Molecular biology and pathophysiology of APC resistance

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Molecular Biology and Pathophysiology of APC Resistance: Current Insights and Clinical Implications

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ABSTRACT APC resistance is often associated with the occurrence of a single point mutation in factor V (factor V_{Leiden}) at a predominant cleavage site for the natural anticoagulant, activated protein C (APC). In this article we will discuss the effects of this mutation (Arg⁵⁰⁶→Gln) on the down-regulation of factor Va cofactor activity and on thrombin formation by APC in model systems and in plasma. Our studies on the effects of APC on thrombin formation in plasma resulted in the development of a new method for the screening of APC resistance that is based on measurement of the effect of APC on the endogenous thrombin potential (the time integral of thrombin generated in clotting plasma). It appeared that sensitivities for APC determined via this method were considerably affected by the use of oral contraceptives (OC) and that women who use OC become acquired APC resistant. The fact that acquired APC resistance in women who use third-generation OC was more pronounced than in users of second-generation OC may explain the further increased risk for venous thrombosis associated with the use of third-generation OC.

Keywords: APC resistance, factor V_{Leiden}, oral contraceptives, venous thrombosis

Some 20 years ago it was recognized that the activated form of the plasma protein protein C, called APC, is a serine protease that down-regulates thrombin forma-

tion via limited proteolysis of factor Va.^{1,2} Soon afterwards, it was reported that efficient inactivation of factor Va by APC required the presence of negatively charged phospholipids³⁻⁶ and another vitamin K-dependent plasma protein, protein S.^{4,5,7,8}

The protein C pathway presently attracts much attention of clinical and research laboratories. This is due to the fact that in 1993 Björn Dahlbäck⁹ discovered a new risk factor for familial venous thrombosis that is characterized by inherited resistance to APC. About 1 year later it was reported¹⁰⁻¹³ that APC resistance is caused by a single point mutation in the factor V gene that results in the replacement of an amino acid at a predominant cleavage site for APC.

In this article we will discuss the effects of this mutation on APC-catalyzed factor Va activation in model systems and on the down-regulation of thrombin formation by APC in plasma. These studies have led to the development of new methods for the screening of hereditary and acquired APC resistance.

INACTIVATION OF FACTOR Va AND FACTOR Va_{Leiden} IN MODEL SYSTEMS

Factor Va, the protein cofactor of the prothrombin-activating complex, is a heterodimer that consists of a heavy and a light chain held together by a single calcium ion. In 1994 the group of Mann¹⁴ reported that APC can cleave three peptide bonds in the heavy chain of human factor Va, which are located at positions Arg³⁰⁶, Arg⁵⁰⁶, and Arg⁶⁷⁹ (Fig. 1). Sequence analysis of the factor V gene has shown that in APC-resistant individuals the arginine at position 506 is replaced by glutamine.¹⁰⁻¹³ To get more insight into the consequences of this mutation, several laboratories¹⁵⁻¹⁹ have purified factor Va from homozygous APC-resistant individuals (factor V_{Leiden}) and compared its sensitivity for APC with that of normal fac-

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FIG. 1. APC cleavage sites in human factor Va. In factor $\text{Va}_{\text{Leiden}}$, Arg^{506} is replaced by Gln.

tor Va in model systems containing purified proteins and artificial phospholipid vesicles.

Figure 2 shows that normal factor Va is inactivated by APC via a biphasic reaction that consists of a rapid phase that yields a reaction intermediate that retains approximately 40% of the cofactor activity of native Va and that is subsequently fully inactivated via a second, slow reaction.¹⁷ Factor $\text{Va}_{\text{Leiden}}$ is inactivated via a slow monophasic reaction, the initial rate of which is some 20-fold lower than that of normal Va. Detailed kinetic analysis showed that inactivation of factor $\text{Va}_{\text{Leiden}}$ proceeds at a rate that is approximately similar to that of the second phase of normal factor Va inactivation.¹⁷

Gel electrophoretic analysis of time courses of inactivation of factor Va and factor $\text{Va}_{\text{Leiden}}$ ¹⁷ indicated that the rapid phase of inactivation of normal factor Va is associated with cleavage at Arg^{506} and that inactivation is completed via cleavage at Arg^{306} . Factor $\text{Va}_{\text{Leiden}}$ is slowly but fully inactivated via a single peptide bond cleavage at Arg^{306} .

FIG. 2. Inactivation of factor Va and factor $\text{Va}_{\text{Leiden}}$ by APC. Time courses of inactivation of 0.3 nM factor Va ($\blacktriangle$) and 0.3 nM factor $\text{Va}_{\text{Leiden}}$ ($\blacksquare$) by 1.5 nM APC were determined in the presence of 25 μM dioleoylphosphatidylserine/dioleoyl phosphatidylcholine (10/90, M/M) vesicles in 25 mM Hepes (pH 7.5), 175 mM NaCl, and 3 mM CaCl_2 . Data from Nicolaes et al¹⁷ (reprinted with permission).

It is interesting to note that the heavy chain fragments formed after cleavage at Arg^{506} remain associated and that factor Va cleaved at Arg^{506} is still active as a cofactor in prothrombin activation.¹⁷ This reaction intermediate has considerably less affinity for factor Xa and requires approximately 50 times more factor Xa for the expression of cofactor activity in prothrombin activation than native factor Va. As a consequence, factor Va cleaved at Arg^{506} can express variable residual cofactor activity in prothrombin activation depending upon the amount of factor Xa present.

Based on these observations we proposed¹⁷ that normal factor Va can be inactivated by APC via two pathways (Fig. 3), the predominant one being pathway 1, which involves rapid cleavage at Arg^{506} and formation of a factor Va intermediate that still has cofactor activity in prothrombin activation, which is subsequently fully inactivated by slow cleavage at Arg^{306} . The latter peptide bond can be cleaved also directly in factor Va. This is a slow reaction that yields a form of factor Va with negligible cofactor activity in prothrombin activation. Due to the mutation at Arg^{506} , pathway 1 cannot occur in factor $\text{Va}_{\text{Leiden}}$, which as a consequence can only be slowly inactivated via pathway 2.

It should be emphasized that this is a model for factor Va inactivation by APC alone. However, it has been known for many years that the activity of the protein C pathway is stimulated by protein S^{7,8} and that factor Xa protects factor Va against inactivation by APC.^{3,20,21} Kinetic analysis¹⁸ of the effects of factor Xa and protein S on time courses of normal factor Va inactivation suggests that factor Xa specifically inhibits the rapid phase of inactivation that is cleavage at Arg^{506} and that protein S accelerates the second phase of inactivation that is associated with cleavage at Arg^{306} . The fact that factor Xa does not protect factor $\text{Va}_{\text{Leiden}}$ and that protein S accelerates its inactivation more than 20-fold¹⁸ confirms that the target site of protein S is Arg^{306} and that factor Xa specifically inhibits cleavage at Arg^{506} . This suggests that in the presence of factor Xa and protein S factor Va and factor $\text{Va}_{\text{Leiden}}$ will be inactivated mainly via pathway 2 (Fig. 3), which diminishes the differences in sensitivity of normal factor Va and factor $\text{Va}_{\text{Leiden}}$ for APC and may explain why APC resistance is only a mild risk factor for venous thrombosis.

SCREENING FOR THE FACTOR V_{LEIDEN} MUTATION BY MEASURING THE EFFECT OF APC ON THROMBIN GENERATION IN PLASMA

On the basis of the information obtained on the down-regulation of factor Va cofactor activity by APC in model systems, we have developed two new methods for the screening of APC resistance.²²⁻²⁴ In one of the accompanying papers in this issue Michiels and Hamulyák

Pathway I

Pathway II

Fig. 3. Pathways of factor Va and factor Va_{Leiden} inactivation by APC (reprinted with permission).

present the clinical application of our prothrombinase-assay. In this article we will discuss the possibility to screen for APC resistance by measuring the effect of APC on the thrombin potential, a valuable diagnostic parameter introduced by Hemker and co-workers some 10 years ago.^{25,26}

When coagulation in a plasma sample is initiated with thromboplastin and thrombin formation is quantified with a thrombin-specific chromogenic substrate, one obtains the so-called thrombin generation curve (Fig. 4). The rising part of this curve probes the activation and in some cases also the inactivation of the coagulation factors that are involved in prothrombin activation. The descending part of the curve mainly reflects the inhibition of thrombin by plasma protease inhibitors, particularly antithrombin and α -2 macroglobulin (α -2M).

Fig. 4. The thrombin generation curve.

Because the α -2M-thrombin (α -2M-IIa) complex is still active on chromogenic substrates, the curve ends with a constant, residual level of amidolytic activity. Using the rate constant of inhibition of thrombin by α -2M, it is possible to calculate the amount of α -2M-IIa present at any moment and correct the thrombin generation curve for the contribution of this complex.²⁵ After this correction, one obtains the amount of free thrombin present in clotting plasma as a function of time. The surface under this curve is defined as the endogenous thrombin potential (ETP), which has a value of $382 \text{ nM} \cdot \text{min} \pm 52 \text{ (SD)}$ in plasma samples of normal individuals.²⁶

It appears that thrombin generation in normal plasma can be almost completely abolished by APC (Fig. 5A). The middle panel of this figure (Fig. 5B) shows the effect of the same APC concentration on thrombin generation in plasma of a heterozygous carrier of factor V_{Leiden}. It is obvious that thrombin generation is resistant towards APC and that approximately two to three times more thrombin is formed than in normal plasma. Thrombin generation in plasma of a homozygous carrier of factor V_{Leiden} is even less affected by APC (Fig. 5C). Visual inspection of the thrombin generation curves indicates that APC decreases the thrombin potential in this plasma by less than 50%.

To quantitate the effect of APC on thrombin generation we defined an APC sensitivity ratio (APC-sr) that is equal to the ratio of thrombin potentials determined in the presence and absence of APC. Because the final level of α -2M-IIa appears to be proportional to the ETP,^{23,27,28} values of the APC-sr calculated from the ETP are the same as those calculated from final levels of α -2M-IIa

FIG. 5. Effect of APC on thrombin generation in plasma. Thrombin generation was initiated in normal plasma (A), in plasma of a heterozygous (B), or homozygous carrier of factor V_{Leiden} (C) with a phospholipid/ $CaCl_2$ /tissue factor mixture either in the absence (Δ) or presence ($\bullet$) of APC. Thrombin formation was determined with the chromogenic substrate D-Phe-(piperidyl)-Arg-pNA (S2238). The amidolytic activity was corrected for the contribution of the α_2 -M-IIa complex. Further details are given in Nicolaes et al²³ (reprinted with permission).

determined after 15–20 min. To increase day-to-day reproducibility, data were expressed as normalized APC-sensitivity ratios (nAPC-sr) by dividing the APC-sr through the same ratio determined in a normal pooled plasma. Hence, the n-APC-sr can be also calculated according the formula:

FIG. 6. n-APC-sr of normal individuals, heterozygous and homozygous carriers of factor V_{Leiden} determined by measuring the effect of APC on the endogenous thrombin potential. Data from Nicolaes et al²³ and Rosing et al.³³

$$nAPC-sr = \frac{(\alpha_2M-IIa_{+APC} / \alpha_2M-IIa_{-APC})_{\text{plasma sample}}}{(\alpha_2M-IIa_{+APC} / \alpha_2M-IIa_{-APC})_{\text{normal plasma pool}}}$$

It should be emphasized that due to the calculation procedure, nAPC-sr determined with the thrombin potential method shows trends that are the reverse of those obtained with assays based on the determination of the effect of APC on the activated partial thromboplastin time (APTT).²⁹ Thus, with the thrombin potential method, normal plasmas will give nAPC-sr ~ 1 , while APC-resistant plasmas will give higher nAPC-sr than normal plasma.

Figure 6 shows the result of a determination of the nAPC-sr with the thrombin potential method of a large number of plasma samples from healthy individuals and

FIG. 7. Effect of OC therapy on n-APC-sr determined by measuring the effect of APC on the endogenous thrombin potential. The populations are: women who do not use OC (no OC), women who use second-generation monophasic OC (MP2), women who use third-generation monophasic OC (MP3), and heterozygous female carriers of the factor V_{Leiden} mutation who do not use OC (FV_{Leiden}). Data from Rosing et al.³³

TABLE 1. APC Sensitivity Ratios and Risks of Venous Thrombosis of OC Users and Heterozygous and Homozygous Carriers of Factor V_{Leiden}

Women		n	nAPC-sr ^a (95–95 percentile)	Risk Increase
No FV _{Leiden}	no OC	53	0.87–2.15	—
No FV _{Leiden}	MP3	40	1.91–3.88	6–8 ^b
Heterozygous FV _{Leiden}	no OC	17	2.09–6.60	7 ^c
Heterozygous FV _{Leiden}	OC	6	4.53–6.45	30–50 ^{d,e}
Homozygous FV _{Leiden}	no OC	5	3.91–6.02	>50 ^f

Data from ^aRosing et al,³³ ^bWHO,³⁶ ^cKoster et al,⁴² ^dVandenbroucke et al,⁴³ ^eBloemenkamp et al,⁴⁴ and ^fRosendaal et al.⁴⁵
MP3, users of third generation monophasic OC.

from heterozygous and homozygous carriers of factor V_{Leiden}. It appears that the majority of the normal plasma samples indeed had a nAPC-sr around 1 and that carriers of the factor V_{Leiden} mutation had nAPC-sr higher than 2. It was, however, not possible to discriminate fully normal individuals from carriers of factor V_{Leiden} because there was some overlap between the nAPC-sr of these populations.

OC THERAPY AND ACQUIRED APC RESISTANCE

The data presented above indicate that the ETP test is not 100% decisive like DNA analysis,³⁰ a prothrombinase-based assay,^{22,24} or an APTT-based test performed in factor V-deficient plasma^{31,32}; and one may question the usefulness of APC-resistance tests that are not 100% specific for the factor V_{Leiden} mutation. We feel, however, that a functional test that is influenced by other plasma components and hence yields a distribution of APC-sr may contain valuable information regarding the prothrombotic status of an individual. This can be illustrated on the basis of an extensive study of plasmas of women using oral contraceptives (OC), which shows that APC-sr determined with the ETP method are strongly influenced by the use of OC and may be a good parameter to investigate the effects of OC on the hemostatic process.³³

During the development of our assay, we observed that women who used OC had significantly higher APC-sr than women who did not take OC, that is, were less sensitive to APC and hence acquired APC resistant (Fig. 7). It is interesting to note that the APC-sr of plasmas of women who take third-generation monophasic OC containing desogestrel or gestodene were significantly higher than those of women who used second-generation monophasic OC containing levonorgestrel of lynestrenol and did not significantly differ from the APC-sr of heterozygous APC-resistant women who were not using OC.

These observations indicate that OC therapy induces acquired APC resistance, the extent of which de-

pends on the kind of OC used. Acquired APC resistance may well explain the higher risk of thrombotic disease of women taking OC³⁴ and the different risks reported for women who use second- or third-generation OC.^{35–38} This proposal is supported by the fact that there appears to be a good correlation between the values of the nAPC-sr determined with the ETP method and the increased risk for deep-vein thrombosis in women who use third-generation OC and in carriers of factor V_{Leiden} (Table 1). During our study we identified six women who were using OC and who were also heterozygous carriers of the factor V_{Leiden} mutation. These women had APC-sr that are normally observed for individuals that are homozygous for the factor V_{Leiden} mutation and who do not use OC. The fact that the risk increase for venous thrombosis of heterozygous pill users and homozygous nonusers are similar further supports our proposal that the increased incidence of venous thrombosis in congenital APC-resistant individuals and in women using OC may originate from a defect in the same physiological pathway.

Unfortunately, we have as yet no information about the molecular mechanism by which OC induce acquired APC resistance. In this respect we would like to emphasize that also according to the APTT-based assay, the use of OC is associated with acquired APC resistance.^{39–41} The effects of OC on APC-sr determined with the APTT-based method are, however, much less pronounced and as yet no significant differences were reported between APC-sr determined with this method in plasma of users of second- and third-generation OC. Because the major difference between the APTT- and ETP-based assays is the fact that in the APTT coagulation is initiated via the intrinsic pathway and in the ETP method is initiated via the extrinsic pathway, this may indicate that the major point of attack of OC likely has to be sought in the activity or regulation of extrinsic coagulation pathway.

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