Coagulation factor and protease pathways in thrombosis and cardiovascular disease

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Summary
The biochemical characterisation of the proteolytic pathways that constitute blood coagulation was one of the most relevant achievements in biomedical research during the second half of the 20th century. Understanding these pathways was of crucial importance for improving global health through application in haemostasis and thrombosis pathologies. Immediately after the cloning of the genes corresponding to these proteins, mutations were discovered in them that were associated with imbalances in haemostasis. Later, the importance of coagulation pathways in other pathological processes was demonstrated, such as in atherosclerosis and inflammation, both essential processes involved in vascular disease. In the present review we evaluate the concepts that have allowed us to reach the integrated vision on coagulation that we have today. The thrombo-inflammation model encompassing these aspects includes a pivotal role for the proteases of the coagulation pathway as well as the regulatory proteins thereof. These concepts illustrate the importance of the coagulation cascade in cardiovascular pathology, not only in thrombotic processes, but also in atherosclerotic processes and in the response to ischaemia-reperfusion injury, making it a central mechanism in cardiovascular disease.

Keywords
Atherothrombosis, coagulation factors, coagulation inhibitors, proteases, protein C/S pathway, thrombosis, TFPI, vitamin K–dependent factors

Introduction
The blood coagulation cascade is a finely tuned system that in concert with cellular elements (platelets, microvesicles and other blood cells) supports haemostasis (1). Blood coagulation factors (proteins) are largely made by the liver and secreted in blood in inactive forms. Several coagulation factors are (also) expressed by extrahepatic tissues like vascular smooth muscle cells, where they may contribute to local processes like angiogenesis. These include, among others, expression of F2, F7 and F10. Others, such as protein S (PS) or tissue factor pathway inhibitor (TFPI) are also produced by vascular endothelial cells (2, 3); for factor VIII this is confined to hepatic vascular bed sinusoidal endothelial cells (4).

Upon binding to tissue factor (TF), the principal driver of coagulation activation (5), factor VIIa is generated initiating a stepwise conversion of coagulation factors (F)X and FIX into enzymes FXa and FIIa through limited proteolysis (Figure 1). Propagation and amplification occurs via the intrinsic route, driven by thrombin (and under certain conditions by FXIIa), to accelerate thrombin and fibrin formation (6). In blood, adequate concentrations and activation of coagulation proteins are essential for maintaining haemostasis; this is illustrated by the bleeding diathesis associated with congenital deficiencies in e.g. FVIII and FIX. Not all coagulation factors are directly linked to haemostasis: FXII, kininogen and prekallikrein, elements of the contact factor system, are not essential for haemostasis; we will not further discuss these.

Physiologically, excess activity of coagulation (risk of thrombosis) is to be avoided and this is achieved by different mechanisms:
- The presence of several anticoagulant mechanisms, comprising:
  - circulating serine protease inhibitors, of which the important antithrombin (AT) directly inhibits thrombin as well as a number of intrinsic factors including Fxa, FIIa and FXIa; this reaction is accelerated by heparin like molecules;
  - the protein C and S system, supported by the cellular receptors thrombomodulin (TM) and endothelial protein C receptor (EPCR); thrombin binding to TM induces activation
of PC to activated PC (APC), which is a potent anticoagulant by inactivating FV and FVIIIa (13); – tissue factor pathway inhibitor (TFPI) that, supported by protein S, inhibits the catalytic activity of the TF/FVIIIa complex in the presence of FXa at low levels of TF (14). Recent data show that TFPIa also attenuates FV activation as well as prothrombinase activity independent of TF (15).

Each of these three modulating mechanisms is critically important in controlling coagulation activity and restricting its location, thereby preventing the occurrence of thrombosis. However, these anticoagulant proteins also serve roles beyond haemostasis. While not the primary focus of this review, it should be noted that the anti-inflammatory properties of AT and APC upon interacting with TM/EPCR provide defence against “thrombo-inflammation”, which is regarded a critical process in, for instance, ischemic stroke (16); this also involves interactions between coagulation proteases and protease activated receptors (PARs), that either protect against (physiologically), or aggravate pathology including atherothrombosis and ischemia/reperfusion injury (17). For the scope of this article we will consider selected cardiovascular diseases from the perspective of a disturbed balance between pro- and anticoagulant forces.

The genetic basis of the anticoagulant defence mechanisms and links to thrombosis

In the field of coagulation research, the study of the genetic (hereditary) component in the pathology of venous thrombosis has been instrumental in our present knowledge of the haemostatic process in general (18–20). Interestingly, most genetic determinants that lead to thrombosis are directly related to the proteases of the coagulation cascade, either because of mutations in their structural genes or mutations in other genes that affect the function of these proteases (19). It is now over 50 years since the first description of a plasma protein deficiency linked to a hereditary thrombotic diathesis was reported, AT deficiency (21). Since then, our knowledge and capacity to study the genome has completely changed the way we understand the gene-environment interaction in health and disease and this genomics revolution has had a tremendous impact in the field of research on thrombosis and cardiovascular disease (18).

The study of the genetic aspects of haemostasis in relation to the predisposition of venous thrombosis has been at the forefront of this field in several aspects. During the decade of the 1980s, most of the genes related to the coagulation cascade and the fibrinolytic system were characterised (22). The major deficiencies associated with thrombophilia were definitively established as risk factors and associated with increasing numbers of loss of function mutations in the genes of the anticoagulant factors protein C, protein S and AT; PROC, PROS1 and SERPINC1 respectively (23). These thrombophilic mutations, i.e. mutations increasing the risk of suffering from a thrombotic event, were characterised as producing a loss of functional properties of the mutated gene, either by diminishing the amount of protein produced or by impairing the expressed protein’s function. These two types of mutations are known as quantitative (or type I) mutations, for instance a nonsense mutation in one of the structural genes; and qualitative (or type II) mutations. These mutations would include for instance mutation in the heparin binding site of AT; mutations causing defects in the interaction of protein C with its substrates, thrombomodulin or phospholipids; and regions crucial for the APC cofactor activity of protein S. In general, these mutations are characterised by a low prevalence in the population and a relative high risk in venous thrombosis, ranging from a 10-fold increase in the case of mutations causing protein S deficiency to an almost 50-fold with AT deficiency (19). However, these figures have to be used carefully, as the major deficiencies associated with thrombosis are very heterogeneous and the risk conferred by each mutation could be very different (24). In the boundary between mutations and polymorphic variants would be those mutations that have a questionable effect on the risk of thrombosis. Examples of this type are protein S Heerlen and protein S Tokushima, AT Cambridge II and protein C Lys150del (25–29). More recently, the EPCR Ser219Gly mutation in PROC was shown to also increase the risk of thrombosis (30). All these mutations contribute to an increased thrombotic risk in different ways and provide interesting subjects of study on the functions of the genes involved.

A second group of mutations affecting thrombosis in genes of the coagulation cascade are gain of function mechanisms, exemplified by factor V Leiden. This mutation was first described as a hereditary trait producing „APC-resistance”, i.e. a characteristic insensitivity to the anticoagulant action of APC (31). The finding that this condition was associated with mutations in the F5 gene modifying one of the cleavage sites of APC in the A2 domain of FVa explained how this mutation could cause this gain of function: a resistance to inactivation by APC on FVa. A different prothrombotic mechanism was proposed for a variant in the F2 gene associated with increased concentration of prothrombin in plasma. In this case, the mechanism is likely a stabilization of the mutated F2 mRNA (32). These mutations are more prevalent than single loss of function mutations and confer a smaller thrombotic risk of, from around 7 in the case of FV Leiden to 1.2 in the case of PROC (19).

Studies on the causative nature of the mutations described so far have been very informative on how the coagulation cascade is regulated and how malfunction of the cascade could lead to thrombotic events. In some cases, the link between mutations and pathology has helped to establish the function of gene products in the coagulation cascade, as in the case of protein S and EPCR. Furthermore, the availability of genetic testing helped to establish the concept of thrombophilia as a common multifactorial disease, where several genetic impairments combine to increase synergistically the risk of thrombosis. However, few studies so far have demonstrated specific mechanistic effects where different prothrombotic traits cooperate to increase synergistically the risk of thrombosis (18).

To date, 12 genes involved in the coagulation pathway have been robustly demonstrated to be associated with an increased risk
on venous thrombosis including those mentioned (PROS1, PROC, PROCr, SERPINC1, F5 and F2) as well as F9 (coagulation factor IX), FGG (fibrinogen), VWF (von Willebrand factor) and THB1 (thrombomodulin), and genes belonging to the contact phase of coagulation (KNG1, F11). Furthermore, the genetically-defined ABO group, which has an important impact on FVIII-VWF levels, has been well established as a thrombotic risk trait. However, despite the enormous advance in our knowledge of the implications of protein pathways in thrombotic disease, much of the heritability for thrombosis remains to be discovered. New studies providing further candidate genes, not related to the coagulation cascade, are showing interesting new mechanisms involved in thrombosis, as those provided by unexpected associations with genes present in platelets (GP6), or other vascular cells, as endothelial cells and leukocytes (19). In contrast to the field of venous thrombosis, genetic mutations related to arterial thrombosis have been much more difficult to establish. In this case, most of the variants found in coagulation and/or protease cascade genes are only associated to minor differences in the risk of arterial thrombosis (33), suggesting that there is a genuine specificity in pathological mechanisms among the two branches of the cardiovascular tree.

Translation of genes to proteins in thrombotic disease

**Venous thromboembolism**

Venous thromboembolism (VTE) occurs upon interaction of the three essential thrombogenic elements from Virchow's postulate: perturbed venous endothelium that expresses TF, binds inflammatory cells (neutrophils releasing extracellular traps), and platelets and generates fibrin at vulnerable venous sites, such as the valves; impaired flow; and systemic hypercoagulability. Multiple genetic and acquired risk factors for VTE have been identified. Many of these translate into inflammatory and/or hypercoagulable effects that perturb vascular endothelium and activate vascular and circulating cells and generate prothrombotic reactions. In the context of this paper, we focus on changes in anticoagulant mechanisms and their causal contribution to VTE.

Key to many prothrombotic mechanisms is a perturbed balance between generation of thrombin and its inactivation. As discussed above all known natural anticoagulant proteins inhibit thrombin (formation), one way or the other. Deficiencies in natural anticoagulants, or functional defects in these proteins, diminish the inhibitory potential in blood and allow excess thrombin to be form-
ed. Excess thrombin will, also by feedback activation of different procoagulant factors (XI, VIII, V), yield more fibrin, but also activate vascular cells via PARs (Figure 2). The basis of the shift in thrombin’s activities from anticoagulant (physiologic: generating APC upon binding to TM) to procoagulant and proinflammatory (pathologic, driving fibrin formation and/or PAR activation) is delicate. Concentration and localisation of thrombin (34), as well as biased signalling mechanisms of the thrombin-TM-EPOR-APC interactions, direct towards protective or offensive routes (35).

Decades of research revealed the molecular basis of VTE risk factors in families with prevalent thromboembolism, as discussed above. Combining genetic and functional tools it became possible to elucidate interactions between genetic and acquired risk factors. One important example is the mechanisms underlying APC resistance. Having established the FV Leiden mutation as a mild and prevalent risk factor for VTE, the group of Rosing and others identified the functional translation of this defect in the biochemical APC resistance assay (36). In this test, the addition of APC to plasma inhibits thrombin generation and this effect is attenuated in the presence of FV Leiden as compared to wild-type FV. Moreover, acquired APC resistance is a highly prevalent condition during pregnancy or due to the use of oral contraceptives (37). In this type of APC resistance, changes in the plasma proteome, including shifts in levels of free TFPI and free protein S, underlie APC resistance (38). The combination of genetic (FV Leiden) and acquired APC resistance works synergistically, concurrently to the increased risk association for VTE. In addition, these studies provided a biochemical basis for the management of contraception in women suffering from VTE: the advice to replace oral contraceptives by methods without prothrombotic effects.

A second and still incompletely understood mechanism involves TFPI and protein S. Based on the observation that protein S also had anticoagulant activity in the absence of APC, it was observed that protein S acts as a cofactor for TFPI at low TF concentrations (± 1 pmol/l) (39). In contrast to protein C, protein S and AT, there is no known association between TFPI deficiency and VTE. This is likely caused by the many forms of TFPI that exist, on the low amount of TFPI (20%) that circulates in the blood. However, changes in concentration and in particular function may relate to thrombosis risk. It is now believed that that lower levels free full length TFPI are associated with risk of recurrent VTE (40, 41). Further study established that particularly reduced TFPI activity associated with increased thrombin generation may be at the basis of this risk of thrombosis, in the presence of normal total TFPI and protein S levels (42) and this concept was recently confirmed in the large MEGA study (Winckers et al., manuscript in preparation).

Atherothrombosis

Atherothrombosis is associated with atherosclerosis, occurring during the process of plaque de-stabilisation and damage due to erosion or rupture. Atherosclerosis is a highly procoagulant process where many coagulation factors co-localise with specific elements in the plaque (43). Part of the coagulation factors may be locally produced by smooth muscle cells (SMC) or macrophages within the plaque, including TF and FVII (44, 45); others may migrate into the plaque upon perturbation of endothelial cell integrity or due to angiogenesis. Similar to venous thrombosis, a shift of thrombin from anticoagulant to procoagulant activity may be one of the early events in atherogenesis. Experimental and clinical studies suggest that during atherogenesis, EPCR and thrombomodulin (TM) are diminished in amount and activity at the vascular surface through shedding, degradation and/or internalization (46). Since inflammation is a hallmark of atherosclerosis (47), inflammatory driven hypercoagulability would be a logical consequence. Lower concentrations of TM and EPCR at the surface of coronary atherosclerotic specimens have been observed as compared to apparently normal vessels (48). A functional defect in TM activity is evident from thrombin infusion studies in atherosclerotic primates (49). One of the effectors is the reduced generation of APC, which in addition to its anticoagulant role, has important anti-inflammatory and endothelial cell protective properties. Reduced endothelial barrier integrity may be one of the results of impaired APC formation. Due to biased cell signalling (see before) thrombin (but also other proteases like FVIIa and FXa) can interact with and activate PAR-1 (and/or PAR-2 in case of other ligands) stimulating various pro-atherogenic pathways (17). The endothelium barrier is an essential antithrombotic defence mechanism. In addition to the loss of anticoagulant receptor molecules, endothelial cell produced protein S may potentially be affected by altered endothelial properties. An intriguing interaction is the binding of protein S to a sub-fraction of HDL, which potentially associates anticoagulant and anti-atherogenic properties (50). Furthermore, protein S and another vitamin K-dependent protein, Gas6, are ligands to the class of TAM receptors (Tyro3, Axl and MerTK), belonging to the family of receptor tyrosine kinases, that may also affect a number of inflammatory, cell growth and apoptosis pathways which may further affect atherogenesis (51). However, human protein S shows low affinity for human TAM receptors, hence the significance of the interactions remains to be elucidated (52, 53). Crucial to these cellular interactions is the capacity of Gas6 and protein S to bind specifically to phosphatidyserine in membranes, a feature shared by the rest of Gla-containing coagulation proteins (54).

TFPI is also localised in plaques and experimental studies show that overexpression of TFPI in mouse SMC reduces plaque progression by inhibiting secretion of macrophage migration inhibitory factor attenuating atherosclerosis and vascular remodelling (55). Hence, TFPI comprises an important anti-atherogenic molecule. Administration of recombinant TFPI reduced fibrin and platelet deposition in neo intima plaques, suggesting a dependency on TF (56). On the other hand, reduced TF does not diminish atherosclerotic plaque burden in susceptible mice. Thus, TF independent actions of TFPI are proposed, which include, in addition to its anticoagulant properties, an interaction with lipids. Full length TFPI binds to the endothelial VLDL receptor (57) while TFPI overexpression in SMC lowered cholesterol levels in mice (58); the relevance of such interactions in humans remains to be demonstrated. Potential roles of TFPI as compared to other coagu-
Coagulation proteases in atherogenesis, such as inhibiting smooth muscle cell proliferation, are currently under discussion (59).

In general, defects in anticoagulant mechanisms and/or hyperreactivity of procoagulant pathways are associated with increased atherogenesis in mouse models, typically apoE null mice (17). Some of these mice show clear indications of atherothrombosis with complex plaque lesions, but the association between procoagulant defect and plaque phenotype is not always consistent and unilateral. Many factors like sex, age, diet and genetic background play roles in mouse experimental models, the effects of which cannot be sufficiently underscored. One example in which age is a crucial factor underlying a phenotypic “inconsistency” is a mouse that combines a defect in TM (point mutation impairing its protein C activation potential) and apoE null background, generating mice with either more stable (60) or more unstable plaques (61).
Ischaemia-reperfusion injury (myocardial infarction, ischaemic stroke, renal failure)

Coagulation proteases appear to be directly linked to key steps in ischaemia-reperfusion injury (I/R injury). Several lines of experimental evidence suggest roles of FVIIa/TF, FXa and thrombin in driving I/R injury, in particular when local anticoagulant defense mechanisms fail. Cardiac injury due to I/R upregulates TF in the heart and I/R damage can be diminished by inhibitors of TF (62) or FVIIa (63). Inhibition of the NFκB pathway as well as reduced neutrophil influx in the ischaemic heart, were suggested protective effects of inhibition of the TF-FVIIa pathway. In addition, APC also protects against I/R injury of the heart by inhibiting apoptosis and inflammation, and modulating glucose metabolism, in part by mechanisms not depending on its catalytic function (64, 65).

In brain and kidney, I/R injury is also affected by PAR activated reactions in which both FXa and APC play key roles. Several seminal studies by Griffin and colleagues, following up on the demonstration that APC was neuroprotective through inhibition of p53 mediated apoptosis in a mouse ischemic stroke model (66) provided evidence for critical roles of APC, PAR-1,3 and EPCR in the defense against ischemia. Specific signalling pathways engaged by APC protect against ischaemic damage (67, 68) also showing that the non-anticoagulant actions of APC are most relevant in this regard (69). The latter provides important advantages for the translation to clinical practice, since one of the main disadvantages of administering pharmacological doses of wild-type APC is bleeding, while the non-anticoagulant APC mutant should be safe in that regard. This concept is currently being tested with the mutant 3K3A-APC in a phase 2 clinical trial in patients with ischaemic stroke (70).

Conclusion

A vast research endeavor has made it possible to establish the current view of coagulation in blood and vessel wall. This view incorporates not only the protein components of the pathways, but also the cellular components and the vesicles that integrate them into the response to vascular damage. Present technologies enable integration of big data in the coagulation field and to modify the genome in new ways to obtain more accurate cellular and animal models. Certainly, these techniques will keep the study on blood coagulation at the forefront of the studies on human pathology. These techniques will ground scientific advances with a sure impact on public health.

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Conflicts of interest

None declared.

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