Coagulant and non-coagulant effects of thrombin

J.J. Posthuma
H.M.H. Spronk

Departments of Biochemistry and Internal Medicine, Cardiovascular Research Institute Maastricht, Maastricht University, The Netherlands

Correspondence:
Henri Spronk
E-mail: henri.spronk@maastrichtuniversity.nl

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A B S T R A C T

Thrombin is a well-known component of the coagulation cascade and has been proven to be a viable target for effective anticoagulation treatment. However, there is growing evidence to suggest that this serine protease is also a crucial modulator of other cellular mechanisms through the activation of protease-activated receptor (PAR)-mediated signaling. The involvement of thrombin and PARs in normal biological and pathophysiological processes has been recognized, and in recent years their potential implications have been explored. Thrombin plays significant roles in mediating cellular signaling effects associated with inflammation, fibrogenesis, cancer, and the initial development of atherosclerosis, a chronic inflammatory vascular disease. For instance, thrombin regulates vascular permeability, migration and proliferation of smooth muscle cells, recruitment of monocytes into atherosclerotic lesions, inflammation, and apoptosis. In addition, more and more evidence indicates that changes in levels of circulating thrombin have a direct impact on atherogenesis. Direct evidence for the role of thrombin in the atherogenic process comes from experiments showing reduced progression of atherosclerosis in apolipoprotein E knockout mice on pharmacological inhibition of thrombin. The association of pathologies with hypercoagulability suggests that inhibition of thrombin through non-vitamin K-antagonist oral anticoagulants (NOACs) not only attenuates fibrin formation, but may also influence pathophysiological processes like atherosclerosis.

Learning goals

At the conclusion of this activity, participants should know that:
- thrombin is the central enzyme in blood coagulation with more than ten known targets;
- thrombin directly activates protease-activated receptors (PARs)-1, -3, and 4, and also transactivates PAR2;
- thrombin regulates inflammation, fibrogenesis, and atherosclerosis through activation of PARs;
- inhibition of thrombin not only attenuates coagulation, but may reduce development and progression of atherosclerosis, thereby opening new potential therapeutic applications for the direct thrombin inhibitors.

Introduction

On top of its primary role in hemostasis, thrombin is known to play a significant role in non-hemostatic (patho)physiological processes such as inflammation, atherosclerosis, angiogenesis, vascular remodelling, fibrogenesis and cancer.[1-6] These cellular responses of thrombin are mainly mediated through activation of protease-activated receptors (PARs), a family of the transmembrane G protein-coupled receptors. So far, four subtypes of PARs have been identified, PAR1 through to PAR4, of which mainly PAR1, -3, and -4 are activated and signal directly in response to thrombin (Figure 1). PAR2 is not activated by thrombin but thrombin-cleaved PAR1 can donate its tethered ligand to transactivate PAR2,[7] thereby facilitating alternative signaling pathways. During late stages of sepsis, PAR1 and PAR2 associate on endothelial cells thereby shifting the thrombin signaling from barrier disruptive to barrier protective.[8] Also, formation of PAR1:PAR4 heterodimers after activation of the receptors by thrombin has been reported.[9]

The PARs are predominantly found within the cellular membranes of platelets, endothelial cells (EC), vascular smooth muscle cells (VSMC), fibroblasts, hepatocytes, T lymphocytes and monocytes[10-14] (Figure 1).

All PARs share the same basic mechanism of activation. Through proteolytic cleavage of their N-terminal extracellular domain by proteases, such as thrombin, a new N-terminal tethered ligand domain becomes exposed which binds and activates the cleaved receptor.[6,15] Depending on the ligand and the location of the receptor, activation of PARs leads to activation of one of the intracellular G-alpha subunit families, $G_{12/13}$, $G_{i/o}$ or G_q, all initiating different cellular responses.[6] Signaling through G_q-activation results in activation of phospholipase C, subsequently leading to activation of mitogen-activated protein kinase (MAPK) and tyrosine kinase trans-activation.[16] Furthermore, activation of intracellular G_i results in ERK/MAPK signaling.[17] Activated PARs are rapidly internalized and targeted to lysosomal degradation. This mechanism involves the phosphorylation of PARs within their C-terminal cytoplasmic domain, which triggers membrane translocation and

internalization by clathrin-mediated endocytosis. Recovery of internalized PARs requires either mobilization from the intracellular pool or synthesis of new receptors.[15]

Thrombin in coagulation

The blood coagulation process can be divided into initiation, amplification and propagation phases (reviewed by Borissoff *et al.*[18] and Versteeg *et al.*[19]). Tissue factor (TF) drives the initiation phase upon its expression on cells or microvesicles, or after exposure of TF to blood upon rupture of an atherosclerotic lesion, containing cells including smooth muscle cells (SMC) and fibroblaststs that express TF at their surface. By interacting with TF, factor VII becomes activated which initiates the formation of the TF-factor VIIa-factor Xa complex. A fraction of factor Xa dissociated from the tenase extrinsic complex can interact with factor Va in the prothrombinase complex, thus generating a small amount of thrombin. The initial small amount of thrombin produced executes the proteolytic cleavage of factors XI, V and VIII into their active forms XIa, Va and VIIa, respectively (Figure 1). In addition, thrombin also activates platelets, ensuring these cells display a properly negatively charged phospholipid membrane, in order to function as a physical platform for the assembly of the intrinsic tenase (factor IXa-factor VIIIa) and prothrombinase (factor Xa-factor Va) complexes. The assembly of the prothrombinase complex on the platelet surface substantially increases factor Xa proteolytic effi-

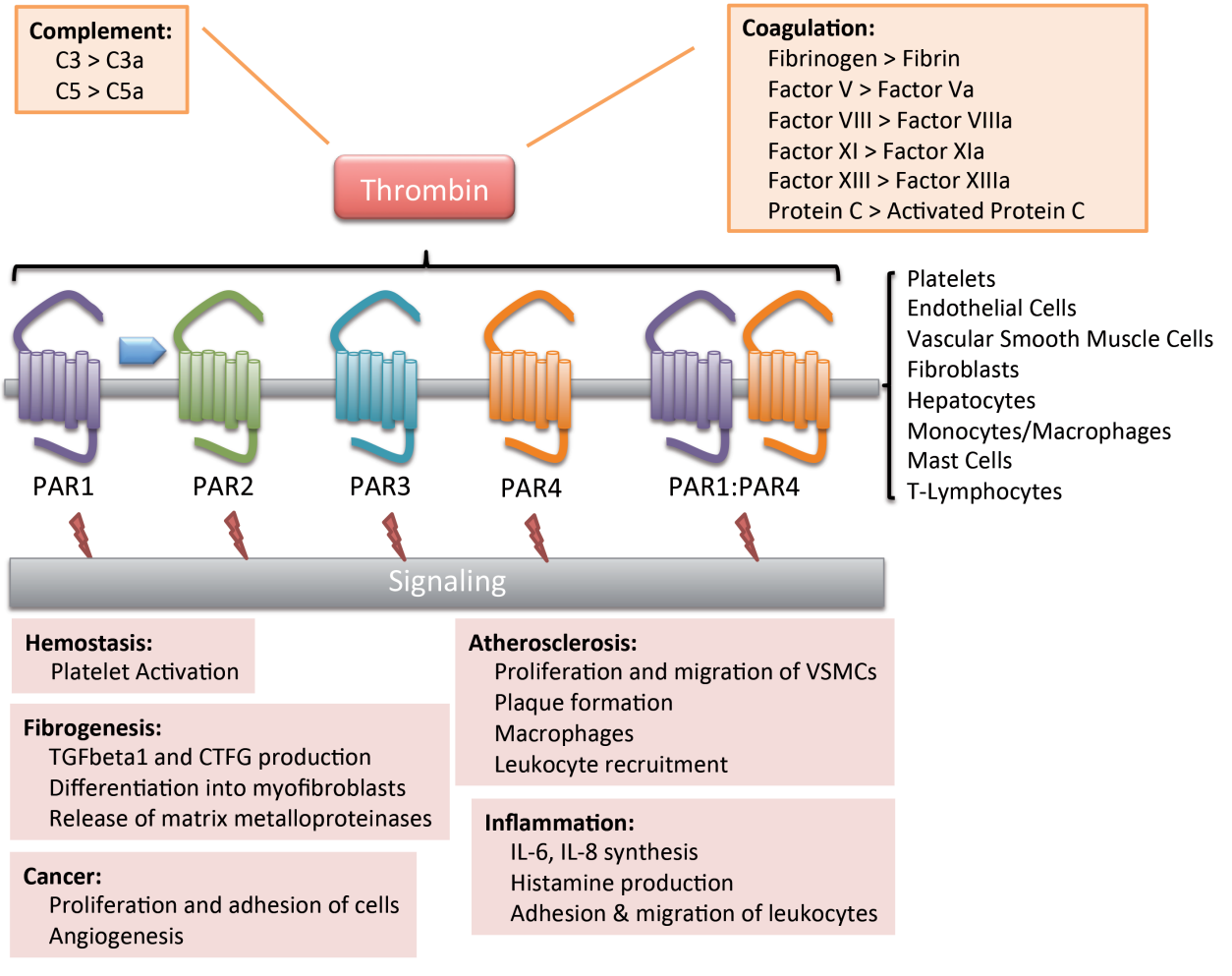

Figure 1. Overview of the coagulant and non-coagulant effects of thrombin. Coagulant functions of thrombin include the conversion of fibrinogen into fibrin, activation of the coagulation proteins factor V, VIII, XI, and XIII, as well as the activation of protein C into the anticoagulant activated protein C. Within the complement system, thrombin can activate complement factors C3 and C5. Thrombin's cellular actions are mediated through activation of protease-activated receptors (PARs), which are expressed on several cell types, including platelets, endothelial cells and vascular smooth muscle cells. PARs are members of the transmembrane G protein-coupled receptors and four sub-types have been identified so far: PAR1 through to PAR4. Thrombin directly activates PAR1, PAR3, and PAR4. Thrombin activated PAR1 can donate its N-terminal domain tethered ligand to transactivate PAR2 (blue arrow). Heterodimers between thrombin activated PAR1 and PAR4 can be formed to facilitate alternative signaling pathways. Activation of PARs by thrombin induces several cellular signaling pathways involved in hemostasis, fibrogenesis, cancer, atherosclerosis, and inflammation.

ciency and the generation of thrombin, which in turn converts fibrinogen to fibrin. Although the initial trace quantity of thrombin is insufficient to produce enough fibrin for a clot, it is crucial for the amplification phase of the coagulation cascade through activation of factor XI and the co-factors V and VIII. Finally, factor XIII activation by thrombin allows this factor to act as a stabilizing agent of the fibrin clot.

Other roles for thrombin besides coagulation

Inflammation and immunity

Predominantly through activation of PARs, a major role has been documented for thrombin in regulating the innate and adaptive immune systems, as well as in other inflammatory processes.[20,21] Under physiological conditions (with an intact endothelium), thrombin attenuates coagulation and inflammation through activation of protein C, a process in which the endothelial proteins endothelial protein C receptor (EPCR) and thrombomodulin (TM) are involved.[22,23] However, at higher concentrations, such as during endothelial damage, atherosclerosis or chronic arterial hypertension, thrombin can initiate pro-inflammatory processes including the downregulation of EPCR and thrombomodulin.[24] Since inflammation reduces the expression of EPCR and thrombomodulin, the competition between thrombomodulin and PAR1 for thrombin might shift towards PAR1, thereby enhancing the pro-inflammatory effects of thrombin.[25] When exposed to the arterial media layer, thrombin can activate PARs on VSMCs, not only resulting in the earlier described release of IL-6, but also in vascular remodeling through proliferation and migration of VSMCs from the media towards the luminal side of the vessel wall, thereby causing vascular stenosis during chronic vascular inflammation.

The complement system is the main pathway of the innate immune system and through activation of one out of three pathways (classical-, lectin- and alternative pathway) the generation of the active mediators C3a, C3b or C5a is established (reviewed by Lachmann[21]). Thrombin can actively generate the complement mediators C3a and C5a through direct proteolytic activation (Figure 1). This suggests that proteases from the coagulation cascade contribute to a fourth pathway of complement activation.[26]

PARs are expressed on many cells of the immune system, including macrophages, monocytes, T cells, dendritic cells, lymphocytes and mast cells, but not on B cells.[27]

The involvement of thrombin in inflammation is based on observation of thrombin-mediated activation of PARs initiating several pro-inflammatory processes, such as the production of interleukin-6 by endothelial cells (ECs) and vascular smooth muscle cells (VMSCs),[18] the induction of interleukin-8 synthesis by monocytes,[11] and the release of histamine from mast cells.[28] Moreover, endothelial activation by thrombin facilitates the adhesion and migration of leukocytes to the endothelium through the release of P-selectin, intercellular adhesion molecule 1 (ICAM-1 or CD45), vascular cell adhesion protein 1 (VCAM-1 or CD106), and chemotaxis of monocytes through the release of monocyte chemotactic protein-1 (MCP-1).[11,29-31] Whether this response induces a shift from physiology to pathophysiology depends, in part, on the balance of the different PAR ligands, such as thrombin, coagulation factor Xa, activated protein C, and trypsin.[32]

In the other direction, specific inflammatory mechanisms can enhance the potential to generate thrombin, for example, mediated by neutrophil extracellular traps (NETs) released by activated neutrophils.[2,33] Activated neutrophils can propagate coagulation and its subsequent thrombin generation via cleavage of the endogenous anticoagulant tissue factor pathway inhibitor (TFPI) and NETosis. The latter is a process characterized by morphological changes of the nucleus of neutrophils upon the release of reactive oxygen species (ROS). The trigger for NETosis and the molecular mechanisms by which ROS drives this process remain unclear. The morphological changes upon the activation of neutrophils include loss of its lobular architecture, resulting in nuclear disassembly and chromatin release into the cytoplasm. Subsequently, the plasma membrane bursts and negatively charged intracellular nuclear and granule components, such as DNA fragments, are released, which contribute to thrombin generation[34] and are critical in inflammatory processes against many microbes, especially those that are protected against phagocytosis.[33,35-37]

Fibrogenesis and wound healing

The inflammatory response (as described earlier) and the subsequent fibrogenesis are critical components for tissue repair after injury in vascular and extra-vascular compartments. During physiological tissue remodeling, these processes are tightly controlled. However, uncontrolled regulation of these processes, such as in chronic injury, can lead to an excessive deposition of extracellular matrix (ECM), resulting in fibrotic disorders. Pathological fibrotic tissue formation is mainly found in the skin, liver, kidney, lung and vasculature.[38-42] In these organs, this process is characterized by a common etiology, the synthesis of profibrotic components (eg, collagen and fibronectin) by myofibroblasts which are derived from differentiation of various potent fibrotic cells, such as fibroblasts, VSMCs, hepatic stellate cells, keratocytes and epithelial-mesenchymal cells.[20,38,43,44] Thrombin can induce the differentiation of fibroblasts into myofibroblasts through activation of PARs (reviewed by Mercer and Chambers[45]) and the downstream profibrotic mediators transforming growth factor beta-1 (TGF-beta1) and connective tissue growth factor (CTGF).[46-49] However, factor Xa is also directly involved in fibrosis through activation of both PAR1 and PAR2, and an experimental study using mouse fibroblasts suggests that thrombin is a less potent stimulator of fibrosis compared to factor Xa.[50] Nevertheless, thrombin mediated PAR1 activation promotes fibroblast proliferation and differentiation into myofibroblasts through PDGF,[51] CTGF,[52] fibronectin,[53] and TGF-beta1.[50] TGF-beta1 is known to induce the differentiation from fibroblasts into the profibrotic myofibroblast. Although fibroblasts are the main precursor cells for myofibroblasts, differentiation of stellate cells, glomerular cells, and VSMCs into profibrotic cells has been described.[54-56]

These effects require a thrombin-dependent cellular proliferation that is mimicked to some extent by a 14 amino acid peptide derived from PAR1.[57] Moreover, several murine models showed decreased fibrotic tissue formation when thrombin was specifically inhibited with hirudin, or

if the thrombin receptors PAR1, -3 or -4 were absent.[58,59]

Furthermore, thrombin is found to participate in fibrogenesis, by the release of matrix metalloproteinases (MMPs), either by direct activation of PARs or through a PAR-independent pathway involving direct activation of epidermal growth factor receptor (EGFR).[60] MMPs are key enzymes in the development of fibrosis, by inducing degradation of proteins in the extracellular matrix of the vessel wall, which along with the earlier described endothelial cell migration and proliferation, is an important process in fibrogenesis.

Overall, a common etiology for pathological fibrogenesis in different organs is most likely. Since a major role of thrombin in pathological fibrotic tissue formation has been described, it is of increasing interest to investigate the possible role of inhibiting thrombin as a therapeutic target.[61] The potential benefit of thrombin inhibition has been suggested by anti-inflammatory and antifibrotic effects of the direct thrombin inhibitor dabigatran in a murine model of lung fibrosis.[62]

Cancer

The association between thrombosis and cancer was first described in the early 19th century by Bouillard *et al*.[63] and later by Trousseau *et al*.[64] Since then, there is growing evidence demonstrating a significant role for thrombin in cancer biology and pathogenesis. Many malignancies are associated with a hypercoagulable state, termed cancer coagulopathy, which is associated with an increased mortality rate.[65] As described earlier, thrombin facilitates cellular proliferation and adhesion of cells to the subendothelial matrix through activation of PARs, processes known to enhance tumor growth and metastasis.[66,67] Upregulation of PAR synthesis was not only observed in fibrotic tissue, but also in several tumors, including breast, prostate, and melanoma.[68] Furthermore, PAR activation by thrombin contributes to the pro-oncogenetic process by promoting angiogenesis through up-regulating vascular endothelial growth factor (VEGF).[69-71] Thrombin also induces the production of integrin $\alpha_v\beta_3$, an angiogenic marker in vascular tissue and in tumor cells. These integrins are important for attachment of cells to the extracellular matrix, which induces migration, proliferation, and apoptosis.[72] Studies showed an upregulation of integrin $\alpha_v\beta_3$ in adenocarcinoma cells.[73] The interaction between thrombin and integrin $\alpha_v\beta_3$ has been shown to induce endothelial cell survival following detachment from basement membranes, a process known to contribute to tumor progression and metastasis.[72,74]

Theoretically, an anticoagulant therapy might be effective in reducing tumor growth and metastasis, but also to reduce the incidence of thrombotic events in cancer patients.[75] Indeed, the majority of animal studies that investigated the possible role of heparin as anti-cancer drugs showed a reduced tumor growth and metastasis.[3,76] Clinically, however, there is only limited evidence of a modifying role of anticoagulants on cancer progression and/or metastasis, although initial large trials like the CLOT study suggested such beneficial effects.[77] Failure to obtain more evidence of clinical efficacy is likely due to patient and tumor heterogeneity and uncertainties about doses and duration of therapy

Atherosclerosis

A potetial role for coagulation proteases, specifically thrombin, in development and progression of atherosclerosis has been suggested from immunohistological studies on atherosclerotic lesions, as well as from animal models applying genetically altered coagulation profiles on an atherogenic background. Initially, Wilcox *et al*. demonstrated the presence of tissue factor and factors VII and X associated with macrophages and VSMCs within atherosclerotic lesions.[78,79] Since then, almost all coagulation proteins, as well as several coagulation enzymes such as thrombin, have been localized in atherosclerotic lesions.[80] Moreover, activities of tissue factor, thrombin, and factors Xa and XIIa were higher in early atherosclerotic lesions compared to lesions in a later stage of atherosclerotic development,[80] suggesting that a local vascular enhanced coagulation potential (hypercoagulability) contributes to the onset or progression of atherosclerosis. This hypothesis of thrombin contributing to the pathophysiology of atherosclerosis is supported by the presence of PAR1 on endothelial cells, VSMCs, and leukocytes, as well as by the fact that thrombin can activate this cellular receptor.

Evidence for a direct role of thrombin in development and progression of atherosclerosis is still limited to observations in experimental studies. Several animal studies have shown that pharmacological attenuation of thrombin activity with the direct thrombin inhibitors melagatran or dabigatran caused a reduction in atherosclerotic burden.[81] Administration of the direct thrombin inhibitor ximelagatran in mice on an atherogenic background ($ApoE^{-/-}$) reduced plaque progression in brachiocephalic arteries and enhanced the stability of advanced atherosclerotic lesions characterized by thicker fibrous caps, smaller necrotic cores and a decrease of matrix-metalloproteinase-9 (MMP-9).[82] Comparable results have been obtained with the new direct thrombin inhibitor dabigatran etexilate (or its active metabolite dabigatran). Treatment of atherosclerotic $ApoE^{-/-}$ mice with dabigatran resulted in impaired formation and reduced size of atherosclerotic lesions.[81] A combined hypercoagulable and atherogenic background increased the atherosclerotic burden with more and larger lesions characterized by a high number of macrophages, a large necrotic core, and an unstable phenotype. Treatment of these hypercoagulable and atherogenic animals with dabigatran reduced the pro-inflammatory and pro-atherogenic phenotype of atherosclerotic lesions, resulting in enhanced plaque stability.[83] Also, in this model, leukocyte recruitment was attenuated and severe progression of atherosclerosis and atherothrombosis was prevented through direct thrombin inhibition. Overall, it can be concluded that thrombin inhibition attenuates atherosclerosis, although a direct relation between thrombin activity and extent of atherosclerosis has yet not been demonstrated. It is, however, clear that coagulation proteases play an important role in development and progression of atherosclerosis. Moreover, inhibition of specific coagulation proteases such as thrombin or factor Xa, or attenuation of coagulation (or decreasing the hypercoagulable state) results in a less pro-atherogenic response in animal models. These effects are most likely mediated through PAR1 and/or PAR2 activation by thrombin/factor Xa or factor Xa, respectively. One player that may also be involved in this mechanism is the platelet that may be activated during activation of the coagulation cascade and which, interact-

ing with leukocytes, is an important contributor to atherosclerosis. The involvement of PAR4 in development of atherosclerosis is less likely, as PAR4 deficient mice on an atherosclerotic background showed no significant differences in the percentage of aortic lumenal surface covered by plaques and in the cross-sectional area of plaques compared to PAR4$^{-/-}$-and ApoE$^{-/-}$ mice after five and ten weeks on a Western diet.[84] Whereas thrombin-dependent cellular responses occur mainly through PAR1 activation, the factor Xa-dependent responses can occur through PAR1 and/or PAR2 cleavage, and these processes may depend on the receptor-specific cell expression pattern, ligand concentration, solubility, or association with other coagulation factors.[25,85] Adding to the complex equation of which coagulation protease is responsible for activation of a given PAR and the subsequent effects on atherosclerosis is the mechanism of PAR1 activation by the natural anticoagulant activated protein C.[86] Activated protein C mediates anticoagulant activity through proteolytical degradation of the co-factors Va and VIIIa, thereby decreasing thrombin generation. It also has anti-inflammatory activity, mediated through PAR1 signaling on several levels, such as enhancing the endothelial barrier integrity, attenuating TF expression and TNF-a release by monocytes, inhibition of cytokine production, and diminished leukocyte endothelial transmigration.[87,88] As described above, thrombin inhibition attenuated atherosclerosis in hypercoagulable mice on an atherogenic background. However, in this study, comparable results were obtained with recombinant activated protein C,[83] suggesting that either reduced thrombin activity, activated protein C directly through inflammation, or attenuation of thrombin (either by dabigatran or through activated protein C) reduces atherosclerosis.

The involvement of one or more components of the coagulation system is also evident from several animal studies using genetically modified mice (reviewed by Loeffen *et al*.[89]). A simplified description divides these animal models into two groups: hypercoagulable (with increased coagulation potential) and hypocoagulable (with decreased capabilities of generating thrombin). A study from our group demonstrated that a hypocoagulable phenotype on an atherogenic background reduced atherosclerosis. Animals with prothrombin levels reduced to 50% (heterozygous prothrombin deletion) on an ApoE$^{-/-}$ background had decreased leukocyte infiltration, as well as altered collagen and VSMC content within atherosclerotic lesions.[83] In agreement with this, hypocoagulable mice deficient in factor VIII on an atherogenic background showed significantly less early-stage atherosclerotic lesions.[90] In contrast, a 50% reduction of tissue factor expression in ApoE$^{-/-}$ mice did not alter the extent of atherosclerosis compared to wild-type mice,[91] suggesting the involvement of downstream coagulation proteins rather than direct effects mediated through TF.

The picture is clearer for hypercoagulability and atherosclerosis, in that an increased potential to generate thrombin results in a pro-atherogenic phenotype. Several studies have demonstrated enhanced atherosclerosis in animals with a hypercoagulable phenotype, including FV Leiden mutation,[92] heterozygous protein C deficiency,[93] thrombomodulin Pro/Pro mutation (TM$^{Pro/Pro}$),[92] and heterozygous tissue factor pathway inhibitor (TFPI) deficiency.[94] In addition, enhanced atherosclerosis with higher plaque vulnerability, spontaneous atherothrombosis, enhanced oxidative stress, neutrophil intraplaque infiltration and apoptosis, and diminished VSMC proliferation was shown in animals on a combined atherogenic and hypercoagulable background (ApoE$^{-/-}$:TM$^{pro/pro}$ animals).[83]

Shifting the focus from animals to humans suggests both a hypercoagulable state and active thrombin generation in association with cardiovascular disease and atherosclerosis as well, although a direct relationship between the coagulation potential and atherosclerosis is not immediately evident from clinical studies (reviewed by Loeffen *et al*.[89]). Overall, the association between common thrombophilic gene defects, such as prothrombin G20210A mutation, FV Leiden mutation, and deficiencies in protein C, protein S, or antithrombin (AT), and cardiovascular disease are modest.[95] One might suggest that hemophilic patients with severe deficiencies in one of the factors VIII, IX, or XI would have reduced cardiovascular risk due to this hypocoagulable state. Whereas hemophilic patients have a 2- to 3-fold increased overall mortality from bleeding disorders, liver diseases, or viral infections, the effects of coagulation factor deficiency on cardiovascular mortality remain controversial.[96] One potential confounder in the differences between reported observations might be the therapeutic administration of coagulation proteins in patients with severe hemophilia, potentially obscuring the effects of the original factor deficiency.

Conclusion

The contribution and importance of thrombin as the key enzyme in coagulation has been known for many decades, whereas its role outside hemostasis has only been recognized over the last ten years. Evidence from experimental studies, including cell culture experiments, animal models, and clinical observations suggest that the pleiotropic effects of thrombin can play a crucial role in inflammation, immunity, fibrogenesis, wound healing, cancer, and atherosclerosis. Although the precise role of thrombin in these biological pathways and pathologies is still not known, the availability of the so-called NOACs for direct (dabigatran) or indirect (factor Xa-inhibitors such as rivaroxaban, apixaban, and edoxaban) thrombin inhibition could provide opportunities to intervene in the described diseases. Treatment of chronic disease involving thrombin, however, requires detailed knowledge of the pathways involved, especially for long-term treatment with the new active-site directed protease inhibitors. Therefore, new scientific approaches are required in order to better understand the relationship between thrombin (and even more so, hypercoagulability) and the pathways and pathologies in which coagulation proteases are involved. Overall, further investigation is needed to fully understand the complexity and possible long-term benefits or drawbacks of inhibition of coagulation proteases.

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