows rapid screening for APC-resistant plasmas by

6.1 Summary

Epidemiological studies have shown that women who use third-generation oral contraceptives (OC) containing desogestrel, gestodene or norgestimate have a higher risk of venous thrombosis than women that use second-generation OC containing levonorgestrel. It is also known that a mutation in factor V (factor V_{Leiden}), which results in resistance to activated protein C (APC) and which is the most common cause of hereditary thrombophilia, potentiates the prothrombotic effect of OC.

Effects of APC on thrombin generation in the plasma of women using OC were compared to the response to APC in non-OC users and in individuals that were heterozygous or homozygous for factor V_{Leiden}. The response towards APC was evaluated on basis of the ratio (APC-sr) of the time integrals of thrombin formation determined in the presence and absence of APC.

Compared with women not using OC, women who used OC exhibited a significantly decreased sensitivity to APC ($P < 0.001$), independent of the kind of OC used. Women who used third-generation monophasic OC were significantly less sensitive to APC than women using second-generation OC ($P < 0.001$) and had APC-sr that did not significantly differ from heterozygous female carriers of factor V_{Leiden} who did not use OC. Women who were heterozygous for factor V_{Leiden} and used OC had APC-sr in the range of homozygous carriers of factor V_{Leiden}. Two women who started OC therapy had significantly elevated APC-sr within 3 days.

Acquired APC resistance may well explain the epidemiological observation of increased risk for venous thrombosis in OC users, especially in women using third-generation OC.

6.2 Introduction

Since their introduction, the use of oral contraceptives (OC) has been linked to an increased incidence of thromboembolic events¹. Compared with non OC users, women that take OC have a risk for venous thrombosis that is increased about 4-fold². Recently, concern has been raised that third-generation OC, which contain the progestagens desogestrel, gestodene or norgestimate, further increase the risk of thromboembolism^{2,3,4}. The small but definite increase of the incidence of venous thromboembolism in OC users becomes more manifest when other thrombosis risk factors are present. This is illustrated by reports that women that are resistant to activated protein C (APC) and that use OC have a considerably increased risk of thrombosis^{5,6}.

APC resistance is a hereditary disorder^{7,8,9}, that results from a mutation in the gene of blood coagulation factor V¹⁰ and that renders the activated form of factor V (factor Va) less susceptible to proteolytic inactivation by APC¹¹. Since APC-catalyzed factor Va inactivation is a crucial step in the down-regulation of thrombin formation¹², impaired inactivation of factor Va likely explains the increased risk for venous thromboembolism in APC-resistant individuals. A remarkable feature of APC resistance is its high prevalence. About 4 % of the West-European population appears to be heterozygous APC resistant^{9,10,13,14} and this abnormality is found in 20-50% of patients with venous thrombosis^{8,9,10,13,14}.

Although the higher risk of idiopathic venous thromboembolic events, deep-vein thrombosis and/or pulmonary embolism in association with the use of OC is well established, no clear causal relations with alterations within the haemostatic system have as yet been reported^{15,16}. In general, the effects of OC therapy on coagulation variables are modest and in several reports it has been suggested that the use of OC induces changes in the procoagulant- and anticoagulant pathways that may counterbalance each other^{15,16}.

In this chapter we report the change of a haemostatic variable that may be indicative for a substantial disturbance of the haemostatic balance and for the existence of a prothrombotic state in OC users. We have measured the effect of APC on thrombin generation in plasma and show that women who use OC are much less sensitive to APC than non users and that differences in sensitivity to APC between women who use second- and third-generation OC correlate with the reported higher risk of thrombosis in third-generation OC users.

6.3 Materials and Methods

This study was performed according to a protocol approved by our institutional ethics committee. The participants in this study were selected from general practitioners offices using criteria described below.

Study cohorts

The following plasmas were used in our study: a normal plasma pool prepared from plasma of healthy volunteers (40 females not using OC and 50 males, mean age 35 years), individual plasma samples from male volunteers ($n = 25$, 18-38 years old, mean age 28 years), women not using OC ($n = 53$, 19-39 year, mean age 31 years), women using triphasic OC ($n = 24$, 19-37 year; mean age 28 years, duration of OC use 0.5-17 year, average duration OC use 8 year), women using second-generation monophasic contraceptives containing levonorgestrel or lynestrenol ($n=32$, 20-39 years, mean age 29 years, duration OC use 0.5-20 years, average duration OC use 9 years), women using third-generation monophasic contraceptives containing desogestrel, gestodene or norgestimate ($n = 40$, 18-36 years; mean age 29 years, duration of OC use 1-20 year, average duration OC use 7 years) and women heterozygous for the factor V_{Leiden} mutation not using OC ($n = 17$, 16-79 year, mean age 41 years).

Women who had ceased OC therapy for more than 6 months were considered as non users. The OC user group consisted of women that were using the same OC for more than 3 months. With the exception of carriers of the factor V_{Leiden} mutation, individuals with a previous episode or a known familial history of venous or arterial thrombosis (~ 10 % of our population), having a chronic or intercurrent acute disease, taking medication that may interfere with coagulation, and pregnant women were excluded from our study.

Collection and Handling of Plasma Samples

Nine parts of blood from consenting volunteers were collected in one part of 0.13 M trisodium citrate (pH 7.8). Platelet poor plasma was obtained by centrifugation for 25 minutes at 3,000 g at room temperature followed by centrifugation for 25 min at 20,000 g at 4 °C and was stored in small aliquots at -80°C until analysis.

The occurrence of heterozygous and homozygous APC-resistance was established by determination of the sensitivity of plasma factor Va for APC¹⁷ and by DNA analysis¹⁸.

Determination of Thrombin Generation in Plasma

Thrombin generation curves were determined at 37°C in defibrinated plasma¹⁹ containing 15 µM phospholipid vesicles^{11,17,20} (dioleoylphosphatidylserine/dioleoyl-phosphatidylethanolamine/dioleoylphosphatidylcholine, 20/20/60, M/M/M; Avanti Polar Lipids, USA), 0.1 ng/ml relipidated tissue factor (Recomboplastin S, Dade, USA), and 15 mM added CaCl₂, with or without 5 nM APC (Immuno AG, Vienna, Austria). At regular time intervals, samples were withdrawn to determine the generation of amidolytic activity¹⁹ (thrombin plus α₂-macroglobulin-thrombin complex [α₂M-IIa]) with D-Phe-pipecolyl-Arg-pNA (S2238, Chromogenix, Sweden). Correction of the time course of generation of amidolytic activity for the contribution of α₂M-IIa yielded the free thrombin concentration as a function of time²¹. These values were used to calculate the ETP, which is defined as the time-integral of free thrombin concentration in a thrombin generation test^{19,21}.

Since ETP values of a particular plasma sample are directly proportional to the residual levels of amidolytic activity (α₂M-IIa)^{20,22}, we quantitated the effect of APC on thrombin generation (i.e. on the ETP) by measuring its effect on the final level of α₂M-IIa. The APC sensitivity ratio (APC-sr) was defined as the ratio of α₂M-IIa determined in the presence and absence of APC divided by the ratio determined in the normal plasma pool²³:

$$\text{APC-sr} = (\alpha_2\text{M-IIa}_{+\text{APC}}/\alpha_2\text{M-IIa}_{-\text{APC}})_{\text{plasma sample}}/(\alpha_2\text{M-IIa}_{+\text{APC}}/\alpha_2\text{M-IIa}_{-\text{APC}})_{\text{normal plasma}}$$

Statistics

Statistical analysis was performed after logarithmic transformation of the data which resulted in normally distributed APC-sr values. P-values were obtained by pairwise comparison of the groups using Student's *t*-test and were corrected for comparison of multiple groups according to the Bonferroni procedure²⁴.

6.4 Results

Effect of APC on Thrombin Generation

Fig 6.1 shows thrombin generation curves (closed symbols) obtained after initiating thrombin formation via the extrinsic pathway in plasma pools of women not using OC, women using third-generation OC and heterozygous APC resistant men and women who did not use OC.

FIG 6.1 Effect of APC on thrombin formation in pooled plasmas. Time courses of thrombin formation were initiated in plasma with a tissue factor/ CaCl_2 /phospholipid mixture in the absence (closed symbols) or presence of 5 nM APC (open symbols). ◆, ◇ plasma pool from women not using OC ($n=27$); ●, ○ plasma pool from women using third-generation monophasic OC ($n=25$) and ▲, △ plasma pool from men ($n=8$) and non OC-using women ($n=15$), heterozygous for the Factor V_{Leiden} mutation.

APC strongly inhibited thrombin formation in the plasma pool of women not taking OC (open symbols) and the residual ETP was 9.5 % of that determined in the absence of APC. Thrombin generation in the plasma pool of woman taking third-generation OC was much less sensitive to APC (residual ETP = 28.0 %) and exhibited a response towards APC that is similar to that observed in a pool of heterozygous APC-resistant individuals (residual ETP = 29.0 %). Thrombin formation in the plasma pools of women using triphasic OC or second-generation OC was also considerably less affected by APC than in women who did not use OC (Table 6.1).

Table 6.1

Effect of APC on thrombin generation in pooled plasmas

Pooled plasmas [*]	n	Mean age years	ETP _{-APC} [†] nM.min	ETP _{+APC} [†] nM.min	ETP _{+APC} [‡] %
normal plasma	90	35	458 ± 6	43 ± 4	9.4%
men	23	28	442 ± 5	39 ± 1	8.8%
women					
no contraceptive	27	29	473 ± 29	45 ± 2	9.5%
triphasic	28	28	533 ± 10	105 ± 3	19.7%
monophasic					
2nd generation	28	29	483 ± 5	85 ± 1	17.6%
3rd generation	25	29	554 ± 4	155 ± 13	28.0%
heterozygous Factor V _{Leiden} [§]	23	45	486 ± 5	141 ± 9	29.0%

^{*}Plasma pools were prepared from individual plasma samples described under 'Materials and Methods'

[†]Thrombin formation was quantified by calculating the time integral of the thrombin generation curve (ETP) as described under 'Materials and Methods'. Values are given as means ± SE.

[‡]% of value determined without APC.

[§]Men (n=8) and women (n=15)

Effects of OC therapy and factor V_{Leiden} on APC-sensitivity ratios

Since determination of the APC-sr in a large number of plasmas is precluded by the fact that measurement of complete thrombin generation curves is very time consuming, we routinely determined the APC-sr from the residual levels of the $\alpha 2M-IIa$ complex obtained in the presence and absence of APC. This procedure yields APC-sr that are identical to values calculated from the ratio ETP_{+APC}/ETP_{-APC} ^{20,22}. It should be emphasized that APC-sr calculated by this method exhibit trends that are the reverse of APC-sr determined with APTT-based clotting assays^{7,9,23} ($APC-sr = APTT_{+APC}/APTT_{-APC}$). Since APC inhibits thrombin generation and prolongs the APTT, decreased sensitivity for APC (APC resistance) yields higher APC-sr in ETP-based assays and lower APC-sr in APTT-based assays.

The APC-sr of individual plasma samples of men, women not using OC, women on OC therapy and heterozygous carriers of the factor V_{Leiden} mutation (women not using OC) determined as described above are summarized in Fig 6.2 and Table 6.2.

FIG 6.2 APC-sr of men, women, women using OC and female heterozygous carriers of Factor V_{Leiden}. Medians (indicated in the figure) and 5th and 95th percentiles are given in Table 6.2. MP2 = second-generation monophasic OC users, TP = triphasic OC users, MP3 = third-generation monophasic OC users.

It appeared that men had significantly lower APC-sr than women that were not using OC, confirming that men are more sensitive to APC than women^{8,25}. Plasmas from women using different kinds OC had APC-sr that were considerably higher than those of non OC users. Independent of the kind of OC used, significant differences were observed between the APC-sr of OC users and of women that were not using OC (Fig 6.2). The APC-sr of plasmas from women who take third-generation monophasic OC were almost equal to the APC-sr of heterozygous APC-resistant women not using OC and was significantly higher than the APC-sr of women that used triphasic ($P < 0.005$) or second-generation monophasic OC ($P < 0.001$).

For heterozygous APC-resistant women not using OC the APC-sr defined by the 5th and 95th percentile fell in the range 2.09-6.60. No men and 3/53 (6%) of the women not using OC had an APC-sr within this range. Among women using OC, 12/32 (37%) taking second-generation monophasic OC, 8/24 (33%) using triphasic OC and 32/40 (80%) on third-generation monophasic OC therapy had APC-sr values within the 5th and 95th percentile of the heterozygous APC-resistant population.

During our study we identified five women who were using OC and were also heterozygous carriers of the factor V_{Leiden} mutation. These women had an APC-sr (4.52-6.40) of the levels normally observed for homozygous APC-resistant individuals (Table 6.2).

Table 6.2

Effect of OC therapy and factor V_{Leiden} on APC-sr.

Group	n	Mean age (yr)	APC-sr Median	5 th -95 th percentile
men	25	28	0.96	0.65-1.28
women				
no OC	53	31	1.22	0.87-2.15
no OC, heterozygous	17	41	3.10	2.09-6.60
FV _{Leiden}				
no OC, homozygous FV _{Leiden}	5	41	4.75	3.91-6.02
triphasic OC	24	28	1.93	1.26-3.29
2 nd generation monophasic	32	29	1.81	1.19-2.87
3 rd generation monophasic	40	29	2.59	1.91-3.88
Heterozygous FV _{Leiden} using OC	5	31	5.41	4.53-6.45

Changes of APC-sr after Starting OC Therapy

The sensitivities to APC of two healthy women, who had not used OC for more than 8 years and who started with OC therapy, were followed over a 4 month period. Prior to the use of OC the women had an APC-sr within the normal range (Fig 6.3).

FIG 6.3 Effect of starting OC therapy on the APC-sr. APC-sr were determined in plasma from two healthy women, age 26 years (○) and 34 years (□), who had not been taking oral contraceptives for more than 8 years and who started OC therapy on day 0 with a third-generation monophasic OC (150 µg desogestrel/30 µg ethinylestradiol). OC free periods are indicated by shaded bars.

Within 3 days of starting OC therapy the APC-sr had significantly increased, reaching values ≥ 3 in the third week of OC use. During the seven OC-free days the APC-sr significantly decreased, rising again during the second and third treatment cycles.

6.5 Discussion

In vivo down-regulation of thrombin formation is achieved by plasma protease inhibitors (e.g. antithrombin III and α_2 -macroglobulin) and by the proteins of the anticoagulant pathway (APC and protein S). The physiological importance of these pathways is demonstrated by the association of venous thrombosis with congenital deficiencies in antithrombin III²⁶, protein C²⁷ or protein S²⁸ and by the occurrence of hereditary thrombophilia in individuals with a genetic defect^{7,10} that makes factor Va less sensitive to proteolytic inactivation by APC (APC resistance).

An increased risk for venous thromboembolism has also been associated with the use of OC by healthy women. Although large retrospective and prospective studies have associated OC therapy with a 4-9 fold increased incidence of venous thrombosis²⁻⁴, the changes in haemostatic parameters in women using OC are minor and generally remain within the normal range^{15,16}. In OC users without other thrombosis risk factors, the increased procoagulant activity of the haemostatic system is at least partially compensated by an increased activity of the anticoagulant- and profibrinolytic pathways.

In this chapter we report a substantial change of a haemostatic variable (the APC-sr) in women using OC which appears to be much more sensitive and that may be indicative for the existence of a prothrombotic state in OC users. We have shown that measurement of the effect of APC on thrombin formation in plasma provides information on the sensitivity of an individual to APC. Screening a population of women who use or do not use OC shows that OC therapy induces acquired APC resistance. Significant differences in sensitivities to APC were observed between women not using OC and women on OC therapy, independent of the kind of OC used. Within the group OC users, women taking third generation monophasic OC were significantly less sensitive to APC than triphasic or second-generation monophasic OC users and exhibit a decreased sensitivity to APC comparable to that observed in heterozygous APC-resistant women who did not use OC (Fig 6.2).

We propose, that decreased sensitivity to APC (acquired APC resistance) in OC users may, at least in part, explain the higher risk of thrombotic disease of women taking OC. The fact that users of OC with third-

generation progestagens, for whom a higher risk of venous thromboembolism has been reported^{2,4}, exhibit significantly lower sensitivities to APC than users of second-generation OC, reinforces this hypothesis.

Until now, the increased risk for venous thrombosis during OC therapy has always been linked to effects of estrogen on the coagulation system^{15,16}. Our study provides a first example of a biological effect of the progestagen component on haemostasis and shows that, with respect to the risk for venous thrombosis, the role of progesterone cannot be ignored.

Whereas women that use third generation OC have APC-sr similar to heterozygous carriers of factor V_{Leiden} not using OC (Fig 6.2), the APC response in women that take OC and who also carry the factor V_{Leiden} mutation is further impaired and similar to that observed in homozygous APC resistant individuals (Table 6.2). Together with the fact that, compared with non-carriers non-users, the increased risk for deep-vein thrombosis of non carriers who use third-generation OC (7-8 fold)^{2,4} is similar to that of heterozygous carriers not using OC (7-fold)⁹ and that heterozygous carriers of factor V_{Leiden} who use OC exhibit a risk increase (50-fold)⁶ that approaches that of homozygous carriers not using OC (80-fold)^{5,14}, this supports our proposal that the increased incidence of venous thrombosis in congenital APC resistant individuals and in women using OC originate from a defect in the same physiological pathway.

The observations that the effect of OC on the APC response takes place within a few days after starting OC therapy and that the APC-sr drops in pill-free periods (Fig 6.3) contains valuable information about the underlying mechanism. Unfortunately, our present data do not allow further conclusions regarding changes in the haemostatic system that may link the impaired APC response and the increased thrombotic risk of OC users. It is unlikely, however, that the decreased sensitivity to APC is due to the reported decreased levels of protein S in OC users^{15,16}, since addition of protein S to the plasma of OC users did not normalize the APC-sr (data not shown). We confirmed that measurement of sensitivity for APC in APTT-based clotting assays yields marginal differences between women using and not using OC^{25,29}. The major difference between ETP-based and APTT-based methods is that initiation of coagulation in the case of the ETP occurs via the extrinsic pathway of coagulation (tissue factor/factor VIIa) whereas in APTT-based systems coagulation is initiated via the intrinsic coagulation pathway. This indicates that the basis of the impaired APC response in women using OC may have to be sought in the activity and/or regulation of the extrinsic pathway of coagulation.

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Chapter 7

General Discussion and Conclusions

In this general discussion the results, described in the previous chapters, will be put in the context of surveys performed by other research groups. Where possible, connections between chapters are made and the mechanistic and physiological implications of our findings will be discussed.

7.1 Meizothrombin-catalyzed activation of Factor V

Activation of the inactive procofactor of human FV is essential for the expression of FVa cofactor activity in prothrombin activation. The pivotal role of FVa in the prothrombinase complex, and hence for the process of thrombin formation is well-established and undisputed. Under *in vitro* conditions it has been shown that activation of FV is the rate-limiting factor during the initiation of plasma prothrombin activation¹. However, both in *in vitro* experiments in plasma and in blood and in the *in vivo* situation the activation of FV has for long been somewhat of a paradox since efficient activation of FV requires thrombin²⁻⁶ - whereas thrombin is only formed in sufficient amounts when FVa is present. However, in 1990 it was shown by Monkovic and Tracy⁷ that in the presence of phospholipids, FXa can also serve as an efficient activator of human FV. Given the kinetic parameters determined in the latter study, FXa is ~2 times less efficient than thrombin in activating plasma concentrations of FV. It should be emphasized however, that FV activation by FXa may be a temporary process since in the presence of procoagulant membranes FXa forms a tight complex with FVa and not with FV. This will reduce the free FXa concentration available for FV activation. Together these data indicate that the physiologic activation of FV is a complex process, which is probably initiated via minute amounts of extrinsically formed FXa, but which is rapidly shifted to activation by thrombin and/or meizothrombin (see below).

In chapter 2 we describe that meizothrombin, an active reaction intermediate in the activation of PT, can activate FV in a model system. Meizothrombin is a potent activator of FV in the presence of phospholipids which activates FV with rate constants that exceed those of thrombin and FXa ~4 fold.

In doing so, meizothrombin must act exclusively at a phospholipid/cell surface (Table 2.1). Binding of meizothrombin to a membrane surface then opens a possible role for meizothrombin in the activation of protein C as well. This activation can be thought to occur together with another membrane-associated protein, thrombomodulin. Meizothrombin thus 'fits' well within the concept of 'the catalytic surface', a general concept within haemostasis⁸⁻¹⁰.

It should be emphasized however, that it is possible that the amounts of meizothrombin formed *in vivo* are insufficient to activate FV. Although it has been shown that, in model systems containing purified proteins, meizothrombin is the preferred PT activation intermediate when FVa is present and the PT concentration is high¹¹⁻¹⁵, it was observed that meizothrombin formation on cultured endothelial cells^{16,17} or in plasma systems^{15,18,19} is rather low or even undetectable. Formation of intact meizothrombin in plasma experiments may be limited for two reasons. First, it has been reported that meizothrombin rapidly converts itself into meizothrombin-desF1 via autocatalysis^{20,21}. This form of meizothrombin hardly activates FV (see chapter 2) since it does not bind to phospholipids due to the fact that it lacks the Gla-containing fragment 1 domain. Secondly, formation of meizothrombin in plasma experiments may also be low because the bulk of prothrombin is activated in plasma at a very high factor Xa-Va concentration. From thrombin generation curves, determined during thromboplastin-induced clotting of plasma (chapter 6,7, refs.^{22,23}) it can be calculated that the factor Xa-Va concentration during the burst of thrombin formation exceeds 300 pM. At such conditions prothrombin activation can become a diffusion-limited process²⁴ and despite the fact that the bulk prothrombin concentration is high, the local PT concentration, in the vicinity of the factor Xa-Va complex, will approach zero. This condition enables rapid conversion of locally-formed meizothrombin into thrombin. The current data do not rule out, however, that meizothrombin may be the physiological FV activator during the early phase of PT activation, i.e. at conditions at which the amount of factor Xa-Va complex is still low.

More data are needed in order to fully comprehend the physiological value of meizothrombin. Close examination of the forms of FVa, which are formed *in vivo* (i.e. presence of the 280,000 kD activation fragment, fig. 2.5) might prove helpful.

7.2 Inactivation of FVa and FVa_{Leiden} by APC in model systems

The inactivation of human FVa by APC in reaction systems containing purified coagulation proteins has been described by many laboratories^{14,25-29} already before 1993. After having overcome the difficulties of purification of the clotting factors used, these first publications showed the essential characteristics of APC-catalyzed FVa inactivation: a) inactivation occurs by multiple cleavages of the heavy chain domain of factor Va, that lead to loss of cofactor activity^{30,31} b) inactivation occurs preferably at a phospholipid

surface^{25,26,32} c) inactivation is stimulated by the presence of protein S^{29,32,33}
 d) Factor Xa can protect factor Va from inactivation by APC^{25,26,34}.

In 1994, Kalafatis et al.³⁰ reported the exact locations of the APC-cleavage sites in the heavy chain domain of human FVa and in the FV molecule. The discovery of the R506Q mutation in FV_{Leiden} patients³⁵ has however been a major incentive for the detailed analysis of FVa inactivation by APC.

Chapter 3 describes a study that was performed to elucidate the order and rates of peptide bond cleavages during the APC-catalyzed inactivation of FVa and FVa_{Leiden}. In reaction systems containing 0-5 nM of FVa and 0.05-5 nM APC time courses of FVa inactivation were obtained in the presence or in the absence of phospholipid vesicles. Under these conditions, reaction kinetics were first-order, both with respect to FVa and APC, which allows calculation of apparent second-order rate constants for FVa inactivation. By the use of the mathematical technique of curve fitting, we were able to obtain rate constants for the peptide bond cleavages that eventually annihilate the cofactor activity of the FVa molecule in prothrombin activation. Identification of cleavage sites was performed by combination of the kinetic analyses with immuno-blotting experiments. Our experiments resulted in a model for the inactivation of human factor Va by APC that is given in figure 7.1 below:

FIG. 7.1 **Inactivation of FVa and FVa_{Leiden} by APC.** Schematic representation of the model for inactivation of FVa and FVa_{Leiden} by activated protein C in the presence of phospholipids. Molecular masses of fragments and rate constants of cleavages are indicated. For explanation of the two alternative pathways, see text below.

In this model, inactivation of membrane-bound FVa can occur via two independent pathways. In pathway I, FVa is initially cleaved at Arg⁵⁰⁶, yielding a partially active reaction intermediate (FVa_{int}) that loses its cofactor activity after cleavage at Arg³⁰⁶. Cleavage at Arg⁵⁰⁶ is much faster than cleavage at Arg³⁰⁶, which causes the 75 kD intermediate (FVa_{int}) to accumulate during FVa inactivation. FVa inactivation via pathway II occurs after initial cleavage at Arg³⁰⁶ and causes the direct loss of all cofactor activity. The slow rate at which cleavage at Arg³⁰⁶ occurs is similar in both the pathways I and II. Since in FV_{Leiden}, the Arg at amino acid position 506 is mutated into Gln, inactivation of FVa_{Leiden} can only occur via the (slow) pathway II. For normal FVa, the distribution of FVa over both pathways is largely governed by the concentration of FVa that is present. We have shown that inactivation at Arg⁵⁰⁶ has K_m and k_{cat} values that are ~10 times lower and ~3 times higher, respectively, than the corresponding parameters for inactivation at Arg³⁰⁶. This implies that differences between normal FVa and FVa_{Leiden} are most pronounced at low concentrations of FVa.

This model contradicts reports by Kalafatis and coworkers in which it is stated that inactivation of FVa is sequential and that cleavage at Arg⁵⁰⁶ is necessary to expose the cleavage sites at Arg³⁰⁶ and Arg⁶⁷⁹ refs 30,31. However, these studies are performed at very high FVa concentrations (~20 times higher than the plasma FV concentration) in combination with a FVa cofactor assay that contains a high amount of FXa (see below).

To unquestionably elucidate this mechanism however, kinetic studies of APC-catalyzed inactivation of mutant FVa molecules with mutations at the APC-cleavage sites, will be necessarily.

At the time of preparation of this thesis, a preliminary study was presented by Egan et al.³⁶ who used site-directed mutagenesis to mutate human factor V at positions 306 and 506. Their findings show that in the absence of 306 and 506 there is no inactivation and that cleavage at 306 and/or 506 are required for efficient factor Va inactivation. A detailed kinetic analysis of these mutants are not provided, however.

Upon cleavage of the Arg⁵⁰⁶-Gly⁵⁰⁷-bond the resulting FVa intermediate possesses a partial FVa cofactor activity in prothrombin activation. We have shown that the partial loss of cofactor activity is particularly due to diminished interaction with FXa, whereas the affinities of FVa_{int} for PT and phospholipid vesicles were virtually the same as for FVa. This finding has important implications for functional assays for APC resistance that are based on measurement of the loss of APC-mediated FVa activity, and which involves determination of the remaining cofactor activity of FVa and FVa_{Leiden} in prothrombin activation. In order to obtain maximal differences between FVa

and FVa_{Leiden}, the determination of FVa cofactor activity in such assays should be performed at low FXa concentration (see section 7.4 below).

The rate constants for APC-catalyzed cleavage of peptide bonds in the heavy chain of FVa and FVa_{Leiden} were also determined in the absence of phospholipid vesicles. These experiments showed that two cleavages, at Arg⁵⁰⁶ and Arg³⁰⁶, are stimulated by the presence of phospholipid vesicles. The order of stimulation is different however, and is ~300-fold and ~625-fold respectively, under the conditions tested. This implies that a shift can occur in the relative amounts of FVa that are inactivated via pathway I or II. What is more important though, is that in the absence of phospholipid vesicles the rate of cleavage at Arg³⁰⁶ is of the order of magnitude as the cleavage-rate at Arg⁶⁷⁹. Especially in the case of inactivation of FVa_{Leiden}, where the fast cleavage at Arg⁵⁰⁶ can not occur, cleavage at Arg³⁰⁶ and at Arg⁶⁷⁹ will both contribute to inactivation in the absence of a membrane surface, which was confirmed by immunoblotting experiments.

Despite the careful explanation of the kinetics approach, it is somewhat surprising to note that Kalafatis et al.³⁷ question our kinetic data. According to their comment, the differences in inactivation rates reported in their and our studies is most likely due to the use of a non-bioactive APC, and to the, relative to FVa, high APC concentrations in our experiments. The concentrations used in our kinetics experiments, however, never exceeded 1.5 nM, which is well below the limit of 5 nM that was ascertained by us for the first-order kinetics under our experimental conditions. Furthermore, the kinetic parameters, K_m and k_{cat} , and the k_{cat}/K_m values that were obtained from Michaelis-Menten kinetic analysis (an analysis that is essentially different from the second-order kinetic analysis based on curve-fitting of time courses of FVa inactivation) were in complete agreement with the rate constants obtained from the curve fitting experiments as well as with published kinetic data by Solymoss and coworkers²⁹. We therefore have full confidence in the kinetic data as were obtained by us and reject this unfounded critique.

Discussion about the quality of an APC preparation would however indeed be helped if it were possible to uniformly define the activity of a preparation. Like in case of thrombin, a consensus on the definition of 1 international unit of APC (e.g. the amount of APC in nM that reduces the APTT of a normal pooled plasma from normal to 90% of its normal value) would be very meaningful. Expression of concentration in IU, possibly in parallel with a unit definition for the clinically applied protein C concentrates, would then avoid the disagreements about the functionality of an APC preparation.

Shortly after the publication of the work that is described in chapter 3 we published a paper in which we further extended the model as is presented in

figure 7.1. In this study we reported that FXa and protein S have very specific effects on the inactivation of FVa by APC. Protein S accelerates FVa inactivation by selectively enhancing the slow cleavage at Arg³⁰⁶ (20-fold), whereas FXa protects FVa from inactivation by selectively blocking cleavage at Arg⁵⁰⁶. The assignment of these target sites were confirmed in experiments with FVa_{Leiden}. Due to the lack of the cleavage site at Arg³⁰⁶, APC-mediated FVa_{Leiden} inactivation is not inhibited by FXa, while the effect of protein S on the loss of cofactor activity of FVa_{Leiden} is much more pronounced than in the case of normal FVa. The implications of these observations, which are of great importance for APC resistance tests and for the inactivation of FVa in plasma, will be discussed in the next section.

7.3 Inactivation of FVa and FVa_{Leiden} by APC in plasma systems

Inactivation of FVa by APC in plasma systems is complicated by several factors that are absent in model systems that utilize purified proteins. Among these are a) the presence of such 'interfering' coagulation factors as FXa, PT and protein S, b) protease inhibitors that inhibit the proteolytic activity of APC, c) FVIII(a), which are also substrates for APC, d) non-activated FV, which forms a pool of procofactor that constantly replenishes FVa activity and that itself is a substrate for APC, e) anticoagulants, which in most experiments are used to prevent clotting of blood, f) fibrinogen, which can easily form fibrin clots that hamper sample handling.

Interference by all these factors was lost in the APC resistance test that we devised (chapter 4). Although the test uses a plasma sample, the 100% specificity and sensitivity that is obtained is largely attributable to the >750 fold dilution that was used. Inactivation of FVa in diluted plasma samples resembles inactivations in model systems more than those in full plasma. This was underscored by the good correlation between the rate constants for FVa inactivation obtained from time courses of FVa inactivation in diluted plasma samples of normals, heterozygous and homozygous APC resistant individuals and those determined in model systems. In agreement with observations in the model systems, we showed that differences between APC-mediated loss of FVa cofactor activity in plasma samples from normal individuals and FV_{Leiden} carriers were maximal when FVa is inactivated at low protein S concentrations and when the loss of FVa cofactor activity is probed at a low FXa concentration.

Studies on the inactivations of FVa, responsible for the inhibition of thrombin formation by APC in tissue-factor triggered full plasma (chapters 5 and 6), occur under conditions that are hampered by interference by the

factors that are mentioned at the beginning of this section. A simple description of how FVa is inactivated in full plasma is therefore not possible. It is likely the relative rates of activation and inactivation of FV(a) that determine the effect of APC on thrombin generation in plasma. As reported in chapters 2 and 3, the catalytic efficiencies (k_{cat}/K_m) of both activation and inactivation are similar. This means that at the low concentrations of activating and inactivating enzymes, as presumably present during the onset of *in vitro* coagulation, both processes can balance each other. When APC concentrations are sufficiently high, the balance is shifted in favour of inactivation and thrombin generation (clot formation) will be inhibited. In that situation APC directly cleaves the heavy chain of FVa (generated after activation by (meizo)thrombin and/or FXa), thereby preventing formation of thrombin, that is necessary for further FV activation through positive feedback reaction. As a result, a shift towards inactivation will cause a lengthening of clotting times.

Interesting in this context are reports by Kalafatis and coworkers^{37,38} who observed that APC can also inactivate the non-activated procofactors FV and FV_{Leiden}. Furthermore, it is shown that addition of low amounts of APC to FV_{Leiden} results in transient accumulation of a FV molecule containing a heavy chain like fragment of amino acids 1-679 which possesses potential cofactor activity. Following cleavage at Arg³⁰⁶, the cofactor activity of this FV derivative is lost. At higher concentrations of APC (> 5.5 nM) cleavage at Arg³⁰⁶ is believed to occur much faster than cleavage at Arg⁶⁷⁹, preventing accumulation of the 1-679 heavy chain-like fragment with procoagulant activity. In plasma containing normal FV, this fragment is believed not to accumulate due to fast inactivation at Arg⁵⁰⁶. The blotting data that are provided in this study are however hampered by the fact that they were performed in 10-fold diluted plasma. This dilution might have consequences for the reaction kinetics that occur and may therefore not reflect the conditions that occur in full plasma.

Not included in this discussion so far are recent observations by Shen et al.³⁹, Varádi et al.⁴⁰ and Lu et al.⁴¹ who reported a cofactor function for FV in the inactivation of FVIIIa and FVIII by APC. This will possibly influence FVa inactivation in plasma. The former study³⁹ showed that when assembled on phospholipid membranes, FV acts in synergy with protein S in the downregulation of FVIIIa by APC. The authors showed in model systems that it is an intrinsic ability of FV, and not FVa, to enhance the APC-catalyzed inactivation of FVIIIa.

This latter observation is seemingly in contradiction with the observations by Lu et al.⁴¹ who observed that APC- or thrombin-treated FV, and not single

chain intact FV, is able to stimulate the inactivation of the procofactor FVIII by APC in the presence of protein S. Western blot analysis showed that upon addition of APC to mixtures of FVIII and FV, in the presence of phospholipids and protein S, FV was completely cleaved by APC at the beginning of the inactivation process. From combination of these experiments with data from experiments using thrombin-treated FV (=FV heavy and light chains + activation peptides), it was concluded that a portion of the B region of FV is involved in the FV cofactor function for FVIII inactivation. Since membrane-bound FVIIIa showed a spontaneous rapid loss of cofactor activity and APC-catalyzed inactivation of the procofactor FVIII was relatively slow compared to inactivation of FV, it is concluded that FV and FVa, rather than FVIII and FVIIIa are the physiological substrates for APC. The difference in FV-cofactor activities between refs 39 and 41 may be explained by a difference in quantitation of FVIIIa activity: whereas the first group used a well-defined model system containing purified clotting factors to assess FVIIIa cofactor activity, the latter group used a one-stage clotting assay. Apart from these observations in model systems, Varádi et al.⁴⁰ confirmed in plasma-based reaction systems that both FV and protein S are required for optimal inactivation of FVIII by APC. It was observed that at a low protein S concentration (2.42 nM) neither FVa, nor thrombin-treated FV or RVV-treated FV, expressed cofactor activity. The most remarkable observation was however, that, FV_{Leiden} almost totally lacked cofactor activity in APC-catalyzed inactivation of FVIII in the presence of protein S.

Down-regulation of thrombin formation in plasma (chapters 5 and 6) is a complex process in which down-regulation of FVa activity plays an equally important role as down-regulation of FV activation: inactivation of FVIIIa will result in reduced FXa and thrombin formation, which in turn reduces FV activation. Therefore, thrombin formation can be controlled by preventing the activation of any factor that can activate FV, or by slowing down the formation of these factors, so that their activities can be down-regulated by plasma-inhibitors. The kinetics of activation of FX may in particular be of importance since APC must compete with FXa for complexation with FVa, when FV becomes activated. Once bound to FXa, FVa is protected against inactivation by APC, especially when FVa is incorporated into PTase complex. Thus, an integrated view of several clotting factors is required when considering limitation of thrombin formation. To what extent FVIIIa contributes to the tissue factor-triggered thrombin generation, as described in chapters 5 and 6, is presently under investigation.

Figure 7.2 below summarises and illustrates in a simplified way the complex reactions that can occur following the addition of tissue factor to

plasma *in vitro*, that were discussed in this section. The reactions may partly represent the reactions that occur *in vivo* after tissue damage.

FIG. 7.2 Schematic representation of reactions in the pro- and anticoagulant pathways that are initiated after occurrence of tissue damage. The presence of tissue factor will elicit both a procoagulant response, eventually leading to fibrin deposition, and an anticoagulant response after thrombin-catalyzed protein C activation. Solid arrows indicate clotting factor conversions, dotted lines indicate participation of respective proteins as a cofactor, double lines denote positive feedback reactions. Grey horizontal bars indicate surface dependent reactions. FVi=inactivated FVa, FVIIIi=inactivated FVIIIa, TM=thrombomodulin, TF=tissue factor, PrS=protein S

Future research is needed to estimate the physiological values of all the *in vitro* APC-related interactions that have been published so far.

7.4 Current view on hypercoagulable states: APC resistance and its assessment in the hematological laboratory

If the inactivation of FVa is not the sole determinant for the down-regulation of thrombin generation, then what determines in a plasma sample the *in vitro* APC resistance assay outcome and what is the outcome's value?

Likewise, what are the causative conditions for the occurrence of thrombophilia as is observed *in vivo* in APC resistant individuals?

In chapters 5 and 6 we have shown that tissue factor triggered thrombin generation, and its inhibition by exogenously added APC, embodies a sensitive assay system for the detection of hypercoagulability. The mechanistic explanation for the power of the assay system in the detection of the tested hypercoagulable states, is possibly different for the groups of plasmas (carriers of FV_{Leiden} mutation and women using OC) tested in chapters 5 and 6 respectively. In chapter 5, the FV mutation will most likely contribute to observed differences between normals and plasmas from individuals carrying the Arg⁵⁰⁶→Gln mutation. Also, plasmas, presenting different APC-sr had no differences in standard laboratory parameters (coagulation factor concentrations, APTT, PT) other than the presence of the FV_{Leiden} mutation. For the experiments described in chapter 6, this explanation cannot account for the observed differences between the groups of plasmas, since all plasmas were tested for the presence of the FV_{Leiden} mutation and only groups of similar FV_{Leiden}-genotypes were compared. Differences in coagulation parameters, as have been reported in users of oral contraceptives and pregnant women, e.g. decreases in protein S levels, antithrombin III and increases in prothrombin, FX, FVII and protein C⁴²⁻⁴⁴ may contribute to the observed hypercoagulability in these plasmas. Notably, not only are the responses to APC in the plasmas tested in both chapters 5 and 6 different from normal plasmas, also the thrombin generations in the absence of APC are significantly enhanced in these plasmas (in particular in case of FV_{Leiden}, OC use and pregnancy). This may reflect a possible contribution of endogenously formed APC, and thus the protein C pathway, during the thrombin generation experiment. Also other factors such as diminished plasma inhibitor concentrations or altered coagulation factor levels could contribute to this enhanced *in vitro* thrombin formation.

Since October 1995, when the United Kingdom Committee on Safety of Medicines recommended that women should use oral contraceptives containing desogestrel or gestodene only if prepared to accept an increased risk of thromboembolism, an increasing amount of papers have been published that focus on the relationship between venous thromboembolic disease and use of oral contraceptives. None of these papers has found a so clear difference in laboratory test outcome between groups of plasmas from users of different generations of contraceptives as was found in our experiments, described in chapter 6. The reason for this is at present unclear, but the possible explanation for why in our system these differences are more marked than in other assay systems may very well give a clue to the

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Summary

Haemostasis and thrombosis are two closely related processes with a common origin. Haemostasis is a physiological process in which blood loss from damaged blood vessels is limited and stopped by formation of a thrombus. In the case of thrombosis, a pathological condition, the haemostatic balance is disturbed. This can lead to formation of thrombi at locations in the body where, and at times when no thrombus formation is needed. Unwanted formation of thrombi can lead to obstruction of the circulation which can have many consequences. Chapter 1 reviews the general hypotheses for the formation of blood clots under both physiological and pathophysiological conditions. Several components of the haemostatic system are described which function in the formation and regulation of thrombin, a serine protease that plays a pivotal role in haemostasis. Furthermore, the structure, activation, function and inactivation of FV, a non-enzymatic protein cofactor which is required for efficient generation of thrombin, is discussed. Particular attention is given to APC-mediated down-regulation of FVa cofactor activity in individuals with a Arg→Gln amino acid replacement at position 506 in the FV molecule (FV_{Leiden}, APC resistance). Acquired APC resistance, a phenomenon that has arisen since the general acceptance of screening for APC resistance by functional methods, is discussed in the last part of this chapter.

Chapter 2 describes the activation of FV by meizothrombin, a reaction intermediate formed during prothrombin activation. In this study, a recombinant meizothrombin (R155A meizothrombin) was used in which Arg¹⁵⁵ was replaced by Ala. This mutation prevents autocatalytic removal of the fragment 1 domain and allows studies on the functional activity of intact meizothrombin. In the presence of negatively charged phospholipid vesicles R155A meizothrombin was a potent activator of FV which activated FV with rate constants similar to those obtained for activation of FV by thrombin. Gel electrophoretic analysis of R155A meizothrombin- and thrombin-catalyzed activation of FV showed differences in the rates of cleavage of the susceptible peptide-bonds of FV by these two activators. However, FVa molecules obtained after full activation by R155A meizothrombin and thrombin were identical. It is concluded that during the initial phase of prothrombin activation, meizothrombin may play an important role in *in vivo* FV activation.

In chapter 3, the kinetics of inactivation of purified normal plasma FVa and FVa from an individual homozygous for the Arg⁵⁰⁶→Gln were studied. To this end, functional assays, probing the FVa cofactor activity, as well as immunoblot analysis were used. This study resulted in a model for the inactivation of FVa in which FVa loses its cofactor activity by APC-catalyzed

cleavage in the heavy chain domain of FVa in the presence of phospholipid vesicles via random cleavages of peptide bonds at positions Arg³⁰⁶ and Arg⁵⁰⁶. Cleavage at Arg⁵⁰⁶ is, however, characterized by a ~ 25 times higher second order rate constant (k_{cat}/K_m). This implies that at low FVa concentrations the peptide bond at Arg⁵⁰⁶ is cleaved at a ~ 20 times faster rate than cleavage at Arg³⁰⁶. Likewise, initial cleavage of a low concentration of FVa by APC is ~ 20 times faster in normal FVa than in FVa^{R506Q} since in the latter FVa, Arg⁵⁰⁶ is not present and cleavage occurs only at Arg³⁰⁶. It also demonstrates that FVa^{R506Q} is not fully resistant to inactivation by APC. Cleavage at Arg⁵⁰⁶ in normal FVa yields a reaction intermediate that possesses 40% cofactor activity in prothrombin activation mixtures containing 5 nM FXa. It is shown that the expression of cofactor activity of the intermediate is a function of the FXa concentration present in the FVa assay system that is used to monitor the loss of cofactor activity in prothrombin activation. This demonstrates that Arg⁵⁰⁶-cleaved FVa is impaired in its ability to interact with FXa. Similar kinetic analyses were performed for reaction systems that did not contain phospholipid vesicles. In these systems it was observed that most prominently in case of FVa^{R506Q}, cleavage at Arg⁶⁷⁹ also contributes to the observed loss of cofactor activity upon incubation with APC.

The knowledge about the mechanism of APC-catalyzed inactivation of FVa, obtained from the experiments presented in chapter 3 was used for the design of a chromogenic prothrombinase-based assay for the detection of APC-resistance (chapter 4). In this method, FV present in 1000-fold diluted plasma sample was activated with thrombin and subsequently incubated with phospholipids in the absence or presence of APC. After a fixed time interval FVa is quantified via determination of its cofactor activity in the prothrombinase complex. The percentage of remaining FVa cofactor activity, in the incubation with APC, relative to the incubation without APC, enables discrimination between plasma samples obtained from normal, heterozygous- or homozygous APC-resistant individuals. The test was optimized for the detection of normal, heterozygous- or homozygous APC-resistant individuals and the sensitivity and specificity obtained were both 100%, implying full correlation with DNA-based methods. The observed residual FVa activities for the different plasmas tested corresponded well with residual activities that were calculated on basis of rate constants of inactivation, presented in chapter 3. The test can be performed on any sample, provided that a sufficient amount of FV is present. Moreover, conditions that are known to hamper or influence functional detection of APC-resistance by clotting time-based methods (such as heparin treatment, oral anticoagulation, coagulation

factor deficiencies, pregnancy, use of oral contraceptives or the presence of lupus anticoagulants) do not interfere with the outcome of our test.

In chapter 5 the focus of research shifts from the study of the anticoagulant function of APC in model systems, to systems which contain platelet poor plasma. The effect of APC on the extrinsic and intrinsic pathways of coagulation in plasma from normal and APC-resistant individuals was studied by measuring the time integral of thrombin generation (endogenous thrombin potential, ETP) determined via chromogenic methods. It is shown that the reduction of the ETP by APC can be inferred from the ratio of endlevels of α_2 -macroglobulin-thrombin complexes, in the presence and absence of 50 nM APC: $\alpha_2\text{M-IIa}_{+\text{APC}} / \alpha_2\text{M-IIa}_{-\text{APC}}$. Since the $\alpha_2\text{M-IIa}$ complex possesses amidolytic activity on small chromogenic substrates, the ratio, called APC-sensitivity ratio can be determined from two amidolytic measurements of the final levels of $\alpha_2\text{M-IIa}$ complex, both in the presence and in the absence of APC. Division of the obtained ratio by the same ratio as determined for a normal plasma resulted in the normalized APC sensitivity ratio (nAPC-sr). Significant differences ($p < 0.001$) were observed between APC-sr of plasmas from normal individuals (nAPC-sr: 0.5 - 1.9, $n=25$) and of plasmas from individuals that were heterozygous (nAPC-sr: 2.1 - 6.7, $n=17$) or homozygous APC resistant (nAPC-sr: 3.9 - 5.9, $n=5$). There was no overlap between APC-sr of normal plasmas and plasma from individuals, bearing the FV mutation. The data show that measurement of the effect of APC on the ETP yields valuable information about the (pro)thrombotic status of a plasma (e.g. APC resistance, pregnancy or the use of oral contraceptives).

In chapter 6 the effect addition of APC on the ETP of a plasma sample, as is described in chapter 5, was further evaluated with plasma samples from women who were using oral contraceptives (OC) and from women not using OC. Compared with women not using OC, women who used OC exhibited a significantly decreased sensitivity to APC ($P < 0.001$), independent of the kind of OC used. Furthermore, it was shown that women who used third-generation monophasic OC were significantly less sensitive to APC than women using second-generation OC ($P < 0.001$) and had APC-sr that did not differ significantly from heterozygous female carriers of FV_{Leiden}, not using OC. During the research, five women were identified who used OC and were heterozygous carrier for the FV_{Leiden} mutation. These women had an nAPC-sr in the range of homozygous carriers for the FV_{Leiden} mutation. Two women were included into the research that started OC therapy. Within 3 days after start of OC therapy the APC-sr was significantly elevated. We propose that the observed acquired resistance to APC may well explain the

epidemiological observation of the increased risk for venous thrombosis in OC users, especially in women using third-generation OC.

Chapter 7 gives a general discussion of the data that are presented in the chapters 2 to 6 and puts these data in the context of experiments that were performed by other research groups. The different chapters are linked to each other, where possible. Chapter 7 ends with a discussion on hypercoagulable states, such as APC resistance and acquired factors such as use of oral contraceptives or pregnancy.

Samenvatting

Dit proefschrift beschrijft het onderzoek dat gedurende de periode van 1 maart 1993 tot 1 maart 1997 door de auteur is uitgevoerd binnen de vakgroep Biochemie van de Faculteit der Geneeskunde van de Universiteit Maastricht. De vakgroep Biochemie vormt een onderdeel van CARIM, het Cardio-vasculair Research Instituut Maastricht. Binnen dit instituut wordt vanuit verschillende wetenschappelijke disciplines onderzoek verricht dat betrekking heeft op de veelheid van processen die met het functioneren van hart- en bloedvaten te maken hebben. Belangrijk onderdeel van deze processen zijn de hemostase en trombose.

Hemostase en trombose zijn twee nauw aan elkaar verwante mechanismes die eenzelfde oorsprong hebben. Hemostase (bloedstolling) is een fysiologisch proces dat als doel heeft bloedverlies uit beschadigde bloedvaten te beperken en te stoppen. Hierbij wordt het beschadigde vat door een bloedpropje afgesloten. Van trombose spreken we wanneer er sprake is van een pathofysiologische toestand ('ziekte') waarin de hemostase niet meer onder controle is. Het gevolg hiervan is dat er bloedpropjes ontstaan in het lichaam op plaatsen en op tijdstippen waarop dit niet noodzakelijk is. Deze ongewenste vorming van bloedpropjes kan leiden tot verstopping van bloedvaten, waardoor de normale bloedcirculatie wordt gehinderd. Trombose kan in verschillende vormen, met ieder verschillende consequenties, voorkomen.

Hoofdstuk 1 geeft een samenvatting van de huidige theorieën over de vorming van bloedstolsels, zowel onder normale, als onder pathofysiologische condities. Verschillende bouwstenen van het hemostasesysteem worden besproken. Centraal staat echter de functie van het enzym trombine (behorend tot de serine protease klasse).

Verder wordt de structuur, de activering, de functie en inactivering van de stollingsfactor V¹ besproken. Factor V is een niet-enzymatisch cofactor molecuul dat essentieel is voor de effectieve vorming van trombine.

In het bijzonder wordt er aandacht geschonken aan het ongedaan maken van de activiteit die ontstaat wanneer de niet-actieve factor V is omgezet in het actieve FVa. Onderzoek naar de regulatie van FVa activiteit in personen waarbij als gevolg van een genetische afwijking een verandering in het gen voor factor V heeft plaats gevonden (de arginine op positie 506 is vervangen door een glutamine) neemt een bijzondere plaats in. Deze afwijking noemt men de FV_{Leiden}-mutatie omdat deze voor het eerst door de onderzoeksgroep van Prof. Bertina uit Leiden is beschreven (synoniem is de term FV^{R506Q}). Mensen met FV_{Leiden} worden APC-resistent genoemd aangezien de regulatie van FVa activiteit in deze individuen sterk belemmerd is en het factor Va ongevoelig is geworden voor APC, het enzym dat onder normale omstandigheden FVa inaktiveert.

Door in het laboratorium te testen of het bloedplasma van een individu wel of niet gevoelig is voor APC kan men bepalen of iemand APC-resistent is. Gebleken is echter dat APC resistentie niet alleen voorkomt bij mensen die de FV_{Leiden}-mutatie bezitten, maar ook bij mensen waar er geen sprake is van een bekende afwijking aan het stolsysteem. In die gevallen wordt gesproken van verworven APC resistentie. Verworven APC-resistentie, besproken in het laatste deel van hoofdstuk 1; komt onder andere voor bij vrouwen die zwanger zijn of die anticonceptie-pillen gebruiken.

Hoofdstuk 2 beschrijft de activering van FV door meizotrombine, een reactie-tuusenprodukt bij de protrombine-activering. In deze studie is een recombinante vorm van meizotrombine gebruikt, R155A meizotrombine. Voor de constructie van deze mutant werd het arginine residu op aminozuur-positie 155 veranderd in een alanine; wat tot gevolg had dat autokatalytische afsplitsing van het fragment 1 domein onmogelijk werd gemaakt. In aanwezigheid van negatief geladen fosfolipide-oppervlakken was R155A meizotrombine een sterke activator van FV, gekarakteriseerd door een tweede orde snelheidsconstante voor de activering van FV die van dezelfde grootte-orde is als die voor de activering van FV door trombine.

¹ Volgens afspraak worden een aantal verschillende stoffactoren met Romeinse cijfers aangeduid. Een hoofdletter 'F' is de afkorting voor stoffactor. Wanneer een stoffactor niet actief is staat er enkel een Romeins cijfer, bijvoorbeeld FV. Wanneer men de actieve vorm van een stoffactor bedoelt, dan wordt deze aangeduid door de letter 'a' bij te plaatsen, bijvoorbeeld FVa.

Gelelektroforetische analyse van FV-activeringen door R155A meizotrombine of trombine toonden aan dat er verschillen zijn in de snelheden waarmee de verschillende peptide-bindingen gesplitst worden tijdens de activering van FV. Na volledige activering van FV door R155A meizotrombine of trombine worden echter FVa moleculen verkregen die identiek zijn. De conclusie wordt getrokken dat gedurende de initiële fase van de protrombineactivering, meizotrombine een belangrijke rol kan spelen in de *in vivo* activering van FV.

In hoofdstuk 3 wordt de inactivering van FVa door APC beschreven voor zowel normaal FVa als voor FVa gezuiverd uit het plasma van iemand die homozygoot voor de Arg⁵⁰⁶→Gln mutatie is. Hierbij is gebruik gemaakt van een assay waarmee de functionele activiteit van FVa getest wordt en waarbij peptide-band splitsingen in de zware keten van FVa worden bestudeerd met behulp van immunoblot-analyse. Een model voor de inactivering van FVa door APC wordt voorgesteld, waarbij in aanwezigheid van een fosfolipide-opervlak de cofactor-activiteit van FVa verdwijnt, na splitsing van de peptide-bindingen achter de aminozuren Arg³⁰⁶ en Arg⁵⁰⁶ (in willekeurige volgorde) in de zware keten van FVa. Splitsing op positie Arg⁵⁰⁶ wordt echter gekarakteriseerd door een ongeveer 25 maal grotere reactiesnelheidsconstante (k_{cat}/K_m) dan splitsing op positie Arg³⁰⁶. Dit verschil wordt met name veroorzaakt door een 10-voudig verschil in de K_m voor de splitsing van beide peptide-bindingen. Dit houdt in dat bij lage FVa concentraties, zoals die in de beschreven experimenten zijn gebruikt, de splitsing achter Arg⁵⁰⁶ 20 keer sneller gaat dan die achter Arg³⁰⁶. Dit betekent dat FVa_{Leiden}, niet volledig resistent is tegen inactivering door APC en via splitsing van de peptideband achter Arg³⁰⁶ 20 keer langzamer dan FVa kan worden geïnactiveerd.

Splitsing achter Arg⁵⁰⁶ van normaal FVa levert een reactie-tussenproduct dat 40% van de initiële cofactor activiteit bezit in protrombine-activerings mengsels die 5 nM FXa bevatten. Aangetoond wordt dat de expressie van cofactor activiteit van het reactie-tussenproduct een functie is van de FXa concentratie die aanwezig is in de FVa cofactor assay. Dit toont aan dat Arg⁵⁰⁶-gesplitst FVa belemmerd is in zijn interactie met FXa.

Soortgelijke kinetische analyses als voor de fosfolipide-bevattende reactie-mengsels zijn uitgevoerd voor reactie-mengsels die geen fosfolipide vesikels bevatten. Experimenten in deze systemen toonden aan dat met name in geval van de inactivering van FVa^{R506Q}, de splitsing achter Arg⁶⁷⁹ ook bijdraagt aan het waargenomen verlies van cofactor-activiteit tijdens incubatie met APC.

Het model voor de door APC gekatalyseerde inactivering van FVa, dat in het vorige hoofdstuk 3 werd voorgesteld is gebruikt bij het ontwerpen van een chromogene test, op basis van protrombinase-activiteit, voor de detectie van

APC-resistentie. Deze methode wordt beschreven in hoofdstuk 4. In deze methode wordt een plasmamonster sterk verdund (1000 keer) in een fosfolipide-bevattende buffer en het aanwezige FV wordt met trombine geactiveerd. De resulterende FVa activiteit wordt vervolgens bepaald in een protrombinase-assay, nadat er een incubatiestap met- of zonder APC heeft plaatsgevonden. Het restpercentage FVa activiteit, in het reactiemengsel met daarin APC, ten opzichte van het reactiemengsel zonder APC, wordt gebruikt als een maat om te bepalen of het plasma sample afkomstig was van een normaal individu ofwel van een heterozygoot of homozygoot APC resistent individu. De methode is geoptimaliseerd en zowel de specificiteit als de sensitiviteit van de test waren 100%, wat inhoudt dat er een volledige correlatie bestaat met methoden die op DNA analyse zijn gebaseerd. De waargenomen FVa restactiviteiten voor de verschillende plasma's corresponderden goed met restactiviteiten die op grond van de kinetische parameters die in hoofdstuk drie zijn gegeven worden berekend voor de verschillende inactiveringen. De test kan worden uitgevoerd op elk plasma monster, op voorwaarde dat er voldoende FV aanwezig is. Bovendien is de test uitvoerbaar in plasmas die via methodes die gebaseerd zijn op de bepaling van stoltijden moeilijk of niet te bepalen zijn (bijvoorbeeld bij aanwezigheid van heparine of lupus anticoagulantia, bij gebruik van orale anticoagulantia of oral contraceptiva of bij zwangerschap).

In hoofdstuk 5 verschuift het onderzoek naar de anticoagulante functie van APC van een basaal onderzoek in model-systemen naar een meer toegepaste vorm, in systemen die bloedplaatjes-arm plasma bevatten. Het effect van de toevoeging van APC aan plasmamonsers waarin de stolling zowel intrinsiek- als extrinsiek gestart is wordt bestudeerd voor plasma's van normale, gezonde individuen en van APC-resistente individuen. Kwantitering van dit effect van APC wordt uitgevoerd via verlaging van de tijdsintegraal van trombinevorming (endogene trombine potentiaal, ETP) die wordt geregistreerd via een chromogene methode. Aangezien wordt dat een verlaging van de ETP door APC eenvoudig kan worden afgeleid uit een simultane vermindering van de eindniveau's van de α_2 -macroglobuline-trombine complexen, zowel in aan- als in afwezigheid van APC: $\alpha_2\text{M-IIa}_{+APC} / \alpha_2\text{M-IIa}_{-APC}$. Aangezien het $\alpha_2\text{M-IIa}$ complex amidolytische activiteit bezit t.o.v. kleine chromogene substraten, kan de voorgenoemde ratio van $\alpha_2\text{M-IIa}$ niveau's worden berekend uit twee amidolytische bepalingen van de eindniveau's van $\alpha_2\text{M-IIa}$ complex, één in aanwezigheid, en één in afwezigheid van APC.

Deling van de verkregen ratio van activiteiten van het $\alpha_2\text{M-IIa}$ complex voor een willekeurig plasmamonster door een soortgelijke ratio die bepaald is

voor een gedefinieerd normaal plasma geeft de genormaliseerde APC-sensitiviteits ratio (nAPC-sr). Tussen de plasma's van normale individuen (nAPC-sr: 0.5 - 1.9, n=25) en heterozygote (nAPC-sr: 2.1 - 6.7, n=17) en homozygote APC-resistente (nAPC-sr: 3.9 - 5.9, n=5) personen zijn significante verschillen ($p < 0.001$) in APC-sr waargenomen. Er was geen overlap tussen de APC-sr van normale plasma's en plasma's van personen die drager van de FV_{Leiden}-mutatie zijn. Onze data tonen aan dat bepaling van het effect van APC-toevoeging op de ETP waardevolle informatie oplevert over de (pro)trombotische status van een plasmamonster (bijvoorbeeld in geval van APC-resistentie, zwangerschap of gebruik van orale contraceptiva).

Hoofdstuk 6 beschrijft het effect van toevoeging van APC op de ETP van plasma-monsters, volgens de methode zoals beschreven in hoofdstuk 5, voor een serie van monsters van vrouwen die wel of niet orale contraceptiva (OC) gebruiken. Vergeleken met vrouwen die geen OC gebruiken, vertoont het plasma van vrouwen die OC gebruiken een significant verminderde gevoeligheid voor APC ($p < 0.001$), onafhankelijk van het soort OC dat wordt gebruikt. Bovendien was het plasma van vrouwen die een derde-generatie monofasische OC gebruiken, significant ongevoeliger voor APC dan het plasma van vrouwen die een tweede-generatie OC gebruiken ($p < 0.001$) en waren de APC-sr niet significant verschillend van de APC-sr voor een groep vrouwelijke heterozygote draagsters van de FV_{Leiden}-mutatie die geen OC gebruiken. Vijf vrouwen die aan het onderzoek als bloeddonor deelnamen en die OC gebruikten bleken heterozygote draagster voor de FV_{Leiden}-mutatie. Deze vrouwen hadden een APC-sr in de orde van grootte van die welke behoort bij homozygote dragers van de FV_{Leiden}-mutatie.

Twee vrouwen, starters van OC-gebruik, werden bij het onderzoek betrokken. Binnen een tijdsbestek van drie dagen na aanvang van de OC therapie was de APC-sr in het plasma van deze vrouwen significant verhoogd. Wij stellen dan ook de hypothese dat de waargenomen verworven APC-resistentie mogelijk een verklaring geeft voor het toegenomen risico op veneuze trombose bij het gebruik van OC en met name derde-generatie OC, zoals dat uit epidemiologisch onderzoek wordt geconcludeerd.

Hoofdstuk 7 bediscussieert in algemene zin de data die in de hoofdstukken 2 tot en met 6 worden gepresenteerd en stelt deze in een context van data die door andere onderzoeksgroepen werden gepubliceerd. Verbanden tussen de verschillende hoofdstukken worden, waar mogelijk, gelegd. Hoofdstuk 7 eindigt met een uiteenzetting over hypercoagulabele toestanden (dat zijn condities waarbij om de één of andere reden er sprake is van een trombotiseneiging) zoals APC- resistentie en verworven factoren zoals bij gebruik van orale contraceptiva of zwangerschap.

Curriculum Vitae

De auteur van dit proefschrift werd op 12 mei 1969 geboren te Maastricht. Na het succesvol doorlopen van de kleuterschool, alwaar een eerste proeve van bekwaamheid werd afgelegd in de vorm van het schoenstrikexamen, ging hij naar de basisschool, de John F. Kennedyschool en later de Petrus en Paulusschool te Wolder-Maastricht. Met een verkeers- en een EHBO-diploma op zak ging Gerry vervolgens naar de brugklas van het Stedelijk Lyceum in Maastricht, alwaar hij zijn opleiding vervolgde in het Gymnasium. Op 27 mei 1987 werd het examen Gymnasium-B met veel ijver behaald en in augustus van datzelfde jaar ging Gerry naar de grote stad Utrecht om er Scheikunde te studeren aan de lokale Rijksuniversiteit.

Na een vlotte propaedeuse en een verplicht basisprogramma werden twee bijvakken gekozen, Biochemie van de Erfelijke Metabole Stoomissen (in het Wilhelmina Kinderziekenhuis te Utrecht onder begeleiding van Dr. M. Duran en Dr. L. Dorland) en een bijvak Klinische Chemie (in het Academisch Ziekenhuis Utrecht onder begeleiding van Dr. M.A. Blankenstein en Prof. Dr. J.H.H. Thijssen). Vervolgens werd Gerry hoofdvakstudent bij de vakgroep Enzymology en Protein Engineering binnen het Centrum voor Biomembranen en Lipide Enzymologie van de Universiteit Utrecht (onder begeleiding van Dr. R.B. Lugtigheid en Prof. Dr. H.M. Verhey). Na een geslaagde afstudeerscriptie over het onderwerp 'HIV-infecties en de mogelijke chemotherapeutische waarde van HIV-protease inhibitors' werd het hoofdvakexamen Biochemie afgenomen door de professoren Prof. Dr. L.L.M. van Deenen[†] en Prof. Dr. B. de Kruijff. Direct gevolg van dit examen was het verkrijgen van de doctorandus-titel alsmede een keuze uit twee AIO-opleidingsplaatsen, waarvan één in Maastricht. Op 25 januari 1993 ontving Gerry, gehesen in een nieuw pak, zijn bul van de Universiteit Utrecht.

Vanaf 1 maart 1993 was Gerry met veel genoegen werkzaam als Assistent In Opleiding bij het Cardiovasculair Research Instituut in het geliefde Maastricht (CARIM). Binnen het CARIM, onderdeel van de Rijksuniversiteit Limburg, een universiteit in beweging, ze heet inmiddels Universiteit Maastricht, werkte hij onder begeleiding van zijn promotor Prof. Dr. H.C. Hemker en de copromotoren, Dr. J. Rosing en Dr. G. Tans aan hetgeen er in dit proefschrift beschreven staat.

List of Publications

1. Acylation of porcine pancreatic PLA₂ influences penetration and substrate headgroup binding, depending on the position of the acylated lysine in the enzyme molecule.
Lugtigheid RB, Nicolaes GAF, Veldhuizen EJA, Slotboom AJ, Verheij HM, de Haas GH. (1993) *European Journal of Biochemistry* **216**(2), 519-525
2. Activation of human factor V by meizothrombin.
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3. Peptide bond cleavages and loss of functional activity during inactivation of factor Va and factor Va^{R506Q} by activated protein C
Nicolaes GAF, Tans G, Thomassen MCLGD, Hemker HC, Pabinger I, Váradi K, Schwarz HP, Rosing J. (1995) *Journal of Biological Chemistry* **270**, 21158-21166
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5. A prothrombinase-based assay for detection of resistance to activated protein C
Nicolaes GAF, Thomassen MCLGD, van Oerle R, Hamulyák K, Hemker HC, Tans G, Rosing J. (1996) *Thrombosis and Haemostasis* **76**(3), 404-410
6. Effect of activated protein C on thrombin generation and on the thrombin potential in the plasma of normal and APC resistant individuals
Nicolaes GAF, Thomassen MCLGD, Tans G, Rosing J, Hemker HC. (1997) *Blood Coagulation & Fibrinolysis* **8**, 28-38
7. Oral Contraceptives and venous thrombosis: different sensitivities to activated protein C in women using second and third generation oral contraceptives.
Rosing J, Tans G, Nicolaes GAF, Thomassen MCLGD, Van Oerle R, Van der Ploeg PMEN, Heijnen P, Hamulyák K, Hemker HC. (1997) *British Journal of Haematology* **97**, 233-238
8. Human factor Va1 and Va2: Properties in the procoagulant- and anticoagulant pathways.
Hoekema L, Nicolaes GAF, Tans G, Hemker HC, Rosing J. (1997) *Biochemistry*, **36**(11), 3331-3335
9. Regulation of thrombin formation by activated protein C: effect of the FV_{Leiden} mutation.
Tans G, Nicolaes GAF, Rosing J. (1997) *Seminars in Hematology*, *accepted for publication*

Abstracts

1. Detection of APC resistance by measurement of the effects of APC on the endogenous thrombin potential and on plasma factor Va cofactor activity
Nicolaes GAF, Thomassen MCLGD, Hemker HC, Tans G, Rosing J. (1996)
Haemostasis 26(suppl 3), 444
2. Human factor Va1 and Va2: different sensitivities for activated protein C (APC) results in different procoagulant activities.
Nicolaes GAF, Hoekema L, Tans G, Rosing J., accepted as oral presentation at the Congress of the International Society on Thrombosis and Haemostasis, Florence, 6-12 June 1997
3. Use of two new functional assays to detect inherited and acquired APC-resistance.
Nicolaes GAF, Thomassen MCLGD, Hemker HC, Tans G, Rosing J. accepted as poster presentation at the Congress of the International Society on Thrombosis and Haemostasis, Florence, 6-12 June 1997
4. APC-mediated regulation of factor Va activity in plasma.
Hoekema L, Nicolaes GAF, Tans G, Rosing J. accepted as poster presentation at the Congress of the International Society on Thrombosis and Haemostasis, Florence, 6-12 June 1997
5. Different sensitivities to activated protein C (APC) in women using second and third generation oral contraceptives.
Tans G, Nicolaes GAF, Thomassen MCLGD, Hamulyák K, Hemker HC, Rosing J. accepted as poster presentation at the Congress of the International Society on Thrombosis and Haemostasis, Florence, 6-12 June 1997

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