Advances in Understanding Mechanisms of Thrombophilic Disorders

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Initiation and Propagation of Blood Coagulation

Circulating platelets adhere to subendothelial structures at sites of vascular damage. A series of reactions is initiated that result in the formation of a platelet plug. Concomitant to these primary hemostatic events, blood coagulation is activated by the exposure of tissue factor (TF), which binds activated factor VII (FVIIa).\(^1,2\) A cascade of proteolytic reactions—referred to as the extrinsic pathway or the TF pathway—follows that culminates in the formation of thrombin (Fig. 1). Thrombin expresses several procoagulant properties, for example, it activates platelets, amplifies the coagulation process by activating factor XI (FXI) and procofactors FV and FVIII, activates factor FXIII (FXIII), and converts fibrinogen into a fibrin network. The FVIIa–TF complex activates both factor IX (FIX) and factor X (FX).\(^3\)–\(^5\) Activated FIX (FIXa) activates FX, which in turn activates prothrombin to thrombin. FIXa and FXa are inefficient enzymes requiring negatively charged phospholipid surfaces, protein cofactors, and calcium ions for expression of full activity. The cofactors are the activated forms of factor VIII (FVIIla) and factor V (FVa), respectively. FV and FVIII are high-molecular-weight proteins sharing the modular arrangement A1, A2, B, A3, C1, and C2.\(^6\)–\(^8\) On activation by thrombin or FXa, peptide bonds surrounding the B-modules are cleaved. In circulation, FVIII is bound to von Willebrand factor (VWF) and the thrombin-mediated activation of FVIII results in the release of FVIIla from VWF.\(^9\)

The TF pathway is the physiological activation mechanism associated with traumatic injury. Coagulation can also be activated through the intrinsic pathway after exposure of blood to negatively charged surfaces.\(^1,10\)–\(^12\) This system comprises the contact phase proteins (FXII, FXI, prekallikrein, and high-molecular-weight kininogen). The intrinsic pathway does not appear to be physiologically important for injury-related coagulation in vivo, illuminated by the lack of bleeding problems in individuals with deficiency of FXII.\(^13\) However, the interest in the intrinsic pathway has increased dramatically in the last decade after the discovery that it may...
be important for the formation of arterial thrombosis, and efforts are being made to develop inhibitors to the system for safe anticoagulation. Another discovery that has sparked the interest in the intrinsic pathway is that platelets contain negatively charged polyphosphates that may serve as a natural activator for the intrinsic system.

**Regulation of Coagulation**

The coagulation system is strictly regulated at each level of the pathway. Membrane-bound molecules on endothelial cells, circulating inhibitors, and negative feedback mechanisms provide efficient control. Pro- and anticoagulant mechanisms are balanced in favor of anticoagulation under physiological conditions, whereas procoagulant forces prevail at sites of vascular damage. Defects in this ingenious system are associated with increased thrombin generation and a hypercoagulable state that increases the risk of thrombosis.

Antithrombin (AT) is the most important serine protease inhibitor (serpin) regulating coagulation. It inhibits thrombin, FXa, FIXa, FXa, and FVIIa. AT is stimulated by heparan sulfate proteoglycans present on the endothelial cell surface. In clinical situations, heparin injections are used to stimulate AT and thus inhibit coagulation.

The TF pathway inhibitor (TFPI) regulates the TF pathway. The full length TFPI (TFPlα) inhibits the FVIIa–TF–FXa complex, thus efficiently turning off the TF pathway. TFPI also directly inhibits FXa, in a phospholipid-bound reaction that is stimulated by protein S and FV. Recently, a splice isoform of FV (FV-Short) having a truncated B-domain, which exposes a high-affinity binding site for TFPlα, is shown to be a carrier for TFPlα in circulation as well as an efficient synergistic TFPlα cofactor in synergy with protein S.

The procoagulant reactions of thrombin are physiologically adequate at sites of vascular injury. However, intact endothelium converts thrombin into an efficient initiator of a natural anticoagulant pathway, the protein C system. Endothelium, thrombin binds thrombomodulin, a potent modulator of thrombin activity and a cofactor to thrombin in the activation of protein C. In addition, the endothelial protein C receptor stimulates the activation of protein C by the thrombin–thrombomodulin complex. Activated protein C (APC) inactivates membrane-bound FVa and FVIIa by limited proteolysis in reactions that are potentiated by a cofactor designated protein S and, in the case of FVIIa degradation, also by the nonactivated form of FV. APC cleaves three peptide bonds in FVa at Arg306, Arg506, and Arg679, whereas FVIIa is cleaved at Arg336 and Arg526. As a consequence of the APC-mediated cleavages, FVa and FVIIa lose their procoagulant properties.

Protein C is a vitamin K-dependent plasma protein homologous to FVII, FIX, and FX and shares with them a common modular organization; a vitamin K-dependent γ-carboxyglutamic acid (Gla)-rich module, two epidermal growth factor (EGF)-like modules, and a serine protease module. Efficient anticoagulant function of APC depends on the presence of its cofactor protein S. The APC-mediated degradation of FVIIa in the FIXa–FVIIa complex in addition to protein S requires the presence of FV, which in this scenario works as an anticoagulant protein.

The synergistic APC cofactor activity of FV and protein S is stimulated by APC-mediated cleavage at Arg506. The Arg506 → Gln mutation impairs the anticoagulant effect of
FV, which is part of the mechanism of APC resistance (FV Leiden [FVL]; see below). FV loses its APC cofactor activity upon proteolysis by thrombin (T) in the activation of PC. The endothelial protein C receptor supports the activation by binding the Gla-domain of PC thus presenting PC to the T/TM complex. The activated protein C (APC) floats along with the blood stream to control reactions of coagulation.

Protein S is a multimodular vitamin K-dependent plasma protein, but not a serine protease. It contains a Gla module, a thrombin-sensitive module, four EGF-like modules, and a large module homologous to sex hormone-binding globulin. In plasma, 60 to 70% of protein S circulates bound to C4b-binding protein (C4BP), a regulator of the complement system.

Fig. 2 Activation of protein C (PC) by thrombin–thrombomodulin. On all endothelial cells, thrombomodulin (TM) serves as a cofactor to thrombin (T) in the activation of PC. The endothelial protein C receptor supports the activation by binding the Gla-domain of PC thus presenting PC to the T/TM complex. The activated protein C (APC) floats along with the blood stream to control reactions of coagulation.

Inhibition of coagulation by APC

Fig. 3 Degradation of factor Va (FVa) and factor VIIIa (FVIIIa) by activated protein C (APC). FVa and FVIIIa are cleaved and inhibited by APC in reactions involving APC cofactors protein S and factor V (FV). Protein S and APC are sufficient to inhibit FVa, whereas FVIIIa regulation additionally involves FV, which in this reaction serves as cofactor to APC.
system demonstrating that protein has functions outside the protein C system. The Gla module of protein S is instrumental in binding to membranes containing phosphatidylserine, for example, exposed on apoptotic cells, which is important for their elimination by phagocytosis.

In the degradation of FVα by APC, the Arg506 cleavage is faster than that at Arg306 but only yields partial loss of FVα activity. In contrast, cleavage at Arg306 results in efficient loss of FVα activity. Protein S serves as cofactor for the cleavage at Arg306 but has minor effects on the Arg506 cleavage.

The synergistic TFPIα-cofactor activity between protein S and FV/FV-Short in the inhibition of FXα is an anticoagulant activity of protein S that is potentially physiologically important.

The importance of the anticoagulant protein S functions in vivo is clearly demonstrated by the association between protein S deficiency and venous thromboembolism (VTE) in humans and by the lethal thrombotic phenotype associated with protein S knockout in mice.

**Genetics of Thrombophilia**

In Western societies, the annual incidence of VTE is approximately 1 to 2 per 1,000. Thrombosis often occurs in conjunction with pregnancy, the use of oral contraceptives, surgery, fractures, and immobilization. Genetic factors are frequently involved and patients often report family histories of VTE. The most common genetic risk factors for VTE are point mutations in the FV gene (F5) and in the prothrombin gene (F2), whereas deficiencies of anticoagulant protein C, protein S, or AT are less common. These inherited causes of thrombophilia result in a lifelong increased risk of thrombosis. However, the individual risk profile is difficult to predict as many other genetic and environmental factors affect the risk.

**Factor V Gene Mutations Causing APC Resistance**

In 1993, we described the APC resistance phenotype as a highly prevalent cause of inherited thrombophilia and in 1994 we reported its association with FV. Shortly thereafter, many laboratories confirmed its high prevalence among thrombosis patients (20–60%). The characteristic feature of APC resistance is that the addition of APC to plasma in a clotting test does not give a normal prolongation of the coagulation time. The F5 gene (GenID 2153, OMIM ID 227400, 188055, 612309) was soon shown to be the involved gene and around 95% of cases carries a single-point mutation in F5; a G → A substitution predicts replacement of R506 with a Q (Gln). The mutant F5 is referred to as FVL, or FVR506Q, or FV:Q506 (R and Q are one-letter codes for Arg and Gln, respectively; Fig. 4). Other rare F5 mutations causing APC resistance include FVNara (W1920R) and FVBonn (A512V). Other cases with APC resistance where F5 mutations have been found but not proven to be the cause include FV E461Q and FV G493E. Two rare mutations affecting the Arg306 cleavage site, FV Cambridge (R306T) and FV Hong Kong (R306G), do not result in APC resistance and are not risk factors for VTE.

The FVL allele is found in individuals with Caucasian genetic background. In southern Sweden, Germany, Greece, Arab countries, and Israel, high prevalence (up to 15%) is found. In the United States, the United Kingdom, and the Netherlands, around 3 to 5% carry the mutant allele. Lower prevalence (around 2%) is found in Hispanics. Its high prevalence suggests a survival advantage, and an associated reduced risk of bleeding after delivery has been shown. The slightly increased risk of VTE associated with the FVL has
likely not been a negative survival factor in the history of humankind as VTE develops relatively late in life and does not negatively influence fertility. In addition, our ancestors were not affected by modern circumstantial risk factors for VTE, such as a sedentary lifestyle, surgery, and the use of oral contraceptives.

The FVL allele is the result of a founder effect, the mutation event estimated to have taken place around 20,000 years ago, after the “Out of Africa Exodus” and the separation of Asians from Europeans. This explains why the FVL is common among European populations but rare in China, Japan, and in the original populations of Africa, Australia, or Americas.

The general consensus is that the FVL is the most common genetic risk factor for VTE in Western societies but is not a risk factor for arterial thrombosis. The odds ratio for VTE has been estimated to be two- to threefold in heterozygous cases, whereas homozygous individuals are at higher risk. FVL is associated with a hypercoagulable state caused by two molecular mechanisms, one being an impaired inhibition of FVa by APC, the other being that FVL functions as a poor APC cofactor in the degradation of FVIII. In FVa degradation, the Arg506 cleavage is approximately 10-fold faster than the cleavage at Arg306 and mutant FVa is therefore inhibited at an approximately 10-fold lower rate than normal FVa. However, degradation of FVa in the prothrombinase (PTase) complex is different from that of free FVa because in the PTase the Arg506 site is protected from degradation by APC by both FXa and prothrombin. Moreover, protein S functions as an APC cofactor primarily for the Arg306 cleavage. Therefore, APC-mediated inhibition of FVa in the PTase follows a different pathway compared with that of free FVa. As a consequence, when mutant and normal FVa are part of assembled PTase complexes, their rates of degradation by APC and protein S are relatively similar.

### Prothrombin Gene Mutations

The second most common genetic risk factor for VTE is a point mutation in the prothrombin gene, F2 (GeneID 2147, OMIM ID 176930.0009), G20210A located in the 3′ untranslated regions (~Fig. 5), which is associated with approximately twofold increased risk of VTE. The mutation is associated with increased plasma levels of prothrombin due to increased efficiency of the F2 gene mRNA. Its prevalence is 0 to 5% in the general population. Similar to the FVL, the F2 20210A variant originated from a single mutational event and the allele is only found in Caucasian populations.

Another F2 gene gain-of-function mutation was found in a Japanese family with VTE. The mutation results in a substitution of Arg for Leu at position 596 and is located in the last exon of the F2 gene (OMIM ID 176930.0015). Arg596 is located in a region containing AT-binding sites and it is anticipated that the mutation causes AT resistance. Two other mutations affecting the same amino acid residue (Arg596Gln and Arg596Trp) have been linked to VTE and AT resistance.

### Antithrombin Deficiency

AT deficiency is caused by mutations in the AT gene SERPINC1 (GeneID 462, OMIM ID 107300, 613118) located at chromosome 1q23–q25. Heterozygous AT deficiency is found in 1 to 2% of VTE patients and in 0.02 to 0.05% of the general population suggesting a 10-fold increased risk of VTE. Type I deficiency is characterized by low AT plasma concentration, whereas type II denotes functional

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**Fig. 5** G20210A mutation in the prothrombin gene. The G to A single-point mutation affects the 3′ untranslated region of the prothrombin gene (F2). The mutation is not affecting the coding sequence but increases the prothrombin plasma concentration and thus the risk of thrombosis.
defects. Type II can be further divided into three subtypes: reactive site (RS) mutants, heparin-binding site (HBS) mutants, or mutants giving pleiotropic effects (PE). According to the Human Gene Mutation Database (www.hgmd.cf.ac.uk/ac), there are several hundred distinct SERPINC1 gene mutations reported. Type II RS variants are functionally defective in protease inactivation often due to mutations in the vicinity of the RS. The type II HBS deficiency carries mutations in the HBS, and type II PE variants are caused by a limited number of mutations between amino acids 402 and 429.

**Protein C Deficiency**

Protein C deficiency is caused by mutations in the protein C gene PROC (GeneID 5624, OMIM ID 176860, 612304) on chromosome 2q13–q21. Type I is characterized by decreased protein C antigen and functional activity. Type II is caused by functional defects and the plasma protein C concentration may be normal. Heterozygous deficiency is found in a few percentage of VTE cases and in approximately 0.3% in the general population, and the relative risk of VTE is similar to that of FVII. Homozygous or compound heterozygous protein C deficiency is a rare condition (1 in 200,000–400,000) which causes severe and fatal microvascular thrombosis in the neonatal period with purpura fulminans, necrotic skin lesions, eye and brain damage, and disseminated intravascular coagulation. Several cases have been successfully treated with fresh frozen plasma or with protein C concentrates followed by lifelong anticoagulant treatment. The Human Gene Mutation Database (www.hgmd.cf.ac.uk/ac) lists several hundred distinct mutations associated with protein C deficiency. Most genetic defects are missense mutations, which lead to single amino acid substitutions, or nonsense mutations, located within the coding region of the gene. Type I mutations are scattered over the entire polypeptide chain. Type II mutations are less common but have been found in almost all the domains of protein C.

**Protein S Deficiency**

Protein S deficiency is caused by mutations in the protein S gene PROS1 (GeneID 5627, OMIM ID 176880, 612336, 614514) located close to the centromere at chromosome 3p11.1–q11.2. A pseudogene (PROSP, GeneID 5628) is located in close vicinity and shares more than 95% similarity with PROS1, which complicates genetic investigations of PROS1. Phenotyping for protein S deficiency is complicated by the presence in plasma of both free and C4BP-bound protein S (27, 33, 35). We have demonstrated that the concentration of free protein S discriminates better between those with and without protein S deficiency than the level of total protein S. The molecular explanation for this is that in individuals with normal PROS1 alleles the plasma concentration of protein S is slightly higher than that of C4BPβ−, which is the protein S-binding isoform of C4BP. The binding affinity of protein S to C4BPβ− is very high and therefore all C4BPβ− molecules are occupied with protein S. The molar surplus of protein S constitutes the free pool of protein S in plasma. In contrast, in protein S-deficient individuals the concentrations of protein S and C4BPβ− are equimolar and most of protein S is bound to C4BPβ−, and as a consequence the free protein S is low. Type I protein S deficiency is characterized by low levels of both free and total protein S. Protein S deficiency demonstrating low free protein S and normal total protein S has been believed to constitute a separate genetic type (type III). However, coexistence of the two types in many protein S-deficient families demonstrates that they represent different phenotypic variants of the same genetic disease. Type II deficiency denotes cases with normal free and total protein S but with low protein S function. To date, very few type II deficiencies have been found, presumably related to poor diagnostic performance of available functional protein S assays. We have recently described a novel functional assay measuring the TTPla cofactor activity of protein S that is expressed in synergy with FV-Short. Hopefully, this assay will help identify more patients with type II deficiency. Heterozygous protein S deficiency is present in a few percentage of VTE patients, whereas the prevalence of protein S deficiency in the general population of Western societies has been estimated to be 0.03 to 0.13%, but higher figures have been reported from Japan and Thailand. Family studies suggest that heterozygous carriers have at least fivefold increased risk of VTE compared with their healthy relatives, which is similar to the rates in thrombophilic families with protein C deficiency and FVL. Homozygous protein S deficiency is extremely rare but appears to give a similar picture to homozygous protein C deficiency, with purpura fulminans in the neonatal period. Protein S knockout mice are embryonic lethal due to uncontrolled coagulation.

In the Human Gene Mutation Database (www.hgmd.cf.ac.uk/ac), there are several hundred different PROS1 mutations that may be associated with a protein S deficiency phenotype. Most of the gene defects are missense or nonsense mutations, and mutations affecting splicing or small insertion/deletion defects are less common. In some families with phenotypically established protein S deficiency, PROS1 gene mutations are not found although linkage between the PROS1 locus and protein S deficiency has been established. One reason may be that common genetic screening techniques miss large deletions.

**Large-Scale Genomic Screening Studies**

The heritability of VTE is very high and patients often report positive family history of VTE. A large study of single-nucleotide polymorphisms (SNPs) of a UK Biobank cohort estimated the heritability of VTE to be $\approx 30\%$, whereas twin studies find even higher numbers ($\approx 50\%$). As the known genetic variants only explain a small fraction of the VTE heritability, additional genetic variants remain to be discovered. Genome-Wide Association Studies (GWAS) have associated several genetic variants to VTE, most of them already known to be involved in hemostasis, such as ABO, F2, F5, F11, FGG, and PROCR. ABO encodes the blood groups; it has been
known for many years that individuals with non-0 blood groups have higher risk of VTE. These individuals have high FVIII and VWF concentrations, which may be the mechanism for the increased risk of VTE. FGG mutations causing functional defects in fibrinogen is a rare VTE risk factor. The mechanism for the PROCR association with VTE may be mediated via the influence of the protein C receptor on protein C activation. Nonhemostatic genes identified were TSPAN15 and SLC44A2 but the mechanisms of their influence on VTE remain to be elucidated. Next generation sequencing (NGS) have been used to identify rare and low-frequency variants. However, a metaanalysis of large-scale exome array analyses of VTE failed to identify novel VTE genes.

Severe Thrombophilia Is a Multigenic Disease

VTE is a typical multifactorial disease involving one or more environmental and/or genetic risk factors. In Western societies, many individuals have more than one genetic risk factor because of the high prevalence of FVL and F220210A alleles. In contrast, in countries where these alleles are rare, fewer individuals carry more than one genetic defect. This probably explains the VTE incidence difference between Western and Asian countries. The frequency of individuals carrying two or more genetic defects can be calculated on the basis of the prevalence of the individual genetic defects in the general population. Those that carry more than one genetic defect have considerably increased risk of thrombosis. The thrombotic tendency in individuals with one or more inherited thrombogenic defects is highly variable and some individuals never develop thrombosis, whereas others develop recurrent severe thrombotic events at an early age. This depends on the particular genotype, the coexistence of other genetic defects, and the presence of environmental risk factors such as oral contraceptives, trauma, surgery, and pregnancy.

Conflict of Interest
None.

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