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REVIEW

Role of Platelets and Serine Proteinases in Coupling of Blood Coagulation and Inflammation

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Abstract—In addition to having a key role in thrombogenesis, platelets are actively involved in acute and chronic inflammation: they induce the release of proinflammatory mediators, expose adhesion molecules, and recruit leukocytes. The inflammation-induced expression of tissue factor by endothelium and monocytes leads to production of hemostatic serine proteinases, which can regulate both blood coagulation and the inflammatory response of the body. Serine proteinases activate blood and connective tissue cells and regulate blood coagulation, inflammation, tissue repair, atherogenesis, etc. This review considers new functions of platelets in thrombogenesis and inflammation, stabilization of platelet–platelet and platelet–leukocyte aggregations, receptor functions of tissue factor, proinflammatory properties of hemostatic serine proteinases mediated by proteinase-activated receptors (PAR), activation of transcriptional factors (NFκB and other), and anti-inflammatory and cytoprotective properties of the anticoagulant proteinase (activated protein C) mediated through binding of the endothelial protein C receptor (EPCR) and cleavage of PAR1.

Key words: adhesive molecules, platelets, tissue factor, proteinase-activated receptors, modulators of inflammation, blood coagulation proteinases

Activation of the blood coagulation and thrombogenesis in the area of atherosclerotic lesions of the coronary artery are crucial factors in the development of acute coronary diseases (unstable angina, myocardial infarction, etc.) [1, 2]. Atherosclerosis is a chronic inflammatory disease, which acquires an acute form on disruption of plaque and thrombogenesis [3, 4]. Formation of arterial thrombi are mainly contributed by platelets that adhere to the damaged vascular wall or destroyed atherosclerotic plaque, aggregate, and generate an active surface for production of hemostatic serine proteinases and blood coagulation. Platelets express adhesion molecules, chemokines, growth factors, cytokines, and other inflammatory mediators, and recruit leukocytes [4]. Platelet adhesion to activated endothelial cells and leukocytes is one of the key events in thrombogenesis, inflammation, and atherogenesis. Understanding of the role of serine proteinases in the blood coagulation mechanisms during inflammation seems fundamental for development of new approaches for prevention of thrombotic complications of cardiovascular diseases. The involvement of thrombin, a key enzyme of the blood coagulation, in inflammation, tissue repair, and atherogenesis has been discussed in some reviews [5-12], but concepts about the role of hemostatic proteinases and their receptors during local and systemic inflammation are very contradictory.

In the present review attention will be mainly concentrated on the role of platelets in the development of inflammation and blood coagulation and also on production and functions of hemostatic serine proteinases as signal molecules that control blood coagulation and inflammation through specific receptors on cells (including the family of proteinase-activated receptors (PAR)). Mechanisms of adhesion, platelet activation, and stabilization of platelet–platelet and platelet–leukocyte aggregates during the development of inflammatory response will be considered. These aggregates form an active surface, which promotes activation of blood coagulation and inflammation by exposing onto the surface and releasing inducers of coagulation and inflammation and microparticles carrying these inducers.

Blood coagulation and production of regulatory serine proteinases are activated in a variety of inflammatory diseases, such as atherosclerosis, septic shock, and also in ischemia/reperfusion and other processes which cause damage or dysfunction of vascular endothelium.

Platelet adhesion and activation during the development of inflammatory response. Endothelium plays a key

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role in response to vascular damage because it regulates platelet adhesion and aggregation, leukocyte adhesion, and blood coagulation. The lesion vascular endothelium under the influence of inflammatory stimuli disturbs the balance of pro- and antithrombotic factors and results in thrombinogenesis [13, 14]. The previously generated adhesive molecules are expressed onto the surface of activated endothelial cells.

Adhesive molecules, von Willebrand factor, and P-selectin. Weibel–Palade bodies of endothelial cells release von Willebrand factor (VWF), which provides for platelet mobilization and platelet (P)-selectin (CD62P, GMP140) responsible for adhesion of leukocytes, their rolling, and also for stabilization of platelet aggregations [15-17]. The lectin-containing N-terminal domain of P-selectin binds the P-selectin glycoprotein ligand (PSGL-1) (P-selectin receptor of neutrophils) on monocytes, neutrophils, platelets, and the cells adhere to endothelium [18, 19].

P-Selectin is split as a soluble P-selectin (sP-selectin) that stimulates expression and exposition on the monocyte surface of tissue factor, an inducer of blood coagulation [20]. P-Selectin binding to PSGL-1 stimulates the release by leukocytes of microparticles, which carry tissue factor. Microparticles (together with sP-selectin) determine the high procoagulant status of blood in inflammation [21].

VWF is a marker of endothelial lesion and is synthesized in endothelial cells, in platelet precursor megakaryocytes, and is deposited in Weibel–Palade bodies of endothelial cells and α-granules of platelets [22]. From the VWF precursor two large polypeptides are synthesized: the mature VWF which mediates adhesion and aggregation of platelets and the VWF propeptide (VWF antigen II) which is necessary for intracellular post-translational multimerization and transfer of VWF into depositing vesicles. VWF is a high-molecular-weight multimer (500-10,000 kD). The fundamental structure of VWF is a dimer of two subunits joined by the C-ends, which form a block for building the large multimer. Each subunit of VWF consists of domains (D′-D3-A1-A2-A3-D4-B-C1-C2) with certain functions. The VWF molecule contains domains responsible for binding blood coagulation factor VIII and platelet receptors, such as glycoprotein transmembrane complexes GP Ib/IX/V and integrin αIIbβ3 (GP IIb/IIIa), and also heparin and collagen.

VWF binds subendothelial collagen and the platelet receptor GP Ib and ensures platelet immobilization and activation [22]. VWF is immobilized on subendothelial collagens through two types of A domains (A1 and A3) [23, 24]. The A3 domain provides a firm immobilization of VWF on type I and III collagens, and the domain A1 mediates the immobilization on type VI collagen. VWF immobilized on subendothelial collagen binds to GP Ib and "attaches" the cells as a "bridge" between collagen and platelets. VWF is necessary for platelet adhesion to collagen in areas of vascular lesions at high shear rate (sec^{-1}) and shear stress (N/m^2). In the normal circulation, shear rates are the greatest in arteries and arterioles (on average, 1700 sec^{-1}). Shear rates increase by more than an order of magnitude in regions of arteries stenosed because of atherosclerotic plaque. At significant (to 90%) occlusion of the coronary artery shear rates can increase to 40,000 sec^{-1} [14]. VWF is a factor of pathogenesis of acute thrombotic occlusion of stenosed arteries.

Integrins as receptors of adhesive molecules. GP Ib consists of two α- and β-chains bound by a disulfide bridge and is stabilized on the surface of intact platelets by a noncovalent bond with two GP IX chains and one GPV chain (at the ratio of 2 : 2 : 2 : 1) [18].

GP Ibα contains sites for binding VWF, P-selectin, the leukocytic integrin Mac-1 (αM/β2, CD11b/CD18), a high-molecular-weight kininogen, and also proteinases—thrombin and the factor XIIa. The N-terminal globular domain of GP Ibα includes a tandem of seven repeated leucine-rich regions (repeats 2-4 bind VWF) and the N- and C-terminal flanking sequences [18].

The major thrombin-binding site, which contains sulfated tyrosines, is located in the N-terminal ligand-binding domain, which is next to the C-flanking sequence and precedes the central glycopeptide region of GP Ibα. Studies on the crystal structure of the N-terminal domain of GP Ibα bound to thrombin have revealed interaction of the receptor with two molecules of the enzyme: one interacts through anion-binding exosite I of thrombin and the other through its exosite II [25, 26]. On binding to GP Ibα by two sites of its recognition center [5], thrombin acts as a bridge between GP Ibα receptors of the same platelet or adjacent cells. Thrombin activates platelets through a specific proteinase-activated receptor (PAR1) [5, 9, 10] and promotes the platelet aggregation inducing multimerization of GP Ibα receptors and increasing the efficiency of the PAR1 cleavage [27]. GP Ibα acts as a cofactor of PAR1 [28].

GP Ibα is similar to PSGL-1 in structure and specifically binds P-selectin through the domain of sulfated tyrosines [29]. GP Ibα is a receptor for β_2-integrin of leukocytes—Mac-1 (CD11b/CD18)—and is responsible for platelet binding to neutrophils [30]. The N-terminal domain of GP Ibα is responsible for the binding of I (insert) domain of Mac-1 homologous to the A1 domain of VWF. The cytoplasmic domain of GP Ibα contains sites for binding some proteins: an actin-binding and two adaptor proteins 14-3-3ζ involved in the regulation of the functional activity of GP Ib/IX/V. The specific recognition of the ligand-binding GP Ibα domain by P-selectin, VWF, and Mac-1 is responsible for platelet binding to endothelium, leukocytes, matrix, and to one another [18].

VWF activates cells through interaction with GP Ib. However, the mechanism of the signal transmission medi-

ated through GP Ib remains unclear. According to some data, the signal is transmitted via the cytoplasmic domain of GP Ibα bound to the actin-binding protein and adaptor proteins 14-3-3ζ [18] and results in increase in the concentration of intracellular calcium ($[Ca^{2+}]_i$), release of a platelet aggregation agonist, ADP [31], and generation of another platelet activator, thromboxane A_2 (TXA_2) [32]. But according to other data, the complex GP Ib/IX/V is associated with two ITAM (immunoreceptor tyrosine-based activating motifs)-containing proteins (FcγRIIa and FcRγ), and the binding with the agonist leads to phosphorylation of the ITAM tyrosine with the family of Syk kinases, activation of phospholipase Cγ (PLC), protein kinase C (PKC), and increase in $[Ca^{2+}]_i$ [18, 32, 33]. These reactions result in the activation and exposition on the cell surface of another VWF receptor—integrin αIIb/β3.

Integrin αIIb/β3 acts as a receptor of VWF and fibrinogen. The majority of αIIb/β3 integrins on activated platelets are occupied by fibrinogen because in human plasma the fibrinogen concentration is two orders of magnitude higher than the concentration of VWF. VWF is responsible for production of stable platelet aggregates in blood vessels at high shear stress and rate (as occurs in arterioles), whereas fibrinogen alone cannot ensure aggregation [18].

αIIb/β3 is a transmembrane heterodimeric protein, with each of its subunits (α and β) consisting of a large extracellular domain, a transmembrane region, and a short cytoplasmic domain [15]. Integrin αIIb/β3 ensures the binding of cells with various adhesive proteins: VWF, fibrinogen, fibronectin, thrombospondin, vitronectin, laminin, collagen, etc. (table).

There are active and inactive conformations of αIIb/β3. For its activation, an inside-out signaling from the cell is required, which is generated upon platelet activation with inducers released, exposed, or produced in the area of vascular injury (ADP, thrombin, collagen, platelet activation factor (PAF), etc.). Conformation of the integrin αIIb/β3 extracellular domain is controlled by interaction of the β3-chain cytoplasmic domain with intracellular mediators and cytoskeleton proteins [27].

Integrins – receptors of adhesive molecules – and their agonists regulate cell functions. Although mechanisms of increase in the concentration of intracellular calcium on activation of integrin αIIb/β3 with adhesive molecules (the outside-in signal) are not quite clear, some findings support the involvement of ITAM-containing receptors (such as GP VI/FcR γ-chain) in this process [34]. It has been shown that Src kinase Fyn, which phosphorylates ITAM tyrosine, also phosphorylates the C-end of the β3-chain cytoplasmic domain of αIIb/β3 [35]. A non-receptor tyrosine Syk kinase, which binds to cytoplasmic domains of ITAM-containing receptors, also interacts with the cytoplasmic domain of β3-chain of integrin αIIb/β3 [36]. These adaptor molecules can accelerate the binding and activation of other signal molecules, first of all, of phospholipase C (PLC) and phosphoinositide 3-kinase (PI3K) that, finally, increases $[Ca^{2+}]_i$ and activates protein kinase C (PKC) [34].

Receptors and ligands of platelet activation during thrombogenesis

Phases of response	Ligands, agonists, substrates	Major receptors
Initiation		
Attachment and adhesion of platelets	Von Willebrand factor	GP Ib/IX/V
	Collagen	α2β1, GP VI
	Fibrinogen/ fibrin	αIIbβ3
	Fibronectin	α5β1
	Laminin	α6β1
Propagation		
Platelet activation	Thrombin	PAR_1, PAR_4, GP Ibα
	ADP	$P2Y_1$, $P2Y_{12}$
	Thromboxane A_2	TR
	Epinephrine	α_{2A}
	Fibrinogen	αIIbβ3
Aggregation	Von Willebrand factor	
	Fibronectin	α5β1, αIIbβ3
Stabilization		
	P-Selectin	PSGL-1
	CD40-ligand	αIIbβ3
Inhibition		
	Prostacyclin (PGI_2)	IP
	Nitric oxide (NO)	
	ADPase (CD39)	

The platelet adhesion through GP Ib or αIIb/β3 to VWF immobilized on the subendothelial collagen seems to be associated with generation of various calcium signals, which cooperatively regulate the behavior of platelets [34]. Separate calcium spikes arising on activation of GP Ib induce a reversible activation of the platelet, exposition of integrin αIIb/β3 onto the surface, and an unstable adhesion of the cell to the substrate. The subsequent resistant calcium oscillations associated with activation of αIIb/β3 provide stable adhesion.

Blood prothrombin also interacts with the platelet αIIb/β3 (in both active and inactive form), and this binding accelerates activation of the proenzyme and thrombinogenesis [37].

Collagen receptors. Collagen is an effective substrate for platelet binding to the damaged area of a blood vessel, and it also activates the cells promoting their stable adhe-

sion, activation, and the thrombus growth via two main receptors, GP VI and integrin α2β1 [34]. GP VI plays the crucial role in platelet binding to collagen of damaged vessels [34, 38]. Monoclonal antibodies to GP VI decreased 89% the platelet adhesion to subendothelium of denuded carotid artery in mice [38]. The adhesion and aggregation of platelets were decreased in GP VI-deficient mice. The binding of GP VI can "shift" integrins α2β1 and αIIb/β3 from the low-affinity to high-affinity state, with resulting platelet activation and stable adhesion of the cells to the damaged vascular wall [34]. Collagen binding to the platelet GP VI leads to clustering and subsequent phosphorylation (by ITAM domains) of FcR γ-chains (associated with the receptor) by Srk kinases Fyn and Lyn [38]. These kinases activate Syk, which in turn phosphorylates adaptor proteins LAT and SLP-76. Finally, the signal cascade results in phosphorylation and activation of PLCγ2, increase in $[Ca^{2+}]_i$, activation of protein kinase C, and promotion of the effective activation of platelets [38].

Platelet adhesion to collagen mediated through integrin α2β1 also stimulates intracellular signaling, which leads to activation of Srk kinases, PLCγ2, increase in $[Ca^{2+}]_i$, and significantly contributes to production of a stable thrombus [39].

The initial stage of thrombogenesis is subdivided into several phases: *initiation*, which includes platelet attachment to subendothelium and their adhesion; *propagation,* which includes platelet activation and aggregation under the influence of specific agonists; and phases of *stabilization* and *inhibition* [14]. The process occurs due to existence or exposition onto the platelet surface of receptors, which bind agonists, ligands, and substrates (table).

Stabilization of platelet aggregates. Stabilization of platelet aggregates and their binding to blood cells and vascular wall cells are significantly contributed by the CD40-ligand (CD40L) (CD154, gp39), which is a transmembrane protein of the II type related to tumor necrosis factor (TNFα). This factor is expressed on immune system cells activated during inflammation (activated CD4+ T cells, basophiles, and mast cells) and on activated platelets [40]. The inducible CD40L of platelets binds to the CD40 receptor (type I transmembrane receptor of the receptor superfamily TNF) on endothelial cells and also on monocytes, macrophages, and smooth muscle cells (SMC) [41]. The CD40/CD40L interaction plays an important role in inflammation and atherothrombosis, which depend on platelet activation. The proinflammatory activity of CD40L is manifested on platelets and other cells by stimulation of expression of chemokines (chemoattractant protein 1 of monocytes (MCP-1)), interleukins (IL-6, IL-8), proinflammatory adhesive molecules (vascular cell adhesive molecules (VCAM-1)), intracellular adhesive molecules (ICAM-1, CD54), and P- and E-selectins; the procoagulant activity of CD40L is manifested by triggering the expression of tissue factor, which is the major inducer of blood coagulation, and by suppressing the expression of thrombomodulin, which is a thrombin cofactor in activation of the protein C anticoagulant system [40, 42, 43]. On binding of CD40L to the CD40 receptor, these proteins are expressed due to induction of intracellular signaling which results in activation of the transcriptional factor NFκB and its translocation into the nucleus. The interaction of CD40 expressed by endothelial cells with CD40L exposed on activated platelets stimulates the synthesis of a powerful proinflammatory mediator platelet activation factor (PAF), which induces platelet aggregation with leukocytes and also contributes to remodeling of vessels, stimulating neoangiogenesis [44].

Platelet activation is associated with splitting of a soluble three-dimensional fragment of the CD40-ligand (sCD40L), but the proteinase responsible for release of the ligand is unknown [44]. The production of sCD40L is stimulated by a positive feedback mechanism. The binding of sCD40L to the receptor CD40, which is constitutively expressed by platelets results in proteolysis of cell-bound CD40L and the further production of sCD40L [45]. The interaction of sCD40L with endothelial cells stimulates the cell proliferation by a VEGF-dependent mechanism [40]. Recently, sCD40L has been shown to promote blood coagulation by two mechanisms: induction (similarly to sP-selectin) of the tissue factor expression on monocytes [43] and activation of platelets as agonist of integrin αIIb/β3 [40]. A motif Lys-Gly-Asp (KGD) specific for adhesive proteins has been detected in the CD40L structure. The sCD40L binding to integrin αIIb/β3 activates platelets at high shear stress and stabilizes arterial thrombi [40].

Using mutation in the KGD sequence (replacement D117E) of the sCD40L structure, Asp117 was shown to play a crucial role in the activation of receptor functions of integrin αIIb/β3 [46]. Unlike antibodies to CD40, integrilin (an antagonist of αIIb/β3) inhibited the ligand binding to the receptor and the platelet aggregation. The binding of the sCD40 ligand KGD domain to αIIb/β3 switches on the outside-in intracellular signaling resulting in phosphorylation of Tyr759 in the cytoplasmic domain of the integrin β3-chain [46].

Levels of CD40L on platelets and of sCD40L in the blood flow were increased in patients with myocardial infarction, angina pectoris, and other cardiovascular diseases [41].

Adhesive proteins provide for the binding and spreading of leukocytes, their rolling, and their further transmigration across endothelium. Binding to the ligand, CD40 induces the inflammatory response independently of cytokines.

Thus, at the early stage of inflammatory response adhesive molecules, such as P-selectin and von Willebrand factor, are exposed on the surface of activated endothelium, platelets are activated, and tissue factor is

exposed, which induces blood coagulation and thrombinogenesis. Thrombin produced in the inflammation area and the endothelium-released PAF rapidly (within seconds) induce platelet activation and secretion [47].

CD40L is expressed on the surface of activated platelets, and their CD40-mediated adhesion to endothelial cells induces the expression of chemokines, cytokines, adhesive molecules, and tissue factor and inhibits the expression of thrombomodulin, thus determining the procoagulant status of endothelium during inflammation. Obviously, platelets play a key role during acute and chronic inflammation owing to exposition of molecules with proinflammatory functions and interaction with leukocytes and endothelium [48, 49].

Platelet aggregation with leukocytes. Platelet activation during inflammation and expression of adhesive proteins, P-selectin (CD62P) and integrins, leads to their aggregation with leukocytes and release of contents of intracellular granules [50]. Platelet–leukocyte aggregates are held by fibrinogen bridges between the platelet integrin αIIb/β3 and the leukocyte β_2-integrin Mac-1 (αM/β2). It has been also shown that leukocyte binding with platelet is provided by interaction of the P201-K217 sequence of domain I in the structure of αM (Mac-1 subunit of leukocyte) with GP Ibα receptor of platelets [51].

P-Selectin expressed by activated platelets interacts with its ligand PSGL-1 on both monocytes and microparticles released from monocytes [52]. In addition to PSGL-1, these microparticles also carry tissue factor, which initiates blood coagulation. Binding of leukocytic microparticles with activated platelets in the region of thrombogenesis confirms the involvement of selectins in blood coagulation, in addition to their known role in leukocyte migration [52].

It has been recently established that in platelets (nucleus-free cells) proteins (including a proinflammatory mediator cytokine IL1β) are synthesized in "secretomas" from preformed (constitutive) mRNAs [53, 54]. Together with other factors, activated platelets secret a transforming growth factor (cytokine TGFβ), which, in particular, inhibits apoptosis of leukocytes and stimulates them for releasing proinflammatory mediator cytokines [55]. Apoptosis of leukocytes is necessary to decrease toxicity of activated cells because apoptotic cells are phagocytized by macrophages without releasing proinflammatory mediators.

Inflammation is actively contributed by chemokines secreted by platelets. RANTES (regulated upon activation normal T-cell expressed and secreted) (CCL5) forms "bridges" between monocytes and endothelium activated during inflammation, platelet factor 4 (PF4, CXCL4) accelerates macrophage differentiation, and MIP1α (macrophage inflammatory protein-1α, CCL3) is a powerful mediator of virus-induced inflammation [49]. Platelets seem to be among the first cells accumulated in the area of lesion and release from their "secretomas" initiators of inflammation, which bind leukocytes, activate the cells, and stimulate the growth and repair of blood vessels [48].

During the development of acute inflammation (in myocardial reperfusion) proinflammatory mediators (including cytokines (IL1β) and tumor necrosis factor (TNFα)) released by activated monocytes activate the endothelium and stimulate the further expression of inducible adhesive molecules (E-selectin, ICAM, VCAM-1), chemokines, and tissue factor, which induces blood coagulation [56]. Platelet–platelet and platelet–leukocyte aggregates produce a surface, providing activation of the blood coagulation and inflammation and release inducers of these processes and microparticles carrying these inducers.

Blood coagulation in inflammation. Exposition of tissue factor on damaged endothelium and monocytes is a key event in the blood coagulation and thrombogenesis in inflammation. Tissue factor binds factor VII and triggers activation of proenzymes (factors of blood coagulation) into serine proteinases of narrow specificity. The coagulation cascade leads to generation of thrombin, which converts blood fibrinogen to fibrin and activates the system of positive and negative feedbacks.

Tissue factor triggers the blood coagulation mechanism. Tissue factor (CD142) belongs to the superfamily of class II cytokine receptors, which are significantly homologous to interferon receptors [57], and has a single polypeptide chain consisting of an extracellular part (219 amino acid residues), a hydrophobic transmembrane region (29 residues), and the C-terminal intracellular "tail" (21 residues). The extracellular part of the tissue factor molecule consists of two domains similar to type III domains of fibronectin. The anchoring of tissue factor on the cell membrane is essential for high affinity binding of factors VII/VIIa, maintaining proteolytic activity of factor VIIa and initiating blood coagulation. Tissue factor has been found in blood vessel adventitium, in the lipid cover of atherosclerotic plaque, disruption of which results in release of tissue factor into blood. This factor is constitutively expressed on fibroblasts and SMC and exposed on leukocytes and endothelial cells in response to inflammatory stimuli [58].

In circulating blood, tissue factor can be in microparticles, released upon cell activation, and inactive and/or in a very low concentration in α-granules of platelets [59, 60]. Tissue factor is likely to be transferred from platelet microparticles (which also carry P-selectin and a specific platelet antigen, CD42a) onto leukocytes in the course of P-selectin-dependent reaction [61]. When the microparticles are concentrated in the area of vascular lesion, tissue factor triggers blood coagulation and activates cells. Adhesive interactions of platelets and microparticles (carrying the tissue factor) with neutrophils and monocytes are responsible for the function of tissue factor in blood as a stimulator of thrombogenesis,

especially in myocardial infarction, acute stroke, pulmonary artery thromboembolism, and sepsis [59]. Unfavorable clinical results of attempts to use integrin αIIb/β3 antagonists seem to be caused by increased expression of tissue factor (and also of P-selectin and CD40L manifesting proinflammatory features) [62, 63].

Tissue factor is found in the blood of patients with myocardial infarction, anti-phospholipid syndrome, sepsis, etc. not only in microparticles and aggregations, but also in a soluble form [64]. The soluble tissue factor (without the transmembrane domain and with a unique C-terminal peptide) is generated by alternative splicing (asHTF). The asHTF form circulates in a patients' blood and displays its procoagulant activity only on binding with phospholipids or incorporation into a thrombus, this promoting growth of the latter.

Tissue factor acts as a cofactor and receptor of serine proteinase (factor VIIa) and proenzyme (factor VII). Factor VIIa complexed with tissue factor (TF/VIIa) triggers the cascade of proenzyme activation into active proteinases (see figure). TF/VIIa activates receptors PAR on fibroblasts and on epithelial and endothelial cells [57, 65, 66].

Production of hemostatic serine proteinases. Factor VII is a vitamin K-dependent single-chained glycosylated multidomain protein of 406 amino acid residues that contains an N-terminal γ-carboxyglutamine domain (Gla-domain), a hydrophobic stack, two domains similar to epidermal growth factor (EGF-domains), and a catalytic (proteinase) domain homologous to trypsin and chymotrypsin in amino acid sequence [57]. The domain structure of the protein molecule and organization of the factor VII gene are like those of other vitamin K-dependent factors—IX, X, protein C (PC), and protein S (PS).

In human blood there is about 1% of factor VIIa with an extremely weak activity, which increases by six orders of magnitude upon binding to tissue factor. Factor VII is converted to active form as a result of cleavage of the peptide bond Arg152–Ile153.

The X-ray analysis of the crystal structure of the complex of the soluble tissue factor (deprived of the transmembrane and cytoplasmic domains) with factor VIIa inhibited at the active site has revealed that the enzyme bound to the membrane through the Gla-domain has elongated contacts with the tissue factor [57]. Tissue factor is reminiscent of a pivot incorporated into phospholipid vesicles of the membrane, with the factor VIIa molecule wound around it. The main sites of factor VIIa contact with tissue factor are located in the first EGF domain and in the catalytic domain, and additional contact points are located in the aromatic stack, Gla-domain, and the second EGF domain. Thus, the TF/VIIa complex is oriented perpendicularly to the membrane surface.

Tissue factor changes the location and orientation of the factor VIIa active site relatively to the membrane: the distance from the enzyme active site to the membrane decreases from 83 to 75 Å, which contributes to an effective cleavage of peptide bonds of the substrates (first of all of factor X) that are also bound to the membrane through Gla-domains [57]. Tissue factor is an allosteric activator of factor VIIa, changing its catalytic properties.

The TF/VIIa complex binds factor X and converts it into factor Xa. The factor Xa molecule retains the association with the complex, and this triple complex activates PAR1 and PAR2 on the cells [65]. The attachment of factor Xa to the TF/VIIa complex intensified fivefold the cell response as compared to the effect of factor Xa alone [67]. The modeling of the TF/VIIa/Xa complex structure by docking (analysis of complementarity of tertiary structures) and site-directed mutagenesis has revealed that factor Xa has an elongated conformation similar to that of the factor VIIa structure in the TF/VIIa complex [68]. The interaction of factor Xa with TF/VIIa involves all domains of factor Xa, including the amino acid residues Glu51 and Asn57 of the first EGF domain, Asp92 and Asp95 of the second EGF domain, and Asp185a, Lys186, and Lys134 of the catalytic domain of factor Xa, as well as the N-end of this domain, which is oriented to provide both activation by factor Xa of the TF-bound factor VII and activation by factor VIIa of the proenzyme, factor X [68].

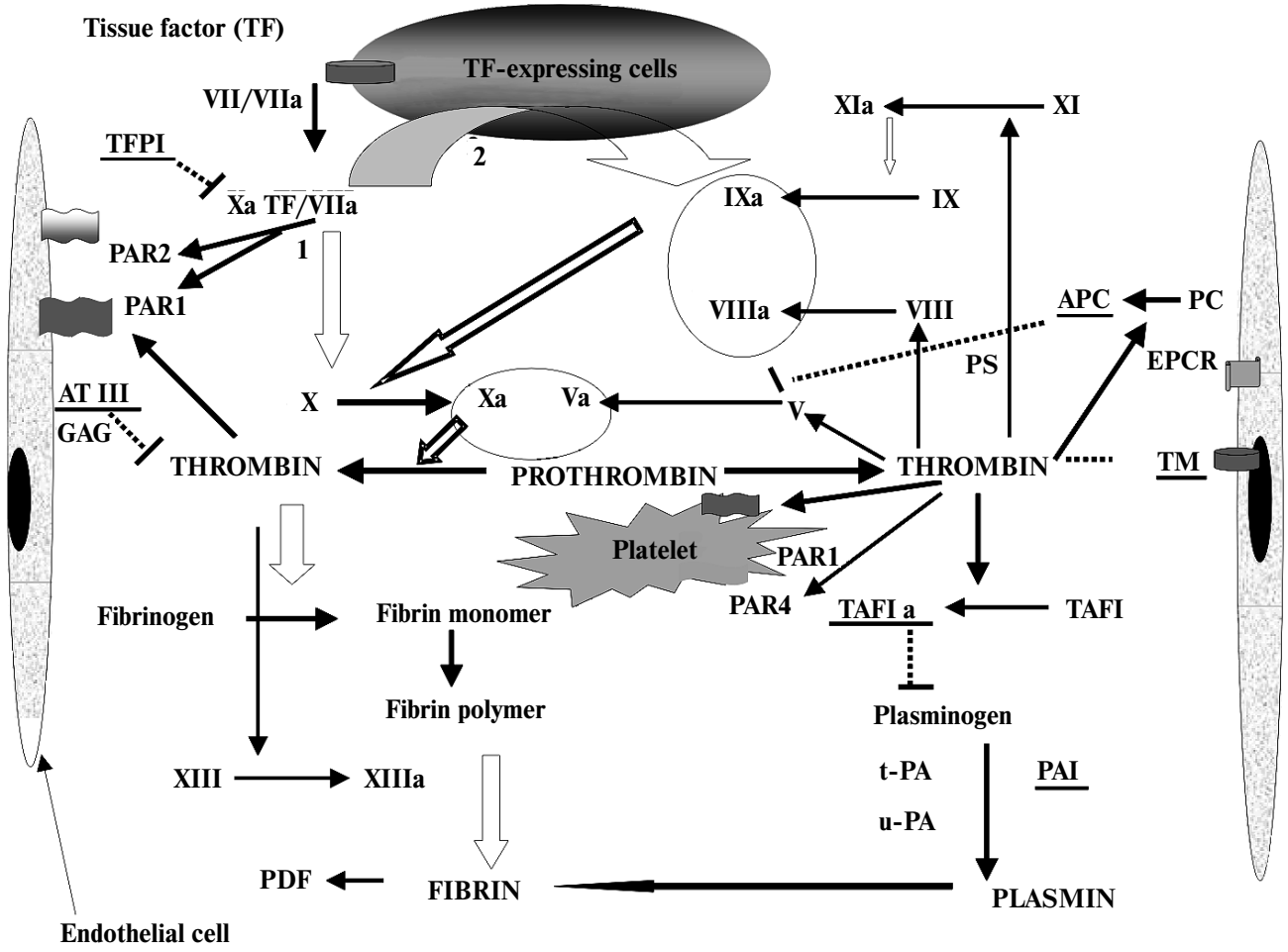
The external pathway of blood coagulation induced by tissue factor has now been shown to be the major mechanism of thrombinogenesis in hemostasis and thrombosis [69-71].

The TF/VIIa complex has extremely narrow substrate specificity and activates by limited proteolysis only factors X and IX. TF/VIIa forms with factor X a triple complex, TF/VIIa/X, and cleaves it at the rate $2 \cdot 10^7$-fold higher than free factor VIIa [71]. Generation of trace picomolar concentrations of factor Xa in the early stage of initiation of blood coagulation (phase 1) stimulates the production of nanomolar concentrations of thrombin sufficient for activating the platelet PAR1, binding thrombomodulin (TM), and activating coagulation cofactors, factors V and VIII, into factors Va and VIIIa (see figure). Activation of both factors is necessary to produce effective coagulation catalysts bound to cell membrane phospholipids and Ca^{2+}, such as complexes: tennase (factor IXa with cofactor VIIIa) and prothrombinase (factor Xa with cofactor Va) at the second stage of coagulation, the propagation stage (phase 2). The early stages of blood coagulation are mainly regulated by tissue factor pathway inhibitor (TFPI), which inhibits activities of factor Xa and of complex TF/VIIa.

During the propagation stage, TF/VIIa activates factor IX on the membrane of activated platelets, microparticles, and endothelium and generates tennase, which activates factor X (see figure). Then prothrombinase is produced, which cleaves prothrombin to thrombin. Both complexes are bound to the cell membrane phospholipids and Ca^{2+} through Gla-domains of factors IXa and Xa and

are 10^5-10^9 times more active than the serine proteinases that are their constituents. The activation of factor X with tennase is 50-100 times more effective than its activation with complex TF/VIIa [71].

Thrombin is generated during the cleavage with prothrombinase of two peptide bonds (Arg320–Ile and Arg271–Tre) in prothrombin. Thrombin consists of a light A-chain (49 amino acid residues) and a heavy catalytic B-chain (259 amino acid residues) bound by a disulfide bond. On the surface of the B-chain of the thrombin molecule, the anion-binding exosite I, responsible for enzyme recognition of specific substrates and receptors (fibrinogen, factor V, thrombomodulin, PAR1, GP Ibα, etc.), and also the anion-binding exosite II,



Generation and functions of serine proteinases of the blood coagulation system. Tissue factor (TF) is a cofactor and receptor of serine proteinase (factor VIIa) and proenzyme (factor VII). Factor VIIa complexed with tissue factor (TF/VIIa) triggers the cascade of proenzyme activation into active proteinases. During phase 1 (initiation of blood coagulation), complex TF/VIIa binds and activates trace concentrations of factor X into factor Xa. Xa stimulates the appearance of nanomolar concentrations of thrombin sufficient for activating platelet PAR1 and factors V and VIII (cofactors Xa and IXa) into the active forms (factors Va and VIIIa) and binding with endothelial thrombomodulin (TM). During phase 2 (propagation), TF/VIIa activates factor IX on the membrane of activated platelets, microparticles, and endothelium. Tennase (complex of factors IXa and VIIIa, Ca^{2+}, and phospholipids) activates factor X into the form Xa. Prothrombinase (complex of factors Xa and Va, Ca^{2+}, and phospholipids) converts prothrombin into thrombin. Thrombin activates factor XI into XIa, which converts factor IX into IXa. Thrombin converts fibrinogen into fibrin monomer by splitting fibrinopeptides A and B and activates factor XIII into XIIIa (Ca^{2+}-dependent transglutaminase), which stabilizes fibrin polymers by covalent bonds. PAR1, PAR4, and TM are thrombin receptors. PAR2 and PAR1 are receptors of TF/VIIa and the triple complex TF/VIIa/Xa. EPCR (endothelial protein C receptor) and PAR1 are receptors of APC (activated protein C), and TFPI is the tissue factor pathway inhibitor and the main regulator of initial stages of blood coagulation; it inhibits activities of Xa and TF/VIIa. ATIII (antithrombin III) is an inhibitor (in complex with endothelial glycosaminoglycans (GAG)) of thrombin and factor Xa. Thrombin in the complex with TM activates TAFI (thrombin-activated fibrinolysis inhibitor) into carboxypeptidase B (TAFIa), which inhibits fibrinolysis by removing the C-terminal lysine residues from fibrin. These residues open after early stages of fibrin cleavage with plasmin and are required for binding plasminogen, which is a proenzyme of plasmin, and plasminogen activators t-PA (tissue plasminogen activator) and u-PA (urokinase). PAI inhibits type 1 plasminogen activators. PDF are products of fibrin/fibrinogen degradation

responsible for binding of thrombin to GP Ibα, heparin, and glycosaminoglycans, are produced [5, 25, 26].

Thrombin, which is a polyfunctional, but narrow-specificity serine proteinase of the trypsin family, converts fibrinogen to fibrin by hydrolysis of only four Arg–Gly bonds. Thrombin also regulates the positive feedback, activating blood coagulation factors V, VIII, XI, and XIII, and the negative feedback, activating (in the complex with thrombomodulin) protein C, which limits thrombinogenesis by cleavage of factors Va and VIIIa [5] (see figure).

Systemic inflammation is associated with hypercoagulation caused by disorders in regulation of thrombinogenesis due to decrease in activity of the protein C anticoagulant system, inhibition of activity of procoagulant proteinase inhibitors (especially TFPI and antithrombin III (ATIII)), decrease in activity of the fibrinolysis system, and increase in the procoagulant activity of endothelium and blood cells as a result of the induced expression of tissue factor [59, 72, 73].

TFPI belongs to the family of three-headed Kunitz inhibitors and consists of a tandem of three Kunitz domains (K1, K2, and K3). The first domain (K1) binds factor VIIa, immobilized on tissue factor, and the second domain (K2) binds factor Xa. The third domain has no inhibitory activity, but in its structure, especially in the positively charged C-terminal sequence of the TFPI molecule, a binding site of endothelial proteoglycans, such as syndecans and glypicans, is located [74, 75]. TFPI specifically binds to thrombospondin-1 of platelets.

For the most part, TFPI is constitutively synthesized by vascular endothelium cells and also by monocytes, fibroblasts, SMC, and cardiomyocytes, and is exposed onto the cell surface and secreted by endothelium and blood cells. In the presence of IL1β and endotoxin the expression of TFPI mRNA is significantly increased in cardiomyocytes, TFPI is exposed on cardiomyocytes isolated from patients with myocardial infarction, myocarditis, and sarcoidosis, and the concentration of TFPI is increased in the blood plasma of patients with myocardial infarction; these data suggest that TFPI plays a role in heart defense during inflammation [75, 76].

When tissue factor appears on the surface of activated cells, TFPI inhibits the blood coagulation cascade as follows. TFPI complexes with factor Xa through the second domain and through the C-end of the third domain binds to negatively charged proteoglycans of the cell membrane. The local concentration of the inhibitor increases, and it binds factor VIIa and inactivates complex TF/VIIa [74, 75]. TFPI inhibits the ability of TF/VIIa for activating factor X and cells through the proteinase-activated receptor 2 (PAR2) (see figure).

During inflammation, matrix metalloproteinases (MMP) are released from pathogen-activated leukocytes. MMP are zinc-dependent endopeptidases that hydrolyze components of the extracellular matrix and can cleave TFPI [75, 76]. MMP 7 (matrilysin) and MMP 9 (gelatinase) can hydrolyze only TFPI, not affecting TF/VIIa and Xa [77]. Serine proteinases, such as thrombin, plasmin, and factor Xa, can cleave TFPI to partially degraded "truncated" states with a decreased affinity for endothelial proteoglycans [75]. Disorders in the structure of TFPI and decrease in its activity promote an uncontrolled activation of the blood coagulation cascade.

The anticoagulant potential of blood is decreased when the protein C system does not work [72]. This failure is caused in inflammation by suppressed expression and exposition on the surface of thrombomodulin, which is a high-affinity receptor of thrombin and changes the enzyme specificity. Thrombin bound to endothelial thrombomodulin activates cleavage of the proenzyme, protein C, to a serine proteinase (the active protein C (APC)), and splits cofactors Va and VIIIa that are required for thrombinogenesis (see figure). In the absence or deficiency of the natural modulator thrombomodulin, thrombin cannot convert protein C into APC and control its own production.

Blood coagulation leads to appearance of proteinases, such as factors VIIa and Xa and thrombin, which in nanomolar concentrations can activate cells of blood and of connective and nervous tissues and be involved in the regulation of inflammation, angiogenesis, and wound healing [5, 6, 7, 10]. Thrombin induces expression on the surface of damaged endothelium of growth factors, adhesion proteins, and selectins that are responsible for blood cell attachment to endothelium [5, 6, 9]. Thrombin is involved in cell migration, aggregation, and proliferation, and changes permeability of the endothelial monolayer [78]. Thrombin mediates inflammation by activating endothelial cells and adhesion of monocytes, increasing the permeability of endothelium [7, 8, 10] and stimulating secretion of mediators (histamine, cytokines) by mast cells [5, 6].

However, thrombin in very low concentrations can regulate inflammation because it releases from endothelial cells nitric oxide, which inhibits the adhesion of monocytes to endothelium and platelet aggregation [5, 6]. Peptide agonist of the thrombin receptor PAR1 (PAR1AP) inhibits the release of inflammatory mediators from activated mast cells due to stimulation of the nitric oxide production [5]. Thrombin in low concentrations is a growth factor, and, by activating its receptors, it stimulates wound healing via accelerating proliferation of endothelial cells, fibroblasts, and epithelial cells [5, 6]. Through receptors on the cells (PARs, thrombomodulin, GP Ib), thrombin connects the proteolytic systems involved in inflammation.

Proinflammatory functions of active factors of blood coagulation. Activation of cells with TF/VIIa/Xa complex precedes the development of the coagulation cascade. Tissue factor binds factors VIIa and Xa and thus stimulates the signal transmission into the cell: it increases the

concentration of intracellular Ca^{2+}, stimulates phosphorylation of mitogen-activated protein kinases (MAP kinases) (p44/42 (Erk-1/2), p38, Jnk), activation of the Src kinase family (cSrc, Lyn, Yes) and phosphatidylinositol 3-kinase, and induction of transcriptional factors, such as members of the NFκB family, the activated protein AP-1/β-Zip, Sp1, and *Egr-1* (early growth response), which is usually induced by cytokines and growth factors. Finally, this results in synthesis of adhesion proteins responsible for adhesion and migration of cells, proinflammatory cytokines, growth factors, etc. [79-82].

Signaling by complexes TF/VIIa and TF/VIIa/Xa needs active proteolytic enzymes, and this suggests an involvement of proteinase-activated receptors (PAR) in realization of the cell response [66]. PAR2, which is a receptor of the nonspecific proteinase of trypsin, mast cell tryptase, and membrane type serine proteinase-1, is considered as the receptor of factors TF/VIIa and Xa. PAR2 cannot be activated with thrombin, in contrast to PAR1, PAR3, and PAR4 [7, 65]. TF/VIIa and Xa have been recently shown to activate cells through both PAR2 and PAR1 [65, 83].

Thrombin activation of PAR1 on cells involved in blood coagulation, inflammation, and wound healing has been considered in a number of reviews [5-7, 9-12, 84]. But the role of other PARs and hemostatic proteinases in coupling of blood coagulation and inflammation remains unclear. However, PAR1 is supposed to be involved in platelet aggregation, inflammation, embryogenesis, regulation of vascular tone, and tissue repair; PAR2 is supposed to be involved in inflammation, regulation of vascular tone, and nociception; PAR4 seems to be involved in platelet aggregation [7].

The potential physiological role of PARs is determined by their location: PAR1 is expressed on platelets, endothelium, leukocytes (monocytes), SMC, cardiomyocytes, fibroblasts, neurons, and mast and epithelial cells; PAR2 is expressed on the same cells, except platelets, and also on keratinocytes; PAR3 is expressed on platelets and SMC of respiratory tracts, whereas the expression of PAR4 has been found on platelets, megakaryocytes, and monocytes, and this list is constantly being extended [7, 85].

The thrombin-activated PAR1 seems to be a highly specific substrate of thrombin: it has a negatively charged sequence D^{49}KYEPFWEDEEKNES similar to the C-end of hirudin and complementary to the anion-binding exosite 1 in the thrombin molecule [86]. Recognition of this site by thrombin facilitates the cleavage of the N-terminal peptide ESKATNATLDPR^{41}SFLLRN of PAR1 and liberates a new N-terminal peptide SFLLRN, the so-called tethered ligand, which activates the receptor. A negatively charged sequence capable of complementary interaction with the anion-binding exosite 1 of the thrombin molecule has been detected in the PAR3 structure (FEEFP), but it is absent in the structures of PAR2 and PAR4. The receptor seems to be activated by an "intramolecular" mechanism with the tethered ligand, and this is verified by activation of the receptors with synthetic peptides similar to these ligands in PAR2 (SLIGKV) and PAR4 (GYPGQV) (PARs agonist peptides (PARsAP)), although specific efficiency of these peptides is some orders of magnitude lower (1-400 μM). Site-directed mutations in the sites of recognition of thrombin and the cleavable bond result in resistance of the PAR1 receptor to thrombin. Substitution of the site LDPR^{41}/S cleaved with thrombin by the enterokinase-specific sequence (DDDDK/S) accelerates the receptor activation with enterokinase compared to its activation with thrombin [87]. The absence of the key proline residue in the position P_2 of the cleavable bond in the N-terminal peptide (GTNRSSKGR/SLIGKV) of PAR2 makes the receptor resistant to thrombin. However, trypsin can activate PARs degrading PAR2 in low concentrations (<20 nM) and PAR1 in high concentrations (>100 nM); but it also can inactivate PARs by hydrolysis [7]. Nonspecific proteinases, such as trypsin, cathepsin G, plasmin, seem to act as agonists or antagonists of PARs.

Activation of PARs with their ligands stimulates signal transduction into the cell and its activation [5, 9, 10, 88, 89]. Mechanisms have been studied of thrombin-induced activation of PAR1 mediated through the family of regulatory guanine nucleotide-binding G-proteins. These proteins are heterotrimers, consisting of α-subunit (including the nucleotide-binding site and sometimes the site of ADP-ribosylation by a bacterial toxin) and βγ-subunit (a heterodimer anchoring the complex on the membrane). Activation of PAR1 results in its interaction with the α-subunit, substitution of the bound GDP by GTP, dissociation of the heterotrimer, and interaction of the α-subunit (or the βγ-subunit) with the target protein. PAR1 interacts with α-subunits of $G_{12/13}$-, G_q-, and G_i-proteins. The binding of PAR1 to $G_{12/13}$ activates the minor G-protein Rho and is responsible for changes in the shape of platelets and for permeability and migration of endothelial cells [89]. Signals mediated through the G_q-protein result in activation of phospholipase Cβ, start-up of hydrolysis of phosphoinositides, mobilization of calcium, activation of protein kinase C, and finally, in phosphorylation of mitogen-activated protein kinases (MAPK), and activation of receptor tyrosine kinases and other proteins. These processes are responsible for a variety of cell reactions, such as secretion of granules, activation of integrins and aggregation of platelets, and transcriptional responses (including stimulation of proliferation) in endothelial and mesenchymal cells [85, 89]. Association of PAR1 with the G_i-protein sensitive to pertussoid toxin is accompanied by inhibition of adenylate cyclase and promotion of platelet responses. Interaction of PAR1 with Gβγ activates phosphoinositide 3-kinase, which modifies

proteins of the plasma membrane to provide recruiting signal complexes (serine/threonine kinases, nonreceptor tyrosine kinases, etc.) involved in transcriptional responses [89].

Multiple responses on PAR1 activation, as well as existence of several receptors of the PAR family and cofactors of these receptors, provide the polyfunctional effects of thrombin and other hemostatic proteinases, including their proinflammatory functions.

The cleavage on human platelets of PAR4, which has no thrombin-binding site, needs higher concentrations of the enzyme than the activation of PAR1. Effective activation with thrombin of PAR4 in mouse platelets requires a cofactor, and its role is played by PAR3, which has in the structure a region complementary to the anion-binding exosite 1 of the thrombin molecule [88]. This seems to explain the inefficiency of peptide analogs of the agonist PAR3 in activation of the receptor [85, 88]. On human platelets, GP Ibα constitutively expressed by the cells acts as a cofactor of PAR1 [89]. Moreover, the tethered ligand of PAR1 has been shown to activate the neighboring PAR2 through an intramolecular mechanism [90].

Tissue factor acts as a cofactor in the activation of PAR2 receptors of endothelial cells because very low (picomolar) concentrations of factor VIIa in the presence of factor X trigger activation of the cells that express both the tissue factor and PAR2 (under the influence of cytokine TNFα) [65]. About 10 pM factor VIIa can circulate in blood plasma, and this concentration is quite sufficient to initiate in the presence of tissue factor and factor X generation of factor Xa and activation of intracellular signaling via PAR2 in the region of tissue injury, atherosclerotic plaque, or inflammation. The presence of tissue factor on the cell surface is absolutely necessary to provide the factor VIIa induction of the signal transduction into the cell via PAR2 and the subsequent gene expression in various cell types, including fibroblasts, macrophages, and epithelial and endothelial cells [83].

Activating PAR1, factor Xa and thrombin (but not factor VIIa) induce the expression of cytokines (IL8, IL6) and MCP-1 (chemotactic protein 1 of monocytes) in skin fibroblasts [85].

The effector cell proteinase receptor (EPR-1) can be a cofactor of PAR2 on endothelial cells and leukocytes during their activation with factor Xa [91]. Desensitization of PAR2 receptors with trypsin (or PAR2AP) inhibited the cell response induced by factor Xa. The catalytically inactive factor Xa retained the ability for binding to EPR-1 of the cells but did not activate them. However, EPR-1 binding by factor Xa was not required for activating PAR2 on isolated rat aorta [92].

Agonist peptides PAR2AP stimulate the rolling of leukocytes and their adhesion and transmigration in postcapillary venules of rat mesentery, and in PAR2-deficient mice inflammation was retarded [93-95].

Antiinflammatory properties of the protein C system. Anticoagulant functions of the protein C system, which controls blood coagulation, are discussed in some reviews [72, 96-98]. The protein C structure is highly homologous to that of other vitamin K-dependent factors—VII, IX, and X. On binding with endothelial thrombomodulin, thrombin acquires properties of a natural anticoagulant and inhibits its own generation. Thrombin immobilized on thrombomodulin activates protein C but cannot coagulate fibrinogen and activate PAR1 on cells. Thrombin cleaves the peptide bond Arg169–Leu170 in the protein C, releases the negatively charged activator peptide Lys158-Arg169, and produces a narrow-specificity serine proteinase APC. APC specifically cleaves factors Va and VIIIa and thus inhibits the production of thrombin in the presence of the protein S cofactor (a vitamin K-dependent protein) immobilized on platelets and/or endothelium.

Antiinflammatory and antiapoptotic properties of APC. In addition to the anticoagulant activity, APC displays antiinflammatory and antiapoptotic properties. Injection into baboons of low concentrations of thrombin insufficient for activating platelets, but provoking the generation of APC, or injection of APC prevented the death of animals given lethal doses of *E. coli*. This protective mechanism functioned independently of the anticoagulant activity of APC [99]. APC decreases damage to pulmonary vessels and NO-dependent hypotension induced in rats by injection of endotoxin due to inhibition of production of cytokine TNFα [100]. TNFα is known to play a crucial role in development of inflammation, disseminated intravascular coagulation (DIC), and other disorders associated with sepsis [101]. The injection of recombinant APC (Drotrecogin-α) significantly decreased the mortality of patients with severe sepsis [102-105].

The inhibition by APC of TNFα synthesis by human monocytes stimulated with lipopolysaccharide is provided by the ability of the enzyme for blocking activation of transcriptional factors NFκB and AP-1 (activator protein-1) [106]. NFκB is a crucial factor in regulation of production of proinflammatory cytokines TNFα and IL-1β by monocytes [107]. NFκB includes a protein family with a predominant heterodimer consisting of subunits p65 (RelA) and p50 [108]. In unstimulated monocytes, inactive NFκB dimers are bound to the inhibitor IκB (p105, p100, Bcl-3, etc.) and located in the cytoplasm. On stimulation with lipopolysaccharide, IκB proteins are phosphorylated with IκB kinases, ubiquitinated, and degraded with 26S proteasomes. Viruses, bacteria, proinflammatory cytokines (TNFα, IL-1β, IL6), and stress can also release NFκB proteins from the inactive cytoplasmic complex [109]. The released NFκB dimers are translocated into the nucleus and initiate the transcription of genes of cytokines, growth factors, adhesion molecules, cell surface receptors, acute phase proteins, and transcriptional factors.

Stimulation of monocytes with lipopolysaccharide increases the transcriptional activity of AP-1 due to activation of mitogen-activated protein kinases (MAPK), c-Jun N-terminal kinase (JNK), and p38 MAPK, which are involved in the expression of cytokine genes (TNFα) [110]. APC inhibits AP-1 binding to the target and activation of the MAPK-pathway and thus prevents the production of TNFα in lipopolysaccharide-stimulated monocytes [106].

Using microarrays, the recombinant APC has been shown to modulate responses of cultured endothelial cells by changing the expression of genes encoding the proteins responsible for development of inflammation and apoptosis [111]. APC suppresses the genes regulating NFκB and thus decreases its expression and functional activity. APC inhibits TNFα-induced expression of adhesive molecules (VCAM, ICAM, E-selectin) and also modulates genes involved in apoptosis, i.e., displays cytoprotective features [111].

Endothelial protein C receptor (EPCR). Although the fine mechanisms of the antiinflammatory effect of APC are not yet elucidated, the specific endothelial protein C receptor (EPCR) seems to play a significant role in this function of APC. The rate of protein C activation with thrombomodulin-immobilized thrombin is several times increased by EPCR, which binds protein C and its active form APC on the cell surface at K_{dis} ~ 30 nM [72, 96-98]. EPCR is an important regulator of coagulation. A mutation in the EPCR gene (23 bp insertion) was detected in patients with myocardial infarction and deep venous thrombosis, and this mutation resulted in expression of a molecule unable to be exposed onto the cell surface and bind protein C [112].

On binding to EPCR, APC manifests antiinflammatory features. In endotoxin-induced inflammation, APC/EPCR inhibits the release of inflammatory cytokines, especially TNFα [113]. The APC/EPCR complex inhibits adhesion of leukocytes, prevents their infiltration into tissue, and protects against a dramatic decrease in arterial pressure in response to injection of endotoxin.

Inflammatory mediators, endotoxin, and also thrombin increase the expression of EPCR mRNA [114]. A soluble form of EPCR (sEPCR) is released from the activated endothelium by inducible metalloproteinase and can be detected in the plasma [115]. Moreover, the release of sEPCR is induced by thrombin, PAR1AP, proinflammatory cytokines, and toxic oxygen oxides.

On binding to sEPCR, activated protein C loses anticoagulant activity, because sEPCR inhibits APC binding to the phospholipid surface and alters the enzyme specificity [96]. To display anticoagulant activity, APC has to dissociate from EPCR and bind to protein S on the cell surface. The complex APC/protein S binds factor Va (or VIIIa) as a substrate and cleaves it [96].

Studies on crystal structure of sEPCR and of its gene have shown a significant similarity of the receptor molecule and the structure of the α1 and α2 domains of CD1 proteins of the MHP family, most of which are involved in inflammation [97, 116].

The EPCR structure is organized on a β-sheet by two α-helical regions, which form a phospholipid-binding pocket responsible for the EPCR ability for binding the Gla-domain of the protein C/APC [97, 117]. The EPCR structure also includes transmembrane and short cytoplasmic domains. By site-directed mutagenesis, ten amino acid residues have been detected that are significant for binding APC (Arg81, Lys82, Val83, Glu86, Arg87, Phe146, Tyr154, Thr157, Arg158, and Glu160). These residues form a cluster in the proximal end of two α-helical domains of EPCR [118]. Remains of the removed end of the α-helices are not involved in the binding of APC.

The expression of EPCR by leukocytes has been recently shown [119, 120]. APC binding to EPCR of leukocyte seems to explain the inhibition by APC of chemotaxis of leukocytes induced by IL8, C5a, and other inflammatory mediators. Some of these features are caused by ability of the APC/EPCR complex to inhibit the endotoxin-induced translocation of transcriptional factor NFκB into the nucleus and decrease the level of NFκB mRNA [96, 121]. The antiinflammatory features of APC bound with EPCR seem to underlie its ability to decrease the lethality of sepsis in patients and experimental animals.

To manifest these features, APC has to retain proteolytic activity. However, the specific degradable receptor of the enzyme was unknown for a long time. APC/EPCR has been recently shown to activate cells, mainly endothelial cells, by splitting PAR1 [65, 122, 123]. PAR1 and EPCR are suggested to be located in cooperation in functional microdomains on endothelial cells [65]. Inhibition of PAR1 completely suppresses the APC-induced phosphorylation of MAP kinases. Using microarray, the genes determining the APC-increased expression were shown to be induced by agonist peptides of PAR1 (PAR1AP). APC and PAR1AP caused a selective induction of chemoattractant protein-1 of monocytes (MCP-1), which displayed regulatory features in the endotoxin-induced sepsis in animals [123]. The EPCR-dependent activation of PAR1 with activated protein C seems to cause the enzyme ability to decrease the lethality of sepsis [123].

However, it is still unclear how antiinflammatory properties of APC/EPCR and proinflammatory properties of thrombin can be mediated through the thrombin receptor PAR1 and also what role is played by receptor cooperation in the specificity of biological responses.

It has been recently established that both the endothelial receptor EPCR and the thrombin receptor PAR1 are required for the cytoprotective and antiinflammatory effects of activated protein C [124, 125].

Activated protein C prevents the hypoxia-caused apoptosis of cultured endothelial cells of brain vessels

[124]. The antiapoptotic effect of APC in ischemic damage to the brain is mediated by inhibition of transcription of p53 (the suppressor tumor protein) through the EPCR-dependent activation of PAR1 and decrease in the proapoptotic ratio Bax/Bcl-2 and the activity of caspase-3. During ischemic shock in mice caused by occlusion of the medial cerebral arteries, APC (in doses insufficient for preventing thrombotic occlusion and deposition of fibrin) decreased the zone of infarction and edema. The protective effect of APC was absent when the activation of PAR1 was inhibited with specific antibodies or the expression of EPCR was decreased by gene engineering approaches. It seems that transmission of the APC–EPCR–PAR1 signal protects the brain vessels against the hypoxia-caused damage [124, 126].

Similarly to activated protein C, its receptor EPCR can display antiinflammatory activity, as it suppresses the entrance of calcium ions into the cell and NFκB translocation into the nucleus and also modulates gene expression by inhibition of synthesis of inflammatory mediators [96, 113]. Soluble EPCR binds the elastase-like enzyme proteinase 3. This complex interacts with the adhesive integrin CD11b/CD18 of the surface of activated leukocytes and thus prevents their binding to endothelium and infiltration into tissues [127]. Appearance of free receptor APC seems to be a component of protective antiinflammatory mechanisms.

Antiinflammatory functions of the thrombin receptor — thrombomodulin. Thrombomodulin, which is the thrombin receptor on endothelium, displays both direct and indirect antiinflammatory effects. Thrombomodulin is a glycosylated transmembrane protein of type I, which consists of an N-terminal large globular domain slightly homogenous to the C-type of animal lectins and is followed by the structures: an elongated sequence of six domains similar to epidermal growth factor (EGF); a region rich with serine/threonine, containing sites of posttranslational N- and O-glycosylation; a transmembrane domain; and a short cytoplasmic domain. The fifth and sixth EGF domains bind thrombin, and the fourth EGF domain is necessary for activating protein C with thrombin. And thrombin immobilized on thrombomodulin accelerates 100-fold the activation of protein C [128].

Thrombomodulin can regulate inflammation independently of thrombin and protein C [129]. In mice with a mutant thrombomodulin gene responsible for synthesis of a lectin-like and domain-deprived protein, the ability for generating APC was unchanged, but the antiinflammatory activity was inhibited. In these mice, the synthesis of proinflammatory cytokines TNFα and IL-1β and mortality in endotoxin-induced sepsis were increased. The expression of the adhesion molecule ICAM-1 on endothelial cells was increased, and the ICAM-1-mediated adhesion of leukocytes was also increased through activation of the MAP-kinase pathway. These inflammatory reactions were inhibited by recombinant thrombomodulin, which contained only the N-terminal lectin-like domain. Moreover, this recombinant thrombomodulin protected cultured endothelial cells subjected to apoptosis in medium depleted of serum by modulating the NFκB pathway [129].

The indirect antiinflammatory effect of thrombomodulin is manifested by acceleration of the thrombin-induced activation of procarbopeptidase into the enzyme carboxypeptidase B, which inhibits the thrombin-activated fibrinolysis inhibitor (TAFIa) (this inhibitor is also called carboxypeptidase U and carboxypeptidase R). This inhibitor splits the C-terminal lysine residues from fibrin and thus makes it resistant to fibrinolysis [130, 131] (see figure). Moreover, TAFIa regulates inflammation because it hydrolyzes bradykinin, anaphylotoxin C5a of the complement system, and component C3a [132, 133]. TAFIa inactivates C5a, splitting the C-terminal arginine and thus depriving the complement C5a of its physiological function [130]. TAFIa modulates the proinflammatory properties of osteopontin, which is degraded by thrombin [132].

The high concentration of thrombomodulin in microcirculation sufficient for the fast activation of TAFI and subsequent suppression of the complement system, together with the antiinflammatory and cytoprotective effects of APC and the lectin domain of thrombomodulin, determines the defense of endothelium against damage in inflammation. Moreover, when binding thrombin through the anion-binding exosite I, thrombomodulin inhibits the ability of thrombin to cleave specific substrates (fibrinogen, factor V) and to activate PAR1 due to suppression of the procoagulant and proinflammatory properties of thrombin [5].

The binding of thrombin with thrombomodulin dramatically increases the rate of its neutralization with plasma inhibitors, especially with antithrombin III, which interacts with the carbohydrate (chondroitin sulfate) chain of thrombomodulin. Thrombomodulin displays an antiproliferative effect inhibiting the mitogenic activity of thrombin mediated by PAR1 of endothelial cells modulating the PAR1-dependent MAP-kinase pathway of intracellular signaling [134].

Thrombomodulin expression on endothelium is decreased in diseases associated with local inflammation (in the region of atherosclerotic injury), such as coronary atherosclerosis [135] or systemic inflammation in sepsis [136]. The recovery of anticoagulant and antiinflammatory functions of thrombomodulin by injection of the recombinant soluble thrombomodulin seems promising as a therapeutic measure in these situations.

Thus, under conditions of endothelium damage by inflammatory stimuli, the endothelium phenotype is converted into prothrombotic and proinflammatory, and adhesive molecules (von Willebrand factor, P-selectin) and subendothelial substrates, in particular collagen, are exposed, which interact with their receptors (von Willebrand factor with integrins GP Ib/IX/V and αIIbβ3;

P-selectin with ligand PSGL-1; collagen with α2/β1, GPVI) and activate platelets and leukocytes and adhere them to the endothelium. The activated platelets expose onto the surface the receptor of von Willebrand factor and fibrinogen, integrin αIIbβ3, and also P-selectin and CD40 ligand, which provide the generation of stable platelet–platelet and platelet–leukocyte aggregates. The binding of CD40 ligand of platelets with the CD40 receptor of endothelial cells intensifies inflammation and coagulation by stimulating the pathway leading to activation of transcriptional factor NFκB. This factor is translocated into the nucleus and induces the expression of genes and synthesis of adhesion molecules, selectins, chemokines, growth factors, inflammatory mediators (cytokines, PAF, etc.), and tissue factor. NFκB also suppresses the expression of thrombomodulin, which acts as a cofactor of thrombin in the activation of the protein C anticoagulant system. Proinflammatory and procoagulant microparticles carrying receptors of adhesion molecules, tissue factor, etc. are shed off from the surface of activated cells. During platelet activation, soluble forms of P-selectin and CD40 ligand are split and interact with their targets, promote inflammation and coagulation, and stabilize thrombi. The detachment of thrombomodulin and suppression of its expression in endothelium decreases the activity of the protein C system, which becomes unable to adequately ensure regulation of blood coagulation. Tissue factor expression initiates the generation of procoagulant hemostatic serine proteinases: factors VIIa and Xa, which in complexes TF/VIIa and TF/VIIa/Xa bind to type PAR2 receptors activated by proteinases, and also thrombin, which binds with PAR1 (see figure). The activation of PARs by proteinases stimulates inflammation by inducing the activation of factor NFκB and synthesis of proinflammatory and procoagulant factors. In addition, on interacting with endothelial thrombomodulin as the receptor, thrombin changes its specificity, acquires features of an anticoagulant, and converts blood protein C into the enzyme APC (active protein C), which prevents thrombinogenesis. APC interacts with the receptor EPCR (APC/EPCR), activates PAR1, and manifests proinflammatory and antiapoptotic properties modulating the factor NFκB pathway. Thrombomodulin and its recombinant form carrying the N-terminal lectin-like domain also manifest proinflammatory and antiapoptotic effects.

Studies of preparations that inhibit the reactions as follows are very promising: platelet–platelet and platelet–leukocyte aggregation dependent on integrins and selectins; CD40-dependent stabilization of thrombi; activation of the NFκB pathway, proinflammatory effects of thrombin, TF/VIIa, and TF/VIIa/Xa (with antagonists of specific PARs); initiation of the pathway of tissue factor/factor VIIa (TFPI and inactivated factor VIIa (VIIai)). Studies of preparations providing selective activation of anticoagulant, antiinflammatory, and cytoprotective functions of APC and thrombomodulin are also promising.

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