Review article

Regulation and Dysregulation of Thrombin Activity

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Abstract

Thrombin is the enzyme of the haemostasis system that stands at the crossroads between the coagulation cascade and coagulation inhibition by protein C, between plasma coagulation factors and cells involved in haemostasis and between haemostasis and the immune system. Allosteric regulation of thrombin and its interaction with various partners in blood plasma and on the surface of the endothelium and platelets provides a wide variety of thrombin functions. The thrombin activity regulation is multifactorial, so a failure of any part of this system leads to serious consequences. An example of this are thrombotic/bleeding complications during endothelial dysfunction, infections, inflammation and uncontrolled treatment.

This review aims to summarize current knowledge about thrombin structure, functions and regulation. Collected data suggest a crucial role of thrombin in different pathologies accompanied by blood coagulation disorders, in particular diseases causing endothelial dysfunction, such as COVID-19.

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Introduction

Platelet haemostasis and blood coagulation have important and strong interactions, such as positive and negative feedback loops and surface-bound enzyme complexes formation. Thrombin is the resulting enzyme produced by the coagulation cascade. It provides not only clot formation, but also a strong enzymatic feedback activity in procoagulant and anticoagulant reactions and platelet reactivity (1).

The main thrombin functions are: 1) turning soluble fibrinogen to fibrin, that is forming the protein-polymer core of the thrombus; 2) activating platelets; 3) activating clotting factors V, VII, VIII and XI, which enhance thrombin formation; and 4) limiting its own production by binding thrombomodulin and converting protein C (PC) to activated protein C, which subsequently inhibits clotting factors Va and VIIIa.

Thrombin functions extend beyond blood coagulation and include vascular functioning (2) and in particular, thrombin regulates inflammation and regeneration (3). Through PAR-receptors, it stimulates monocytes, T lymphocytes, leukocytes, endothelial cells, fibroblasts, tissue macrophages, neutrophils and mast cells (4-6). This way, thrombin mediates the crosstalk between the coagulation system and the adaptive immune system at the sites of vascular injury (6). Thrombin also enhances proliferation of endothelial cells, epithelium, fibroblasts, smooth muscle and neuromuscular cells and provides an antiviral response (4-6); participates in the development of malignant tumours (stimulates the adhesive and metastatic capacity of tumour cells and activates angiogenesis in the tumour area); regulates the development and apoptosis of nerve cells and neuralgia in the embryonic period and after birth; and controls the survival of the myoblast, the development of the placenta and the embryo as a whole (2-4, 7, 8).

Thrombin is acknowledged as the central enzyme of haemostasis because of the numerous and multi-directional functions it possesses. When it is present in the bloodstream in pathological conditions, it directly triggers intravascular thrombosis and is a marker thereof at the same time. This is why understanding molecular mechanisms of thrombin regulation is important for both laboratory diagnostics and correction of imbalance in the haemostasis system. Thus, the situation with thrombin studies and application is a bit of a paradox. Generation of active thrombin is the main factor of intravascular clotting. However, tests indicating thrombin activity or detecting prothrombin activation products are not used as routine laboratory diagnostic tests. Also, thrombin is the main target of anticoagulant therapy and at the same time, it can provide both pro-coagulant and anticoagulant action. Only a few research groups are working to find ways to switch thrombin activity and direct it to the anticoagulant pathway. This is a promising direction in the search for a fundamentally new treatment of haemostasis disorders.

This article is focused on summarizing current knowledge about the diversification of thrombin functions and substrates selection for the purpose of drawing the attention of medical professionals to the importance of using thrombin-specific tests in routine laboratory diagnostics both with regard to haemostasis disorders and infectious diseases.

Thrombin sites

Thrombin is a multifunctional serine protease of the haemostasis system. It cleaves more than 10 substrates, but remains highly specific to each one of them. Functional plasticity of thrombin is associated with the presence of effector sites (exosites I and II), which together with the active site are involved in the recognition and binding of the substrate; action directionality modulated by Na^+ coordination; and significant conformational mobility and plasticity of the molecule (9, 10). In addition, it should be noted that the multi-directionality of thrombin is ensured by its interaction with different partners, so thrombin is characterized by homotropic

allosteric regulation. In this context, two populations of thrombin can be determined: free and membrane-bound.

The thrombin active site is typical for enzymes of the chymotrypsin family of serine proteases. Thrombin substrates have a positively charged amino acid (usually arginine) on the N-terminus in the cleavage bond (11) (Figure 1).

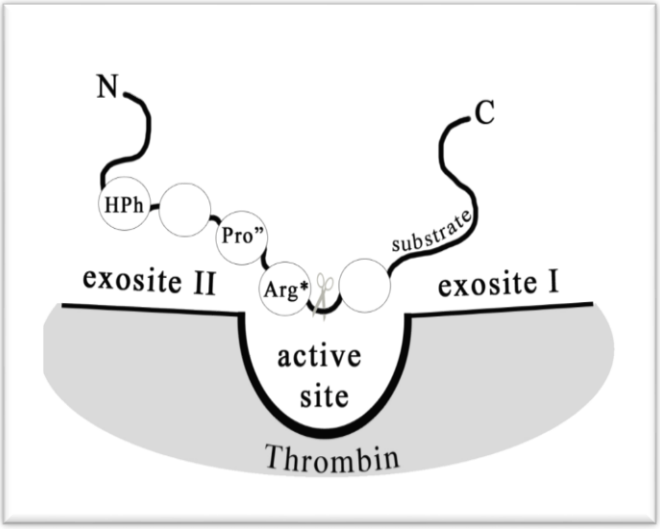

Figure 1. Thrombin substrate recognition scheme (based on [11, 12]).

HPh - hydrophobic amino acid residue

"– Pro or monoamine monocarbon amino acid residue

* - only for heparin cofactor II this position is occupied by Leu

The active site cleft is formed by two loops around the active site, which are longer than such loops in chymotrypsin (12). The 60-loop is hydrophobic and rigid. It forms a cap over an active site and provides interaction with N-terminal hydrophobic substrate residues. The γ -loop is more hydrophilic and flexible in nature. It is adjacent to the active site cleft of thrombin, it can contact substrate residues C-terminal to the scissile bond and can make contacts with the body of the substrate protein. Conformational changes in the thrombin molecule and changes in its catalytic activity result in the binding of exosites I and II of thrombin to the ligands (Table 1). This is confirmed by a functional analysis that demonstrates the relationship between the exosites and the position of the γ -loop of the catalytic centre. The γ -loop can block access to the "gap" of the active site. Active site environments for the individual binary complexes or the ternary thrombin complex are different, such that each binary and ternary thrombin complex could be expected to display unique catalytic properties. At the same time, there is no linkage between exosites I and II structures. Exosite I is formed by Lys36, His71, Arg73, Arg75, Tyr76, Arg77, Lys109 and Lys110; exosite II by Arg93, Lys236, Lys240, Arg101 and Arg233 (11). Exosite I interacts with the C-terminus of the substrate, and exosite II with the N-terminus of the substrate or cofactor

It is suggested that the interaction of ligands with the exosites of thrombin results in the displacement of ions from the exosites. In particular, it is known that the efficiency of interaction with exosite II is strongly dependent on the concentration of NaCl, in contrast to the interaction with exosite I (13, 14). Both exosites require peptides with a high percentage of acidic residues, 50 % for exosite I and 72 % for exosite II, but the hydrophobic content is lower for exosite II. J. Huntington (11) analyzed how thrombin chooses to bind ligands to exosite I or II. The main difference between the exosites is the ratio of negatively charged and hydrophobic amino acids. All exosite I-interacting peptides have a ratio below two and all exosite II-interacting peptides have a ratio above two. Therefore, exosite II is the only true anion-binding site of thrombin and exosite I is actually the "apolar-binding exosite".

Thrombin can interact with ligands by active site only (low-weight substrate), by active site and one of the exosites or by active site and both exosites (thrombomodulin binds simultaneously to thrombin exosite I through its growth factor domains and to exosite II through a chondroitin sulphate moiety). Such interaction variability provides specific recognition of a large range of substrates and their unique rearrangements provide thrombin enzymatic specificity due to

such recognition. The role of exosites in substrate recognition may not be equal. In particular, fibrinogen recognition is dominated by exosite I binding and exosite II plays a secondary role in this process. Eventually, fibrinogen successfully binds to exosite I of active-site-blocked thrombin (15).

Table 1. Thrombin ligands and characteristics of interaction

Ligand	Bound site	Kinetic parameters without Cofactor	Cofactor	Kinetic parameters with Cofactor
Fibrin [87]	exosite I	Kd= 0.52 $*10^{-6}$M (higher affinity site) Kd= 180$*10^{-6}$M (lower affinity site)	-	-
Fibrinogen [88]	exosite I exosite II active site	k_d= 7$*10^{-6}$ M kcat/Km = 13.9 $*10^{6}M^{-1}s^{-1}$ (for FpA release) kcat/Km = 4.0 $*10^{6}M^{-1}s^{-1}$ (for FpB release)	-	-
factor V [89]	exosite I exosite II active site	kcat/Km = 6.3 $*10^{6}M^{-1}s^{-1}$	-	-
factor VIII [90]	exosite I exosite II active site	Km = 12.6 $*10^{-9}$M	-	-
factor XIII [59]	active site	kcat/Km = 1.4$*10^{5}M^{-1}s^{-1}$	Fibrin (in exosite I)	kcat/Km = 1.2$*10^{7}M^{-1}s^{-1}$
GRIb [91]	exosite II	Kd = 5$*10^{-7}$-10^{-8} M	-	-
PAR-1 [91, 92]	exosite I active site	kcat/Km = 3$*10^{6}M^{-1}s^{-1}$	GPIb	kcat/Km = 1.5$*10^{7}M^{-1}s^{-1}$ Kd=10^{-9}-10^{-10} M
Protein C [65, 93]	active site	kcat/Km = 5.6$*10^{2}$ $M^{-1}s^{-1}$	TM (in exosite I, exosite II)	kcat/Km = 5.9$*10^{6}$ $M^{-1}s^{-1}$
TAFI [94, 95]	active site	kcat/Km = 0.96$*10^{3}$ $M^{-1}s^{-1}$	TM (in exosite I, exosite II)	kcat/Km = 1.2$*10^{6}$ $M^{-1}s^{-1}$ Kd = 6.6 $*$ 10^{-9}M
Thrombomodulin [24,96]	exosite I, exosite II	Kd = 1 - 4.9 $*$ 10^{-9}M	-	-
Antithrombin III [97]	active site	kcat/Km = 6.8$*10^{3}$ $M^{-1}s^{-1}$	heparin	kcat/Km = 1.2$*10^{8}$ $M^{-1}s^{-1}$
Heparin cofactor II [20]	active site	-	heparin, dermatan sulphate	Kd = 4$*10^{-8}$M (heparin) Kd =1.9 10^{-6}M (dermatan sulphate)
Heparin [20]	exosite II	Kd = 1.1$*10^{-7}$M (low molecular weight) Kd =0.9 10^{-7}M (high molecular weight)	-	-
Dermatan sulphate [20]	exosite II	Kd = 3.6$*10^{-6}$M	-	-

An interesting example of the regulation significance of thrombin interaction by exosites is fibrinogen γ' binding. Fibrinogen γ' is a product of alternative splicing. It has an alternative chain with the final four C-terminal residues replaced with 20 different residues, with a high proportion of negatively charged residues. Fibrinogen γ' has an average plasma concentration from 8 % to 15 %. [16]. Fibrinogen γ' chain carboxyl terminus strongly binds to thrombin exosite II (16). As a result, fibrinogen γ' reduces thrombin inhibition by antithrombin III (AT III) and heparin cofactor II (HC II) and competes for binding to thrombin with factor VIII. On the other hand, binding to γ' fibrinogen reduces thrombin blood circulation. In addition, fibrinogen γ' forms fibrin clots that are resistant to fibrinolysis. So, even a change in the strength of thrombin exosite II binding to fibrinogen translates into a dramatically different way of clot formation, its content, density and strength of the fibrin network.

It should be noted that there is a competition between cofactors for thrombin exosites binding, which has a significant effect on thrombin's enzymatic orientation. Thrombomodulin (TM) and fibrin have been shown to compete for exosite I thrombin binding. However, thrombin has a 1,000 times greater affinity for TM (Kd = 1 nM) compared to fibrin (Kd = 1 μM), so it always prefers to bind to TM [13]. As the epitopes of binding of TM and fibrinogen, fibrin and PAR-1 overlap and the binding of thrombin to TM interferes with its procoagulant functions (2). Hirudin peptides and fibrinogen bind competitively to exosite I (15).

Exosite II binds to the sulphated region (268-282) of Glycoprotein Ib (GpIb) and heparin with an almost equal affinity, but the thrombin prefers to bind to heparin because its concentration in the intact vascular endothelium is much greater than GpIb (13). This balance can be changed by vascular damage, endothelial dysfunction, inflammation or platelet activation. After coagulation, full clotting factor VIII activation requires cleavage at Arg372, a process involving thrombin exosite II. The sulphated region of GpIb binds to thrombin exosite II and is responsible for the inhibition of the Arg372-Ser373 bond cleavage and activation of FVIII (17).

Another regulative site of thrombin is the Na+-binding site. Na+ is octahedrally coordinated with the three water molecules and the three oxygen atoms of the carboxyl groups of thrombin [14, 18]. The effect is exclusively allosteric because the Na+-binding site is distant from the residues of the catalytic triad (10). The binding of Na+ ions dramatically changes the thrombin's substrate specificity. The "fast" Na+-bound form of thrombin actively cleaves fibrinogen, fibrin, clotting factors V and VIII and PAR. The "slow" Na+-free form has a high affinity for protein C. The transition from "slow" to "fast" thrombin results in the formation of the Arg187:Asp222 ion pair, the optimal orientation of Asp189 and Ser195 for substrate binding, and a significant shift of the side chain of Glu192 linked to the rearrangement of the network of water molecules that connect the bound Na+ to Ser195 in the active site. Under physiological conditions, the concentration of Na+ is 140 mM. The Kd for Na+ binding to thrombin is 110 mM, which implies that nearly half of the thrombin molecules generated in vivo from prothrombin are in the Na+-free, "slow" form (18).

It is suggested that the Na+-binding thrombin loop is directly involved in protein C interaction (2, 14). The binding of Na+ ions plays an important role in fibrinopeptide cleavage by thrombin, since the fibrinogen Aα-chain interacts more closely with the thrombin in the transition condition and, by stabilizing the Na+-bound form of thrombin, makes catalysis more effective (18).

Hypernatremia (Na+ plasma concentration > 145 mM) or hyponatremia (Na+ plasma concentration < 135 mM), which are the most common electrolyte disorders, are often associated with thrombosis or bleeding. Even under physiological conditions, the concentration of Na+ in the blood drops drastically close to platelet clot in vivo, proving the importance of Na+ in controlling the participation of thrombin and other enzymes (clotting factor Xa and protein C) in blood coagulation and thrombosis (18).

Thrombin action during coagulation

Dissociation constants and second-order rate constants of thrombin are quite different for different partners (Table 1). The sequence and speed of cleaving of a substrate depend on the dynamic equilibrium between the concentration of free thrombin, thrombin bound to thrombomodulin, thrombin bound to platelets, thrombin in the antithrombin III-thrombin complex, etc. The thrombin affinity to SERPINs (serine protease inhibitors) is not very high, unlike the thrombin affinity to glycosaminoglycans (dissociation constants of 110 nM and 90 nM for low and high molecular weight, respectively (19, 20)). This is why the only thrombin inhibitors – antithrombin III (ATIII) and heparin cofactor II (HC II) – use heparin and dermatan sulphate to achieve strong interaction with thrombin. Glycosaminoglycans accelerate thrombin inhibition by AT III or HC II 20,000- and 70,000-fold, respectively. They decorate proteoglycans in vascular and extravascular spaces and bind to thrombin exosite II by ionic forces. Thrombin inhibition by SERPINs themselves in the absence of heparin is insignificant. At the same time, heparin presence advances thrombin inhibition and provides strong suppression of coagulation in resting vessels.

Blood clotting cascade activation

In the initial stage of the activation of blood coagulation, subnanomolar amounts of thrombin activate factors V and VIII. Both exosites are involved in thrombin recognition of these factors. This is how thrombin promotes the formation of the prothrombinase complex and thus enhances the process of its own formation through positive feedback regulation. As a result, a few thrombin molecules quickly raise thrombin production (13, 21).

The picomolar concentration of thrombin also activates factor XI (22). The thrombin-GpIb complex can activate FXI on the surface of platelets, where thrombin and FXI are colocalized through separate GpIb interactions. Factor Va also was shown as a cofactor in the activation of FXI by thrombin both in a purified system and in blood plasma and requires phospholipid surfaces (23). But it is not clear whether GPIb and FVa work together or compete.

Thrombin-cells interaction

Membrane-bound enzyme complexes are critical for haemostasis (1). Complexes bound to the sub-endothelium or activated platelets provide blood coagulation activation, while complexes formed on the surface of the endothelium serve for coagulation inhibition. The density of the binding sites on different surfaces can direct haemostasis and change the status of the coagulation system. For example, about 1 % of fVII circulating in blood is normally activated, but it does not lead to coagulation or exceed the activation threshold because of the absence of a binding surface.

While platelet-bound thrombin plays a procoagulant role, thrombin that is bound to endothelial cells provides anticoagulant action (24).

Endothelium binding

Thrombin recognizes three partners on the surface of the endothelium. Interaction of thrombin with heparan sulphate and thrombomodulin (TM) on the endothelium membrane is rapid, reversible and occurs with a high affinity (Table 1), whereas its binding to the membrane protein R-30 is slow, irreversible and occurs with a low affinity (25). This is the way to trigger thrombin activity from coagulant to anticoagulant (Figure 2).

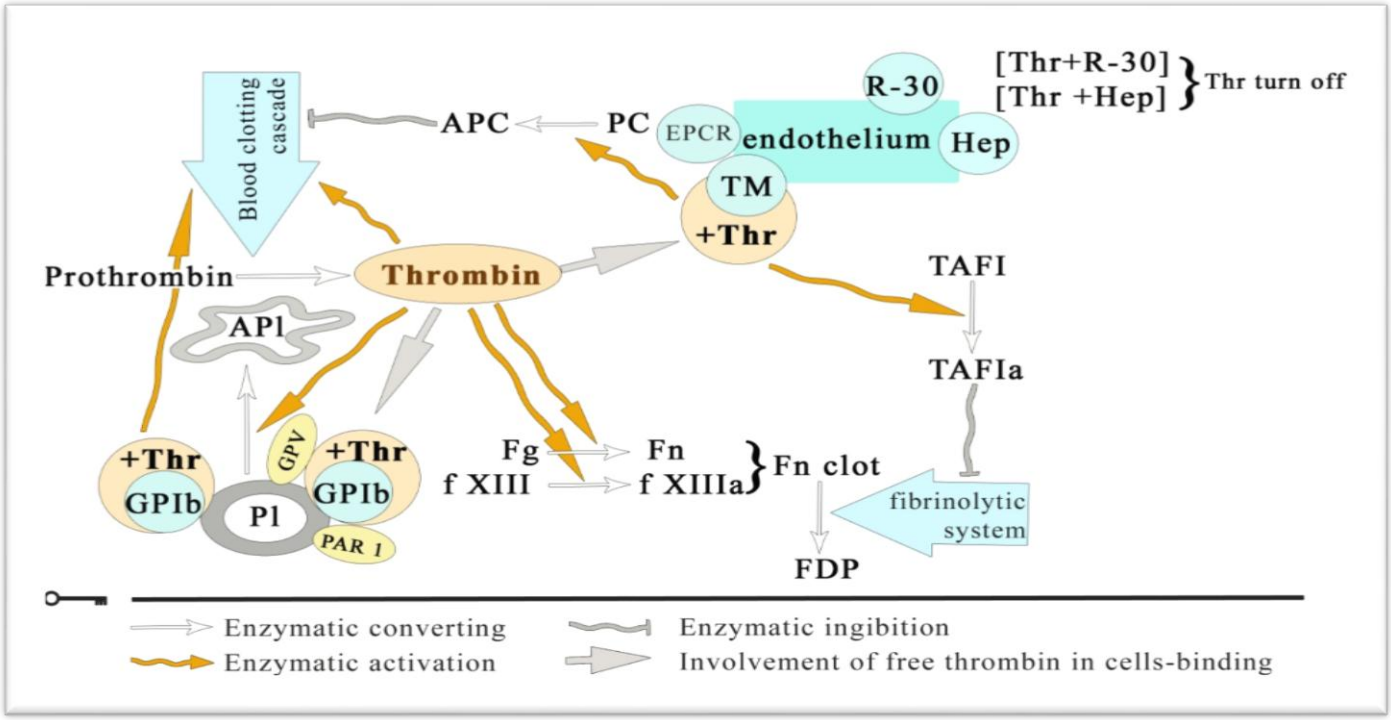

Figure 2. Enzymatic activity points of free thrombin and membrane-bound enzyme

Pl – platelet; APl – activated platelet; Fg – fibrinogen; Fn – fibrin; FDP – fibrin degradation products; TM – thrombomodulin; Hep – heparin; Thr – thrombin; PC – protein C; APC – activated protein C; EPCR – endothelial PC receptor; TAFI - thrombin-activated fibrinolysis inhibitor.© Korolova, 2021

The most striking example of allosteric regulation of thrombin is the change in its enzymatic activity due to its binding to TM. Thrombin recognizes TM by the highly charged regions of exosite I and exosite II. Binding of the chondroitin sulphate moiety enhances the affinity of this interaction. The recognition event also involves conformational changes of the thrombin in the "slow" form, mediated by binding of the EGF-like domains 5-6 to exosite I (24).

Protein C interacts weakly with thrombin due to the placement of polar amino acid residues of protein C near the apolar region of the "slit" of the active site of thrombin. TM interacts with thrombin exosite I by the protein part and with thrombin exosite II by the carbohydrate part. This way, it screens the "unfavourable" amino acid residues of protein C (26). As a result, TM significantly reduces the energy barrier and increases the rate of complex formation [thrombin + protein C] and thrombin activity, in relation to protein C, increases 10,000 times (2, 27). In vivo, the reaction is enhanced approximately tenfold by the endothelial protein C receptor, which binds and localizes PC to the endothelium near TM (Figure 2) (28).

Thrombomodulin is present in an amount of 100,000 copies per endothelial cell. The concentration of free thrombomodulin in large vessels is approximately 0.1-0.2 nM. After interaction with thrombin, its local concentration increases up to 10 nM. It should be noted that in microvessels, the concentration of thrombomodulin reaches 500 nM. This is a prime example of how thrombin-binding sites density can direct haemostasis and provide a significant increase in protein C activity in the microcirculatory system (26). The thrombin-TM complex also activates the carboxypeptidase TAFI (thrombin-activated fibrinolysis inhibitor). Activated TAFI is able to cleave C-terminal lysine residues of fibrin, which typically form fibrinolytic protein binding sites. Thus, the activated TAFI protects the fibrin clot against lysis [29, 30]. This interaction is mediated by thrombomodulin-exosite I interaction, chondroitin sulphate-exosite II interaction and TAFI-active site interaction. Thrombin at high

concentrations binds to the low-affinity R-30 protein on the endothelium surface (25). Thrombin has a low affinity to R-30 and this reaction is slow, but the binding of these proteins is covalent and irreversible. Thrombin R-30 complex serves for thrombin uptake, internalization and degradation.

Platelet binding

Circulating thrombin levels under 100 pM maintain platelets in an inactivated state. Concentration increasing to 1 nM is enough for platelets activation. Thrombin partners on the platelet surface are PARs (protein activated receptors) and GPIb. Platelets provide negatively charged surfaces for coagulation factor assembly. Prothrombinase complex (factor Xa+Va assembled on a phospholipid in the presence of Ca2+) also works on the platelet surface, so thrombin formation is accelerated by acidic phospholipids and platelet activation is upregulated (Figure 2). At low thrombin concentrations, the platelet membrane glycoprotein Ib is involved in the interaction between platelets and thrombin. The binding of thrombin to GPIb is the responsibility of thrombin exosite II on one side and the N-terminal domain and negatively charged GPIb region on the other. Some experimental data (31) suggest that exosite I may also be another site for GPIb interaction. The high-affinity thrombin binding site is located in the α -subunit of the GPIb (268–287 amino acid residues), but only a small fraction of the GPIb exposed on the platelet surface can bind thrombin and most of the receptors specifically bind only to the von Willebrand factor (32, 33). The thrombin-GpIb complex can enhance cleavage of GpV, resulting in hyperactivation of platelets. GPIb/IX/V signalling pathway mediates PI3k/Akt activation and protein phosphorylation (33, 34). It also mediates the increasing intracellular Ca2+ in thrombin-activated platelets. The result of thrombin-GPIb interaction is the stimulation of energy metabolism (glycolysis and oxidative phosphorylation) (34).

GPIb can also be a cofactor in case of factor XI (FXI) and PAR activation by thrombin. Thrombin bound to platelet GpIb via exosite II is brought into proximity to PAR-1 and enhances its activation using exosite I to make contact (11, 13).

Thrombin signalling in platelets depends principally on PARs. Thrombin binds PAR through exosite I and the active site. Thrombin cleavage of PAR is also exosite II-dependent due to the cofactor effect of GPIba, which accelerates the rate of reaction six- to sevenfold (11, 35, 36). Only PAR-1 and PAR-4 are exposed on human platelets and endothelium. PAR-1 is the primary thrombin receptor on platelets, requiring picomolar thrombin concentrations for effective activation, while PAR-4 cleavage is only relevant at high thrombin concentrations (PAR-4 contributes to thrombin-induced platelet aggregation at low thrombin concentrations in the range from ~ 0.4 nM to ~ 0.8 nM (37)). PAR-1 mediates a rapid but transient platelet Ca2+ signalling response to thrombin, whereas PAR-4 mediates a slower, sustained rise, producing the majority of calcium response (38). PAR-4 also plays a more important role in thrombin generation than PAR-1 (39). Both activation of PAR-4 and activation of PAR-1 induce granule release as a feedback mechanism to enhance and stabilize platelet aggregation, with PAR-1 producing reversible aggregation and PAR-4 producing irreversible aggregation (37).

It is probable that the full response of human platelets requires the formation of a pair of receptors PAR-1 and PAR-4 [40]. PAR-4 does not have a sequence complementary to the thrombin exosites. Cleavage of PAR-4 platelets of humans requires a higher concentration of thrombin (~ 100 times) compared to the splitting of PAR-1. Activation of the PAR by thrombin begins with binding and cleavage (Arg41-Ser42) of the N-terminal region of the receptor, which results in the exposure of a new N-terminal region. This amino-terminus serves as a ligand for surfactants, intramolecularly binding to the extracellular portion of the receptor and inducing an intracellular signal (4, 41, 42).

Thrombin activation is associated with G proteins by PAR activation. PARs signalling

mediates the activation of phospholipase C isoform β, PI3- and RhoA/Rho kinases (43-46). PARs also mediate increases in intracellular calcium in thrombin-activated platelets. This leads to changes in cytoskeletal actin and platelet surface exposure of fibrinogen-binding integrins IIbIIIa and activates further signalling and platelet aggregation.

The prothrombinase complex converts prothrombin to thrombin by two pathways. Initial cleavage at Arg320 between the A and B chains generates meizothrombin, which is an active intermediate. The alternative initial cleavage at Arg271 cleaves off the Gla-domain and the two kringles and generates inactive prethrombin-2. Active thrombin is formed as a result of further cleavage of intermediates. Both ways take place in vivo (47–50).

As previously shown, during the first minute of clotting system activation, the in vitro levels of both thrombin and meizothrombin are equal and can reach 0.8 μM (49). Moreover, meizothrombin can bind to the platelet membrane (51), so the local concentration of meizothrombin near the platelet surface may be significantly increased during the initial activation stage. We showed (52) that meizothrombin was able to enhance platelet aggregation induced by ADP, collagen or adrenalin. At the same time, being membrane-bound, meizothrombin is not sensitive to antithrombin III in contradistinction to thrombin (51).

Thrombin can also enhance platelet adhesion through cleavage of ADAMTS13, a proteinase responsible for the von Willebrand factor processing. Since the von Willebrand factor is a key platelet adhesion glycoprotein, by inactivating ADAMTS13, thrombin promotes platelet involvement at the site of injury (53).

Fibrin clot formation

The next step of thrombin activity is fibrinogen cleavage. Thrombin increasing to μM starts the conversion-of-fibrinogen-to-fibrin thrombin activity (Km for fibrinogen cleavage is 7.5 μM). Thrombin cleaves four fibrinogen bonds, cleaving two fibrinopeptides: fibrinopeptide A (fpA: 16-amino acid N-terminal peptide from the Aα chain) and fibrinopeptide B (fpB: 14-amino acid peptide from the Bβ chain) (54).

During cleavage of fpA, thrombin binds directly to the Aα-chain of fibrinogen via exosite I and the active site. The release of fpA is sufficient for the polymerization of both fibrin and this form of monomeric fibrin desA, which spontaneously polymerizes to form protofibrils. When fpB is cleaved, fibrin desAB is formed and lateral association of protofibrils begins. However, the removal of fibrinopeptides B alone does not cause the polymerization of fibrin desB (2, 54, 55). It should be noted that the concentration of thrombin determines the features of the fibrin clot and its resistance to the action of the fibrinolytic system (56).

Thrombin does not react with fibrin through exosite II. This allows the release of fibrin from the active site for clot formation and it is the reason why cleavage of fibrinopeptides is not the main thrombin activity. Thrombin interacts with the N-terminal E region of fibrin to release FpA and FpB, likely disrupting the interaction of the αC terminus with the E-region and exposing knobs A and B in the E-region, which interact with their specific binding pockets in the D-regions of another fibrin molecule, leading to protofibril formation (16).

By converting fibrinogen to fibrin, thrombin provides not only clot material, but also its own cofactor. Being bound to the fibrin E-region, thrombin uses fibrin as a cofactor for cleavage of factor XIII. Factor XIII is bound to the fibrin C-terminal D-region. The fibrin monomers spontaneously polymerize and the E-region of one fibrin molecule is located closely to the D-regions of two other fibrin molecules, moving factor XIII closer to thrombin. Thrombin activates clotting factor XIII by limited proteolysis of its subunit A. Factor XIIIa cross-links neighbouring fibrin molecules by covalent intermolecular bonds and cross-links inhibitors of fibrinolysis to fibrin (13, 57, 58).

The newly generated polymer will provide the cofactor required for 80-fold accelerated factor

XIII activation (59). This ensures that factor XIIIa is generated when it is needed and where it is needed – on the fibrin clot surface.

Interestingly, purified E-regions of fibrin interact with prothrombin, resulting in thrombin-like active site formation in the prothrombin molecule (60, 61). Thus, we can speculate that thrombin-E-region interaction provides not only a thrombin-based approach to factor XIII, but also provides changes in the active site of thrombin and accelerates factor XIII activation.

Role of thrombin in pathology

Historically, the procoagulant function of thrombin (the conversion of fibrinogen to fibrin) has been investigated earlier and more extensively. Based on the aforementioned facts, we can conclude that thrombin has a much wider range of action. Thrombin has a number of functions that can be activated depending on its concentration, localization and accessibility of partner molecules. So, the diversity of thrombin action provides regulation of haemostasis in general and its local concentration influences the thrombus microenvironment and architecture (62). Due to a great variety of protein partners, thrombin is a connector between plasma and platelet coagulation, between coagulation and anticoagulation, between haemostasis and the immune system and between endothelium pathology and coagulation disorders.

Nowadays, thrombin remains a major target of antithrombotic and anticoagulant therapies in cardiovascular medicine. Heparins and direct thrombin inhibitors are currently used in the treatment of acute thrombotic complications, but a strategy that inhibits thrombin at the active site reduces not only procoagulant and prothrombotic functions, but also shuts down activity toward the anticoagulant protein C.

Many researchers have been trying to convert thrombin into a potent and safe anticoagulant for in vivo applications. A new strategy aims at modulating thrombin function, rather than inhibiting it (10, 63). Some authors propose that mutant thrombin be used as a safe physiological anticoagulant (64) or apply a fusion protein, where thrombin and the TM domain are connected through a peptide linker (65).

The shift of balance in pro- and anticoagulant action of thrombin becomes crucial for the development of haemostatic pathologies during inflammation and endothelial dysfunction. It should be emphasized that only the native intact endothelium provides the anticoagulant function of thrombin. So, any endothelium dysfunctions lead to triggering thrombin activity toward coagulation, which appears as thrombotic complications during some diseases.

High levels of angiotensin II cause arterial hypertension by a complex vascular inflammatory pathway that requires leukocyte recruitment and reactive oxygen species production and is followed by vascular dysfunction. The resulting vascular inflammation and dysfunction are mediated by the activation of thrombin-driven FXI feedback, independent of factor XII. FXI receptor GPIb on platelets is required for this thrombin feedback activation [66]. Inhibition of this feedback loop with an antisense molecule against factor XI reduced both vascular pathology and hypertension. At the same time, there are doubts about the importance of feedback activation of factor XI (67).

During bacterial and viral infections, there is an interplay between blood coagulation, immune cells and platelets to restrict the dissemination of pathogens within the body. Endothelial disturbance switches over coagulation and induces thrombotic complications, excessive inflammation and tissue damage. With regard to potential clinical significance, it is possible that interference with the PAR1 pathway by direct thrombin inhibitors or PAR1 inhibitors may increase the risk and severity of viral infection (6).

Thrombin activity leads to endothelial cell activation in Klebsiella infection (bacterial pneumonia). Extrinsic pathway generated thrombin mediates fibrin polymerization and platelet-neutrophil interactions essential for protective immune responses in at least Klebsiella pneumonia–derived sepsis [68]. TF is induced in the lung after an H1N1 IAV infection in

mice, which led to thrombin-induced hyperactivation of coagulation (6). At the same time, activated protein C seems to be involved in coagulation regulation during an H1N1 IAV infection. HIV infection is associated with increased [thrombin-antithrombin III]. Thrombin mediates the crosstalk between the coagulation system and the adaptive immune system at the sites of vascular injury through increased T-cell motility and production of proinflammatory cytokines during an HIV infection (6, 69).

An infection that strongly affects coagulation is the coronavirus disease 2019 (COVID-19). It induces an immune response within the endothelium in blood vessels in several organs (70, 71). Studies of COVID-19 patients demonstrate the presence of fibrin thrombi within distended small vessels and capillaries and extensive extracellular fibrin deposition (72). Disseminated intravascular coagulation (DIC) has been reported to develop in 70 % of patients who succumb to the infection. D-dimer levels were increased far out of proportion to any abnormalities in the prothrombin time (PT/INR), activated partial thromboplastin time (APPT), fibrinogen level, or platelet count; these findings are uncharacteristic of DIC as currently understood.

Hanny Al-Samkari et al. stated that thrombosis is primarily associated with inflammatory markers, rather than coagulation parameters during a COVID-19 infection (73). Marco Ranucci et al. also demonstrated that coagulation is triggered by the release of IL-6 and other cytokines and the consequent release of tissue factor (74).

Thrombin in laboratory diagnostics

In recent times, the most widely used laboratory tests that indicate the imbalance of haemostasis have been the D-dimer test and thromboelastography. D-dimer is the fibrin degradation product appearing in the bloodstream as a result of stabilized fibrin cleavage by fibrinolysis. So, this parameter mainly indicates the existence of stabilized fibrin in the bloodstream and also the balance between coagulation and fibrinolysis (73, 75-77).

The thromboelastography characterizes the clotting process in whole blood, providing information on clot formation and lysis overall (78). The thromboelastography testing has expanded to include managing extracorporeal membrane oxygenation therapy, assessing bleeding and assessing hypercoagulable conditions. In addition, thromboelastography platelet mapping has been utilized to monitor antiplatelet therapy (78).

In particular, in patients with COVID-19, elevated circulating D-dimer levels are associated with mortality (79, 80). But the increasing of D-dimer levels occurs in both thrombosis and bleeding complications, so this parameter cannot be used as a thrombosis marker (81). Management of the thrombotic risk associated with COVID-19 is complicated by heparin treatment (82) and one of the approaches is modification of the thrombin generation assays conditions by adding heparinase as a heparin neutralizing agent.

Markers that directly indicate the appearance of active thrombin in the bloodstream are prothrombin fragment 1+2 (F1+2), prethrombin-1 and soluble fibrin. Unfortunately, they are not incorporated in the routine laboratory practice.

F1+2 is formed during the activation of prothrombin by prothrombinase complex (47), which is why it indicates prothrombinase activity. The appearance of this prothrombin derivative indicates the prothrombin activation to thrombin, so it is a direct method of thrombin detection. The nature of F1+2 makes it a more informative marker of procoagulant changes than clotting tests. Marco Ranucci et al. were able to show the direct appearance of thrombin by measuring F1+2 in the blood of patients with COVID-19 (74). Moreover, the F1+2 level was significantly reduced, whereas it increased in non-survivors.

Another prothrombin derivative is Prethrombin-1, which appears as a result of prothrombin autolysis by thrombin (9). It is present in the bloodstream in case of intensive production of active non-inhibited thrombin, so it is a result of a strong activation of blood coagulation. A high

level of prethrombin-1 evidences the danger of intravascular clot formation (73, 83).

On the other hand, thrombin activity is a useful predictor of the bleeding risk (73). Thrombin acts at the very end of the coagulation cascade and the decrease of its activity is associated with a high risk of bleeding. Several studies indicated the correlation between impaired thrombin generation and the severity of the disease (84). The tendency to bleeding was observed when thrombin generation fell below 20 % of the normal range (73). Similarly, a correlation between the generation of thrombin and the clinical bleeding phenotype in patients with deficiencies in blood coagulation factors FII, FV, FVII, FX and FIX has been observed.

In the same way, as prethrombin-1 is a product of thrombin action on prothrombin, the soluble fibrin appears in the bloodstream as a result of the action of small amounts of thrombin on fibrinogen. Accumulating in small concentrations that are insufficient for clotting, fibrin desA forms oligomers and macromolecular complexes with fibrinogen, circulating in the bloodstream as non-cross-linked soluble fibrin monomeric complexes. Being the direct result of thrombin action, its parameter clearly indicates the activation of blood coagulation even before intravascular clotting (76, 77, 85, 86).

Thus, the level of thrombin as a diagnostic marker has not been neglected during infectious diseases, including COVID-19, and tests that can indicate it directly or indirectly are potentially useful in the prediction of haemostatic abnormalities pertaining to these diseases.

Conclusion

The presence of exosites, active site and Na+ site in the thrombin molecule allows thrombin to recognize a large number of substrates specifically. Thrombin substrates are important for platelet and fibrin clots formation, for activation and inhibition of the blood clotting cascade, for realization of the endothelial anticoagulant function and for the interaction of haemostasis and the immune system. Therefore, thrombin provides wide-scope and multidirectional action in haemostasis. On the other hand, imbalances of various links of haemostasis are reflected in thrombin activity and focus.

This is the reason why a significant number of antithrombotic drugs are aimed at inhibiting thrombin activity. At the same time, inhibition of the general and not procoagulant thrombin activity is not the right approach from the point of view of the current understanding of haemostasis.

Determination of thrombin activity, prothrombin concentration or concentration of prothrombin activation products are not traditional laboratory methods in hemostaseology, despite the fact that thrombin activity is one of the main factors of thrombotic disorders. In our opinion, characterization of the functional state of prothrombin/thrombin should be a required component of everyday laboratory practices.

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