Review

Is thrombin a key player in the ‘coagulation-atherogenesis’ maze?

Julian Ilcheff Borissoff¹, Henri M.H. Spronk¹, Sylvia Heeneman², and Hugo ten Cate¹*

¹Laboratory for Clinical Thrombosis and Hemostasis, Department of Internal Medicine, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University Medical Center+ (MUMC+), Universiteitsingel 50, PO Box 616, Box 8, 6200 MD Maastricht, The Netherlands; and ²Department of Pathology, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University Medical Center+ (MUMC+), Maastricht, The Netherlands

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In addition to its established roles in the haemostatic system, thrombin is an intriguing coagulation protease demonstrating an array of effects on endothelial cells, vascular smooth muscle cells (VSMC), monocytes, and platelets, all of which are involved in the pathophysiology of atherosclerosis. There is mounting evidence that thrombin acts as a powerful modulator of many processes like regulation of vascular tone, permeability, migration and proliferation of VSMC, recruitment of monocytes into the atherosclerotic lesions, induction of diverse pro-inflammatory markers, and all of these are related to the progression of cardiovascular disease. Recent studies in transgenic mice models indicate that the deletion of the natural thrombin inhibitor heparin cofactor II promotes an accelerated atherogenic state. Moreover, the reduction of thrombin activity levels in apolipoprotein E-deficient mice, because of the administration of the direct thrombin inhibitor melagatran, attenuates plaque progression and promotes stability in advanced atherosclerotic lesions. The combined evidence points to thrombin as a pivotal contributor to vascular pathophysiology. Considering the clinical development of selective anticoagulants including direct thrombin inhibitors, it is a relevant moment to review the different thrombin-induced mechanisms that contribute to the initiation, formation, progression, and destabilization of atherosclerotic plaques.

KEYWORDS
Thrombin; FIIa; Coagulation; Atherogenesis; Atherosclerosis

1. Introduction

There is abundant evidence for a close interaction between inflammation and coagulation systems and a bidirectional cooperation between these mechanisms has been proposed.¹,² Although the important contribution of blood cells involved in coagulation, particularly platelets and leucocytes, to atherothrombosis is beyond dispute, the properties of several coagulation proteins and their expression in atherosclerotic lesions suggest that they might also contribute to the pathogenesis of cardiovascular disease (CVD).

With the current development of highly specific antithrombotic agents including thrombin inhibitors aimed for long-term use in patients with CVD it seemed appropriate to focus on the pleiotropic actions of thrombin, in order to better appreciate possible long-term sequelae related to thrombin inhibition. This is even more important considering a number of physiological functions of thrombin (anticoagulant, vasodilating properties) that are of importance in a healthy vascular system. Taking physiology as a starting point for this review we next focus on the different mechanisms by which thrombin may modulate the formation of the atherosclerotic lesion and the course of atherogenesis.

2. Thrombin’s functional roles in physiology

In the coagulation cascade, thrombin is one of the key players. It is a central enzyme generated upon the exposure of tissue factor (TF) which binds and activates circulating factor VII and subsequently enters into the formation of a complex with factor X. The formed prothrombinase complex of factor Xa, factor Va, calcium (Ca²⁺) cleaves prothrombin into thrombin. Thus the coagulation pathways are amplified by thrombin feedback activation of the cofactors V and factor VIII and the activation of the factor XI zymogen. Hence, generated thrombin leads to the conversion of fibrinogen into fibrin and ultimately to the formation of a fibrin clot.

Thrombin activates a subfamily of G protein-coupled receptors named protease-activated receptors (PARs)—1, 3, and 4, affecting processes such as vasomotor regulation. Thrombin depicts a two-faceted role at the level of vascular reactivity, showing diverse vasoactive features, not only...
with regard to the type of vascular bed but also to the physiological condition of the vessel—whether healthy or diseased one. Several reports indicate that thrombin predominantly causes endothelium-dependent vasorelaxation in different species in vitro. In addition, recent published data show that thrombin induces PAR-1-mediated forearm arterial vasodilatation in humans in vivo. These endothelium-dependent dilating effects are generally attributed to a PAR-1-mediated production of various vaso-protective factors such as prostacyclin (PGI₂), endothelium-derived hyperpolarizing factor, and mainly nitric oxide (NO).

Similarly to its contrasting functional effects on vasoreactivity, thrombin demonstrates antagonizing actions in haemostasis also, e.g. the procoagulant action of converting fibrinogen into fibrin vs. the anticoagulant action of activating protein C (APC) after binding of thrombin to thrombomodulin (TM). Moreover, systemically generated thrombin, not captured by receptors is rapidly inactivated by inhibitors such as antithrombin (AT), APC, or heparin-cofactor II (HCII).

### Figure 1

Antagonizing actions of thrombin in coagulation cascade. Platelets get activated by the collagen that is exposed at sites of vessel damage, leading to the formation of a haemostatic plug. (1, 2) Thrombin (FIIa) is generated upon tissue factor (TF) exposure but the reaction is relatively slow. (3) Once formed, thrombin activates factor V, factor VIII, and factor XI, which results in a 300 000-fold acceleration, amplification, and thrombin propagation. (4a) To prevent a massive conversion of fibrinogen into fibrin and thereby leading to the formation of a stable clot, all natural anticoagulant pathways get activated. Thrombin gets involved into these actions by binding thrombomodulin (TM), which results in the activation of protein C (PC) into activated protein C (APC), which by proteolytic cleavage of activated factors V and VIII reduces the rate of thrombin generation. In addition, antithrombin (ATIII) forms a thrombin–antithrombin (TAT) complex, which irreversibly inhibits thrombin, in association with heparin and heparin cofactor II. (4b) In case the procoagulant stimulus overpowers the capacity of the anticoagulant pathways, this would result in more production of fibrin and would lead to the formation of a thrombus. Thrombin–thrombomodulin (T-TM) complex could additionally support the procoagulant actions of thrombin by activating thrombin-activatable fibrinolysis inhibitor (TAFI), thereby inhibiting fibrinolysis. (5) Except for the exposed collagen at the site of injury, platelets also get activated by thrombin via PAR-1- and PAR-4-mediated mechanisms but also by cleavage of glycoprotein V (GPV). Thrombin also prevents destabilization of the platelet plug by inhibiting ADAMTS13 action (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13). Thrombin facilitates clot stabilization by activating factor XIII (fibrin stabilizing factor) which has the capacity to crosslink fibrin.
dysfunction results in increased interactions of circulating cells with the endothelium contributing to enhanced permeability. Thrombin signalling in the endothelium, mediated by PARs, might interlace with some of these pathophysiological pathways by triggering a multitude of phenotypic drifts, including changes in vascular tone, EC shape, haemostasis, permeability, downstream gene transcription, and angiogenesis.

3.1. Thrombin, vascular tone, and phenotypic alterations of the endothelium

Despite the evolving experimental evidence on the molecular mechanisms of thrombin’s signalling in endothelium, mediated via PAR-1 and -4, thrombin’s actions in terms of vasomotor physiology are partly elucidated. However, it is known that thrombin induces contrary effects such as endothelium-dependent or direct smooth muscle contraction in healthy animal arteries in vitro.1,10 Endothelium-independent vasoconstriction is observed in humans in vivo.9 Thrombin mediates its vasoconstrictive actions by the secretion of prostaglandin H2 (PGH2) or thromboxane A2 (TxA2).7,10 NO has a critical impact on the mediation of vascular relaxation and endothelial function. It is synthesized by an endothelial nitric oxide synthase (eNOS) and this enzyme competes with arginase for L-arginine as a substrate. It has been indicated that PARs could regulate eNOS activity by phosphorylating the enzyme at several sites.11 Ser1177, Ser615, Ser633, and Tyr81 enhances the production of NO, whereas Thr495 inhibits. Thrombin mediates eNOS–Ser1177 phosphorylation through Gq and a calcium and protein kinase C (PKC)-delta sensitive, but phosphatidylinositol 3-kinase (PI3K)/Akt-independent pathway. The phosphorylation of eNOS–Thr495 and inhibition of NO synthesis is thought to be directed via the activation of the Rho/ROCK pathway.11,12 Prolonged incubation with thrombin has been reported to inhibit the synthesis of eNOS in EC.12,13 Moreover, multiple in vitro studies report that thrombin increases arginase activity, thereby suppressing NO production.14-16 In addition, the overexpression of arginase by thrombin leads to the depletion of the L-arginine pool, reducing NO production and inducing reactive oxygen species (ROS) synthesis owing to the eNOS uncoupling, which eventually compromises the endothelial function.17 Endothelin-1, a powerful natural vasoconstrictor, also showed an increased expression upon stimulation with thrombin.18

The antagonizing effects of thrombin on vasoactivity seem relevant to the type of vascular bed and the severity of atherosclerotic burden is also dependent on thrombin concentration and continuance of action. In normal arteries, the short-term effect of thrombin is shown to support predominantly the action of vasorelaxants such as NO and PGI2. On the other hand, increased thrombin generation is usually...
concentrated at the sites of vascular injury or within formed thrombus in vivo, but also in patients with advanced CVD or suffering acute coronary syndromes. In vascular lesions, thrombin promotes a pro-inflammatory response, characterized by increased production of diverse chemokines and cytokines, cell adhesion molecules (CAMs), enhanced vascular permeability, VSMC migration and proliferation, wall thickening and vasoconstriction. This might be a result of the combination of a diminished TM and endothelial protein C receptor (EPCR) capacity coupled to an overexpression of PAR-1 and PAR-2 receptors in vascular lesions. Various mechanisms have been reported linked to PARs upregulation. First, thrombin-induced activation of PAR-1 in cultured human EC in vitro upregulates PAR-1 gene expression by signalling via Gα1/2 coupled to Src and PI-3K, thus inducing the downstream Ras/MAPK pathway. Selective augmentation of PAR-2 and -4 gene expression is indicated upon treatment with inflammatory stimuli such as interleukin (IL)-1α, IL-1β, tumour necrosis factor (TNF)-α, and lipopolysaccharide (LPS). Finally, high shear stress, also characterized by reduced expression of various atherogenesis-related genes, inhibits PAR-1 expression in human EC in vitro. Thus, the alterations in the vascular tone and the degree of expression of PARs in the vessel wall might have additional impact on the potency of thrombin’s cell signalling activity and the progression of atherosclerotic disease.

3.2. Impairing the barrier function and other thrombin-mediated effects on the endothelium

Rabiet et al. proposed a mechanism in which thrombin stimulates the intracellular accumulation of Ca2+, consecutively activating the PKC pathway, and causing eventual disruption of (VE)-cadherin–catenin complexes at the EC-cell junctions. Further in vitro studies consolidated the participation of PKC in this pathophysiological process. Moreover, Nobe et al. suggested that thrombin-induced endothelial barrier impairment is a biphasic process in which the Rho/Rho kinase pathway is also involved leading to rearrangement of actin stress fibres. A recent study elicits a new mechanism which gives input to a better comprehension of the thrombin-induced endothelial gap formation and permeability. It was proposed that thrombin activates metalloprotease ADAM10, which mediates VE-cadherin proteolysis by specifically cleaving its ectodomain.

Thrombin could also promote the generation of endothelial microparticles (MPs) via ROCK-II activation. Increase levels of endothelial MPs have been correlated with the morphology and severity of stenosis in patients with CVD.

3.3. Thrombin-induced oxidative stress

Aside from the induction of pro-inflammatory responses, elevated ROS levels are presumably associated with the promotion of endothelial dysfunction, combined most likely with diminished NO bioavailability. The majority of risk factors of atherosclerosis positively correlate with an enhanced ROS synthesis, which tends to initiate multiple pro-atherogenic effects. ROS are implicated in cellular signalling mechanisms, such as gene expression, proliferation, migration or apoptosis. Several reports indicate the potentiating effect of thrombin on ROS production in human VSMCs and platelets. Different enzymatic systems take part in the production of ROS in the vasculature, such as xanthine oxidase, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, and NOS. Nevertheless, NADPH oxidases have been indicated as a major source of superoxide in vascular cells and myocytes. The importance of NADPH oxidases in thrombin-induced ROS synthesis was studied by the depletion of p22phox subunit, which suppressed ROS formation in VSMCs. Thrombin also triggers the activation of p38 mitogen-activated protein kinases (MAPK) in a NADPH oxidase-dependent manner, which establishes a link between thrombin and the MAPK/ERK pathway, suggesting that it is also indirectly involved in processes like cell differentiation, cell survival, and apoptosis. Djordjevic et al. demonstrated that thrombin induces elevated ROS production in EC in vitro by activating p38 MAPK and PI3K/Akt, inducing enhanced proliferation.

Intriguingly, thrombin induces its PAR-1 de novo re-expression via Src-dependent mechanism, including G proteins, PI3K, p38 MAPK, suggesting that redox pathways are also implicated in the regulation of PAR-1 expression. The latter was consolidated by two reports indicating that treating VSMCs with either flavin inhibitor diphenyleneiodonium or antioxidants prevents PAR-1 upregulation upon stimulation by cyclic strain or oxidative agents. Hawkins et al. indicated a thrombin-induced mechanism, causing the production of mitochondrial-derived superoxide (mROS), which is an outcome of a Ca2+-mobilization via inositol (1,4,5)-trisphosphate receptor (InsP3R), leading to a subsequent mitochondrial uptake of Ca2+, triggering mROS expression and nuclear factor-kappa B pathway signalling, which strongly promotes the overexpression of intercellular cell adhesion molecule (ICAM)-1 and the adhesion of leucocytes to the vascular endothelium.

4. Thrombin in the early stage of atherosclerotic plaque formation

Although several more coagulation serine proteases could function as activators of PARs by cleaving the N-terminal extracellular domain (Figure 3) abundant in vitro experimental data suggest that thrombin is a critical mediator in the coagulation, inflammation, vessel wall crosstalk. Thrombin enhances ROS production in the arterial vessel wall facilitating lipid peroxidation and apoptotic processes. Thrombin also induces a plethora of pro-inflammatory mediators, causing alterations in gene transcription of IL-6, IL-8, monocyte chemoattractant protein 1 (MCP-1, CCL2), vascular cell adhesion molecule (VCAM)-1, and ICAM-1, etc., facilitating the recruitment of blood circulating monocytes into the arterial vessel wall and encourages early plaque formation. Its signalling mechanisms with a pro-atherogenic impact on the arterial vessel wall are mostly established via PARs.

4.1. Thrombin-induced pro-inflammatory responses in blood and vascular wall

Thrombin participates in the selective recruitment of monocytes and T-cells into the vessel wall by inducing the synthesis of MCP-1 in EC and monocytes. MCP-1 is a well-characterized chemokine which is abundant in human macrophage-rich atherosclerotic plaques. Thrombin has been shown to augment mRNA levels encoding for MCP-1,
IL-1β, IL-6, and TNF-α in human VSMC and less effectively, at high concentrations, in monocytes. It was stated that MCP-1 synthesis in monocytes in vitro, co-cultured with EC, is mediated by a thrombin-induced production of fractalkine (FK, CX3CL1), a cytokine which effectively chemoattracts T-cells and monocytes and has definite roles in CVD progression. In addition, in human EC in vitro, other inflammatory genes such as macrophage inflammatory protein 2-alpha, and neutrophil-activating protein 3, CD69, were reported to be overexpressed upon treatment with thrombin.

Some of the pro-inflammatory properties of thrombin have been inferred from models of inflammation such as a peritonitis mouse model, in which the administration of the potent thrombin inhibitor hirudin suppressed the antigen- or LPS-stimulated activation of macrophage adhesion. In the same model, the intraperitoneal injection of purified thrombin stimulated the adhesion of macrophages and the accumulation of IL-6 and MCP-1 in a fibrinogen-dependent manner and independently from PAR-1 activation. In a mouse heart-to-rat transplant model, a crucial role of PAR-1 activation by thrombin was shown in the initiation of leukocyte cell recruitment in vivo.

As stated earlier, thrombin is known to potentiate the production of IL-6 both in EC and VSMC in vitro. IL-6 is an important cytokine with recognized impact on inflammation and is known to exacerbate atherosclerosis. Thrombin upregulates IL-8 expression in the endothelium via p38 MAPK signalling pathway in vitro. Similarly, IL-8 triggers monocyte adhesion to the endothelium under flow conditions in vitro and is considered a possible biomarker to predict subclinical atherosclerosis based on data from multiple clinical trials.

Finally, thrombin induces the secretion of macrophage migration inhibiting factor in EC and VSMC.

### 4.2. Thrombin-mediated leucocyte adhesion, rolling, and migration on the activated endothelium

Once the endothelium has been activated, various molecules get entangled in a molecular network of capture, activation, and rolling. Selectins comprise a family of CAM of transmembrane glycoproteins. L-, P-, and E-selectins are known to act as main mediator molecules for rolling of monocytes, neutrophils, T cells, and B cells upon binding to the activated endothelium. E- and P-selectins, in particular, play a substantial role in the initial capturing, tethering, and rolling of the leucocyte, relevant to the atherosclerotic development and progression.

E-selectin, present on EC only, was expressed on the surface upon thrombin stimulation. Much interest was devoted to the mechanism of this thrombin-mediated expression and it was indicated that thrombin intervenes in the phosphorylation and activation of p38 MAPK, thereby inducing NF-κB-dependent and -independent pathways. Moreover, thrombin has the potential to promptly release P-selectin from the Weibel–Palade bodies in the EC. It was recently demonstrated that there is a differential regulation of endothelial exocytosis of P-selectin and von Willebrand factor (vWF) by PARs and cAMP.

Thrombin is not the only potent mediator for the expression of selectins on the endothelium. Many more factors like, e.g. TNF-α and IL-1α intervene in the E- and P-selectin synthesis. Elevated expression of adhesion molecules on activated EC is considered a significant feature in the initiation of vascular lesions. These pro-inflammatory responses additionally increase the overall expression of PARs, facilitating the endothelial reaction to thrombin, both with regard to endothelial dysfunction and further atherosclerotic progression.

Thrombin has a powerful potential to activate the endothelium, especially via its PAR-1 and -2 receptors, but also incites the overexpression of important pro-atherogenic factors.
immunoglobulin superfamily molecules such as ICAM-1 and VCAM-1.\textsuperscript{3,30,69,70} Rolling activated leucocytes are exposed to the influence of various chemoattractants, mediated by diverse integrins, and captured to cell adhesion glycoproteins. This eventually leads to the so called ‘leucocyte arrest’.

Thrombin enhances VCAM- and ICAM-1 synthesis in cultured human EC. NF-κB- and GATA-dependency was observed with regard to VCAM-1 expression.\textsuperscript{71} Other \textit{in vitro} studies indicated that PKC-δ and RhoA/ROCK activation independently lead to thrombin-induced NF-κB-dependent ICAM-1 upregulation.\textsuperscript{72,73} Moreover, the inhibition of both c-Jun N-terminal kinase (JNK) and NF-κB pathways showed additive inhibitory effect on ICAM-1 expression on the endothelium and highlighted a significant role for JNK signalling.\textsuperscript{74}

The actual process of transmigration of leucocytes usually occurs on activated endothelial regions thus facilitating the leucocytes to pass through. Thrombin seems to interlace by increasing the release of Ca\textsuperscript{2+} from the intracellular stores,\textsuperscript{75,76} favouring the ligation of ICAM-1, activating Rho family GTPases,\textsuperscript{77,78} which increases the myosin contractility of EC impairing the inter-endothelial junctions by disrupting VE-cadherin complexes.\textsuperscript{79}

4.3. Thrombin and monocytes/macrophages in atherosclerosis

The effects of thrombin on monocytes and monocyte-derived macrophages during atherosclerotic progression remain less elucidated compared with other blood cells such as platelets. Initially, it was indicated that VSMC may be more sensitive to thrombin activation than monocytes and macrophages \textit{in vitro}, the latter needing much higher concentrations of thrombin to achieve increased IL-6, IL-1β, MCP-1, or TNF-α mRNA expression.\textsuperscript{48} Human monocytes, macrophages, and dendritic cells \textit{in vitro} express PARs. PAR-1 was expressed in all cell types, whereas PAR-3 mRNA was less detected in monocytes and macrophages. PAR-1, -2, and -3 levels were upregulated upon thrombin treatment subsequently inducing MCP-1 expression. IL-4 downregulated PAR-1, -2, and -3 expression in dendritic cells derived from monocytes by granulocyte–macrophage colony-stimulating factor (GM-CSF).\textsuperscript{80} Li \textit{et al.} found PAR-4 protein expression on monocytes, though they failed to detect PAR-4 transcripts. They also showed that IL-6 was released upon treatment with agonist peptides of PAR-1 and PAR-4, but not of PAR-3 which was associated with PAR-3 incapability of mediating transmembrane signalling.\textsuperscript{81}

Finally, there are multiple pro-inflammatory effects of thrombin on other cell types which indirectly induce pro-atherogenic reactions in monocytes (as discussed in the text).

5. Thrombin in the advanced stage of atherosclerosis

Intimal thickening, derangement of the arterial vessel wall anatomy in concert with accumulation of lipids, infiltration of cells, and matrix degradation, presented by a necrotic core are the basic histological features of the advanced atherosclerotic lesion.\textsuperscript{82} Thrombin is implicated throughout plaque progression and destabilization events (Figure 4).

5.1. Thrombin and platelet-mediated effects in plaque progression and destabilization

Besides being a major activator of platelets, thrombin likely induces platelet-mediated atherogenic signals by boosting the synthesis and release of multiple pro-inflammatory mediators by platelets and deploying their interaction with leucocytes to favour chemotaxis, adhesion, and migration into the arterial vessel wall. Platelet activation by thrombin is accomplished exclusively by targeting PAR-1 and -4 receptors, expressed on their surface in humans.\textsuperscript{83} Platelets interfere in atherosclerosis in each of its phases—initiation, progression, and late complications.\textsuperscript{84-86}

\textit{In vivo}, thrombin-activation of human platelets results in the rapid activation and maximal expression of CD40 ligand (CD40L) on their surface.\textsuperscript{87} CD40L is a TNF family protein, expressed on many cell types including platelets, and it binds to CD40 thus forming a trimer, named CD40/CD40L dyad. This established system potentiates downstream of atherogenic signals in the arterial vessel wall constituents, such as EC, VSMC, and monocytes. Downstream signalling of CD40 is mediated by the so called TNF receptor-associated factors which are able to recruit kinases and other effectors, which subsequently lead to the activation of NF-κB pathway, and thus induce the upregulation of various adhesion molecules, matrix metalloproteinases (such as MMPs 1,2,3,9,11,13), cytokines, and growth factors.\textsuperscript{88}

MCP-1 is induced upon transient interactions of thrombin-stimulated platelets with the endothelium.\textsuperscript{89} These pro-inflammatory events, related to MCP-1 production, are observed in VSMC \textit{in vitro} too, probably contributing to VSMC migration and proliferation into the atherosclerotic plaques.\textsuperscript{90} Thrombin is also known to induce IL-1β expression under \textit{in vitro} conditions, both by EC\textsuperscript{91} and platelets.\textsuperscript{92}

An additional number of thrombin-induced platelet mediators, such as platelet factor-4 (PF-4), RANTES (Regulated upon Activation, Normal T-cell Expressed, and Secreted/CCL5),\textsuperscript{93} and neutrophil-activating peptide (NAP)-2\textsuperscript{46} are deposited by activated platelets on the endothelium to support leucocyte arrest and to favour the subsequent transmigration events. PF-4 (CXCL4) is a small chemokine also found in atherosclerotic lesions where its concentration correlates with severity of the plaques.\textsuperscript{95} PF-4 protects monocytes against apoptosis and induces their differentiation,\textsuperscript{96} whereas it serves as a stimulator of oxidative stress in macrophages.\textsuperscript{97}

Aside from its vessel wall-related oxidative activities, a recent study provides evidence for the role of thrombin in evoking apoptosis in human platelets \textit{in vitro}.\textsuperscript{98} It was demonstrated that apoptosis was induced via H\textsubscript{2}O\textsubscript{2} production, mediated by mitochondrial cytochrome c release and the activation of caspase-9, leading to caspase-3 activation and ultimately to phosphatidylserine (PS) exposure. On the other hand, it is well known that MPs are mainly released from cells upon activation or apoptosis. Moreover, increased number of circulation procoagulant MPs are positively associated with the initiation and dissemination of pro-inflammatory processes but also with the severity of CVD.\textsuperscript{99}

In conclusion, thrombin appears to have an important role in platelet-mediated pro-inflammatory cascades, resulting in a stimulation of ICAM-1, VCAM-1, E-selectin, and MMPs
production, all processes that contribute to plaque progression, subsequent destabilization, and rupture. 88,100–102

5.2. Thrombin and VSMC migration and proliferation

Besides its functions in the regulation of vascular tone, thrombin mediates migration, proliferation, and hypertrophy of VSMC. VSMC are known to express PAR-1, -2, and -4 thus potentiating the effect of thrombin in the activation of VSMC proliferation and migration. 103 Multiple studies report on situations associated with changes in the expression of PARs in VSMC. We have to take into consideration that the upregulation of these receptors might be as crucial as the direct effect of thrombin alone, because of the fact that they are the main mediators for its further actions. Hence, an upregulation of PAR-1 in human and rat VSMC in vivo is demonstrated upon the release of multiple platelet-derived products (PDP) such as transforming growth factor (TGF)-β1, platelet-derived growth factor (PDGF) and to a lesser extent, serotonin. 104 Thus a long-term generation of new thrombin receptors at sites of vascular injury might consolidate that thrombin amplifies its pro-atherogenic actions throughout the development of a vascular lesion. Moreover, PAR-1 expression seems responsive to physical stress in both human and rat aortic VSMCs in vitro—being enhanced when cyclic strain is applied 43 and being inhibited upon stimulation with high shear stress. 105 This substantiates the idea that VSMC requires physical stimulation (flow or strain) in order to maintain vessel wall homeostasis, and perturbation of this process may be involved in atherosclerosis where an overexpression of PAR-1 and PAR-2 receptors has been demonstrated. 21–23

Figure 4 Proposed mechanism for thrombin-induced atherogenesis. All known thrombin-induced pro-atherogenic actions are depicted in a consecutive way, showing its impact throughout the different stages of atherosclerotic development. Square with inverted ‘V’ indicates activation; encircled plus symbol indicates induction; upward arrow indicates elevated levels; MCP-1, monocyte chemotactic protein-1; PDGF, platelet-derived growth factor; EDN-1, endothelin-1 gene; ECE-1, endothelin converting enzyme-1 gene; COX-2, cyclooxygenase-2; MIF, migration inhibiting factor; ADAM10, A Disintegrin And Metalloproteinase protein-10; ROS, reactive oxygen species; mROS, mitochondrial-derived reactive oxygen species; IL, interleukin; TNF-α, tumour necrosis factor-α; Mo, monocyte; NO, nitric oxide; ICAM-1, intercellular cell adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; MPs, microparticles; CD40L, CD40 Ligand; MMP, matrix metalloproteinases; PF-4, platelet factor-4; RANTES, Regulated upon Activation, Normal T-Cell Expressed, and Secreted; NAP-2, neutrophil-activating peptide-2; NOR-1, neuron-derived orphan receptor-1; VEGF, vascular endothelial growth factor; PARs, protease-activated receptors; TF, tissue factor; PAI-1, plasminogen activator inhibitor-1.
Wang et al. studied thrombin-induced VSMC migration in cultured VSMC and demonstrated that the process is p38-MAPK-mediated upon the generation of ROS. Maruyama et al. indicated that thrombin-induced proliferation in cultured human VSMC is regulated by NF-κB. VSMC proliferation appears to be regulated by neuron-derived orphan receptor-1 (NOR-1), a transcription factor overexpressed in human atherosclerotic plaques upon stimulation with thrombin.

Finally, the regulation of PDGF in the endothelium also appears to be linked to thrombin. PDGF is related to atherosclerosis for its properties to stimulate VSMC migration and proliferation. PDGF levels rise upon treatment with thrombin of human umbilical vein EC, together with monocyte transmigration and E-selectin expression.

5.3. Thrombin and its pro-angiogenic responses

Neoangiogenesis is closely associated with plaque progression. Intraplaque haemorrhage is currently considered a critical factor for plaque destabilization and is predominantly attributed to the neovascularization of the intima and media by disorganized and immature 'leaky' microvessels. Thrombin promotes angiogenesis both in vitro and in vivo. It is indicated that it reduces the ability of EC to affix to their anchorage on the basement membrane, thereby promoting early angiogenic events. Furthermore, it has been stated that thrombin increases the mRNA and protein levels of α,β3-integrin in a concentration-dependent manner in EC. α,β3-integrin is a known angiogenic marker in vascular tissue and it directly interacts with thrombin, thereby facilitating EC attachment, migration, and survival. α,β3-integrin also mediates progelatinase A (MMP-2) activation. Stimulation with thrombin has shown the induction of MMP-2 release in both human EC and rat aorta in a dose-dependent mode in vitro. In addition, thrombin augments the expression of vascular endothelial growth factor (VEGF) and angiopoietin-2 via PAR-1-mediated mechanism. Finally, various studies indicate a relevant role for hypoxia-inducible factor-1a signalling pathway in the thrombin-induced VEGF gene expression and angiogenesis.

6. Thrombin and atherosclerosis—in vivo animal studies

Despite the wealth of existing data on thrombin's pro-atherogenic actions in vitro, we should point out that many of these studies have been carried out with cell cultures and purified thrombin, in the absence of receptors and inhibitors, such that the relevance of any of these outcomes may be debated. However, the critical role of thrombin in atherogenesis is supported by recent in vivo studies.

Indirect evidence shows that heterozygous tissue factor pathway inhibitor (TFPI)-deficient ApoE−/− mice exhibited a significantly greater atherosclerotic burden compared with TFPI wild-type genotype. TFPI is a potent inhibitor of TF-mediated thrombin generation. Direct evidence for the involvement of thrombin comes from experiments in which the administration of the direct thrombin inhibitor melagatran to ApoE−/− mice reduced lesion progression in brachiocephalic arteries. Total lesion area was significantly decreased in melagatran-treated animals. Thrombin inhibition also contributed to plaque stability (significant increase of immunohistochemical staining against VSMC α-actin), characterized by thicker fibrous caps, increased media thickness, smaller necrotic cores, and a significant decrease of staining against MMP-9. MMP-9 is considered an important catalyst of plaque rupture.

Finally, in a study employing transgenic double knock-out mice, deficient for HCII, a natural thrombin inhibitor, on a ApoE−/− background, HCII deficiency was associated with approximately 64% larger total plaque area and increased neointimal formation than in wild-type mice. In support of these findings, the administration of dermatan sulfate, which potentiates the inhibitory function of HCII about 10 000-fold, showed a HCII-dependent antiproliferative effect in wild-type animals.

7. Clinical studies

Thrombin's impact on atherosclerotic development is a relatively novel topic to investigate and no specific clinical trials have been conducted yet. However, several reports indirectly demonstrate its importance with regard to CVD progression.

Aihara et al. found a negative correlation between plasma HCII activity and ultrasound imaged plaque thickness of the carotid arteries in 306 elderly Japanese patients and suggested that HCII inhibits atherogenesis, thereby also showing a possible indirect link between higher thrombin generation and atherosclerosis progression.

Moreover, various thrombotic markers measured upon progressive CVD, indicate an indirect link for thrombin and atherosclerosis. The Cardiovascular Health Study (CHS) showed that prothrombin fragments F1-2 (F1-2) and fibrinopeptide A measured in 5201 individuals (399 free of CVD), which are markers for thrombin generation in vivo, correlated with various CVD risk factors such as triglycerides, C-reactive protein, low ankle-brachial pressure index (ABPI), etc. F1-2 plasma levels were also independently associated with carotid intima-media thickness in a population of 181 middle-aged adults, free of clinically overt atherosclerosis. Moreover, Nylaende et al. studied the relationship of prothrombotic activity and the severity of peripheral arterial occlusive disease (PAD). Multiple haemostatic markers such as vWF, soluble TM, soluble TF, TAT complex, and D-dimer were determined in a cross-sectional study of 127 patients, diagnosed with PAD. Plasma levels of D-dimer, TAT complex, and fibrinogen significantly correlated with the severity of atherosclerotic burden, evaluated by maximum treadmill walking distance and ABPI. A recent meta-analysis of 191 studies, investigating seven common haemostatic gene polymorphisms in CVD, indicated that the 1691A variant of the factor V gene and 20210A variant of the prothrombin gene, both of which promote thrombin generation in blood, might be associated with the risk of CAD. Moreover, it was recently shown that long after acute myocardial infarction, patients generate higher, earlier, and faster thrombin in comparison with chronic CAD patients. This strengthens the concept of vulnerable atherosclerotic plaques contributing to the propagation of thrombin generation, thereby leading to aggravation of CVD.

Several more indirect cross-relations might be of interest in this context. Numerous clinical trials postulate that
haemostatic factors such as fibrinogen, C-reactive protein, plasminogen activator inhibitor-1 (PAI-1) are risk factors for CVD progression. A recent study associated the progression of symptomatic intracranial large artery atherosclerosis with a pro-inflammatory state and impaired fibrinolysis, characterized with elevated concentrations of the endogenous fibrinolysis inhibitor PAI-1. Despite the fact that thrombin is not a sole mediator of PAI-1 it induces its expression together with TF in in vitro. TF and PAI-1 are already recognized for their pro-inflammatory features. In addition, many studies demonstrate a relationship between elevated PAI-1 levels and the development of atherosclerosis, not only systemically but also locally. Leucocytosis, and high neutrophil count in particular, may represent another intriguing mechanism for enhancing chronic atherosclerosis via maintaining a hypercoagulable state in CVD patients. Neutrophils are a pivotal link in CVD patients.131 Neutrophils have a pivotal role in the liberation of TF-laden MPs into the blood stream upon stimulation with cytokines and consequent platelet adhesion via P-selectin. This seems another potential mechanism for a continuous thrombin generation in vivo, facilitating the amplification of thrombin's pro-atherogenic features.

8. Summary and Perspectives

From histological studies an intense interaction between coagulation, inflammation, and the complex process of atherosclerosis has emerged. Advanced atherosclerotic lesions show evidence of the presence of active coagulation products including fibrin and fibrin cleavage products. Hence, the presence of an active coagulation cascade within the arterial vessel wall seems likely and our recent immunohistochemical data show that essentially all coagulation proteins are detectable in the atherosclerotic lesion. In the coagulation cascade we and others consider the generation of thrombin as one of the key regulating events. In vivo, thrombin is thought to be continuously generated as indicated by measurable quantities of F1-2 and TAT complexes in the plasma of normal individuals. Physiologically, the generation of thrombin is the product of the synthesis under influence of TF and inhibition by several inhibitors including AT and HCII. The net amount of thrombin will be determined by the rate of synthesis and inactivation, the localization (free or bound to surfaces), and its associated binding to receptors including PARs and TM. Upon progressive atherosclerosis, there is a diminution in the level of TM at the endothelium, which impairs the anticoagulant action of thrombin and the increased production of thrombin because of TF exposure allows interactions of thrombin with components of the arterial vessel wall, including dysfunctional EC on both initial and advanced lesions and other cell types in ruptured (thrombotic) plaques.

The continuous generation of mostly procoagulant thrombin may contribute to a vicious circle in the thrombin-induced atherogenesis process. As discussed, thrombin acts mostly via PARs, inducing multiple vascular pro-inflammatory reactions. The authors are aware that also other coagulation proteases including factor VIIa, factor Xa, and APC contain PAR-activation properties that may interfere with or add to the actions of thrombin. There has indeed been a public debate on the preference of thrombin vis-à-vis APC in their binding to PAR-1 and this debate has not yet been settled. Atherosclerotic alterations in the vessel wall are known to increase the level of expressed PARs on the surface of most vessel wall constituents. Thrombin-mediated pro-inflammatory events are a powerful trigger for more thrombin formation, which may eventually amplify its contribution to further atherosclerotic progression.

Finally, from a clinical perspective the introduction of a number of selective oral anticoagulants that will also be aimed for long-term administration makes it of actual importance to consider the effects and possible side-effects of thrombin inhibition on the extent and nature of atherosclerosis. Hopefully, thrombin inhibition is, as predicted from animal experiments, associated with a favourable change in atherosclerosis phenotype. However, the typical Janus face of many clotting proteases should warn against overt enthusiasm and calls for prospective clinical studies.

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