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Blood coagulation-dependent inflammation. Coagulation-dependent inflammation and inflammation-dependent thrombosis

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1. ABSTRACT

Hemostatic serine proteinases –thrombin, Factor VIIa , Factor Xa , play the central role in blood coagulation and thrombosis. Activation of coagulation and generation of active proteinases is initiated by tissue factor (TF) that is expressed by cells of the innate immune system and endothelial cells after tissue damage and cell activation induced by trauma, infection, hypoxia and other cell injury. Coagulation and inflammation are the essential part of the defensive host response . These processes have several connecting points account for the associate and/or the interaction between coagulation and inflammation pathways. The first link between these processes is endothelium, which after damage expresses the adhesive proteins (vWF,P-selectin),inductors and receptors, involved in both coagulation and inflammation. The second link is platelets, which stored in and after activation release proteins with procoagulant and proinflammatory properties. The third link is the serine proteinases, which produced for blood coagulation and activate via its specifical receptors - PARs (proteinase activated receptors) the cells of both coagulation and inflammation system thereby controlling these prosesses. The generation of these proteinases is initiated by tissue factor (TF) which triggers blood coagulation at sites of tissue injury by selective binding of FVIIa. TF/VIIa complexes with substrate – FX that is activated to FXa. TF/VIIa/Xa can activate both the inflammatory responses of endothelial and other cells and also blood coagulation through stimulation of thrombin generation. This review summarizes the latest data on the blood coagulation activation that include generation of active surface for coagulation, generation of hemostatic serine proteinases and its role as signalling molecules that via PARs and other receptors involved in regulation and control of the interaction of blood coagulation and inflammation and illustrates the potential for therapeutic intervention.

2. INTRODUCTION

Inflammation and blood coagulation are the part of the innate host defence mechanism on vascular injury induced by mechanical trauma (such as balloon angioplasty and stenting), infection or many other reasons. Not only the cell of the innate immune system but also activated endothelial cells and platelets are actively involved in acute and chronic inflammation: they release of proinflammatory mediators, expose adhesion molecules and receptors, proteases and its inhibitors, clotting factors and associated proteins, and recruit leukocytes. There is a growing body of evidence to the integration between these systems whereby inflammation leads not only to stimulation of coagulation, but coagulation also considerably activates inflammation (1-4). The extensive cross-talk of inflammation and coagulation contributes the high impact in the initiation and progress of cardiovascular diseases (atherosclerosis and acute coronary diseases as unstable angina, myocardial infarction, etc.), peripheral vascular disease and also to the major complications of sepsis (as disseminated intravascular coagulation (DIC) and multiple organ failure). Both local and systemic vascular inflammation is associated with atherogenesis and increased thrombogenesis (5). Now the concept that atherosclerosis is developed in response to vascular injury as a chronic inflammatory disease, which acquires an acute form on disruption of plaque, is well accepted. Inflammation –induced coagulation has been extensively reviewed (5,6-8). However, the molecular connections between coagulation and following inflammation have only recently gained attention and are become to better understood in the last years (1,3, 9-13). The first of all a link between these processes is mediated by endothelium, which after damage expresses the adhesive proteins (vWF,P-selectin (PS), ICAM-1,VCAM-1 etc),inductor and receptors, involved in both coagulation and inflammation,as tissue factor (TF), thrombomodulin (TM), proteinase activated receptors (PARs),effector cell protease receptors (EPR-1),endothelial protein C receptor (EPCR),urokinase-type plasminogen activator receptor (u-PAR)etc . Then platelets, which stored in and after activation release proteins with procoagulant and proinflammatory properties,as adhesive proteins,growth factors,cytokines, clotting factors,receptors,are included in this link. The third (and least known) link is the serine proteinases of blood coagulation, which via its specifical receptors – PARs, EPCR, EPR-1,TF might activate the cells of both coagulation and inflammation system thereby controlling these prosesses and connection with them angiogenesis and tissue repair.

The inflammation- or injury -induced expression of tissue factor, receptor of factor VII/VIIa, 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. These proteinases bind to cell surface receptors (including proteinase activated receptors (PARs)) to perform its key roles in coupling of blood coagulation with inflammation and wound healing . Many physiological and pathophysiological events as vasoregulation, angiogenesis, gastrointestinal disorders, airway hyperreactivity, hyperalgesia, neuroinflammatory and pulmonary diseases, also as atherosclerosis and other cardiovascular diseases may be regulate by PAR agonists (4,14-20). In spite of the fact that PARs involvement in blood coagulation and inflammation has been considered in a number of reviews (12,14-24), the role PARs and another cell receptors in coupling of blood coagulation and inflammation remains unclear. Understanding of the role of platelets and serine proteinases in the integration of blood coagulation and inflammation seems fundamental for development of new approaches for prevention thrombotic complications of cardiovascular and other diseases.

In the present review attention will be mainly concentrated on the blood coagulation activation that include generation of active surface for coagulation, generation of hemostatic serine proteinases and its role as signalling molecules that through specific receptors on cells (including the family of proteinase-activated receptors (PAR))involved in regulation inflammation and tissue repair and control of the interaction of blood coagulation and inflammation and illustrates the potential for therapeutic intervention.

3. PLATELETS AND ENDOTHELIUM ARE THE SOURCE OF PROTEINS FOR COAGULATION AND INFLAMMATION

3.1. Endothelial cell adhesion molecules

In a normal artery endothelium creates a non-thrombogenic surface that functions as a selectively permeable barrier, which control transport of solutions into the arterial wall. Endothelium plays a key role in response to vascular injury included regulation of leukocyte adhesion, platelet activation and adhesion, and blood coagulation/ thrombosis. To promotion these functions endothelium expresses and responds to multiple active substances, including cell adhesive molecules, cytokines, chemokines (8,25,26). Mechanical or infection injury of a vessel results in the propagation of inflammatory and coagulation events. The first of all cell adhesion molecules (CAM) are expressed onto the surface of activated endothelial cells that attach leukocytes and platelets. Adhesive proteins provide for the binding and spreading of leukocytes, their rolling, and their further transmigration across endothelium.

There are three major classes of CAM : selectins, the immunoglobulin superfamily CAM, integrins. Some integrins can be receptors of CAM and endothelial adhesion molecule , von Willebrand factor (VWF), that binds platelets.Weibel–Palade bodies of endothelial cells release platelet (P)-selectin (CD62P, GMP140) responsible for adhesion of leukocytes, their rolling, and for stabilization of platelet aggregations (27-29). Von Willebrand factor, which provides for platelet mobilization, also is stored and release from Weibel–Palade bodies . The lectin-containing N-terminal domain of P-selectin binds the P-selectin glycoprotein ligand (PSGL-

1) on monocytes, neutrophils and platelets (30,31).Platelet integrin GP Ibα is another ligand of P-selectin and may also support platelet rolling on activated endothelium. P-Selectin binding to PSGL-1 stimulates the release by leukocytes of microparticles, which carry tissue factor, an inducer of blood coagulation (32). P-Selectin is splited as a soluble P-selectin (sP-selectin) that stimulates expression and exposition on the monocyte surface of tissue factor (33).Microparticles (together with sP-selectin) determine the high procoagulant status of blood in inflammation (32). E-selectin (CD62E) is not constitutively expressed on endothelial cells. It is transcribed and exposed on the cell surface and than bind PSGL-1 in response to mechanical injury and inflammatory mediators as IL-1β, TNF-α (tumor necrosis factor- α), bacterial toxins and oxidants (8). P- and E-selectin mediate rolling of activated and nonactivated platelets on activated endothelium similar to mechanism of leukocyte rolling (13). The immunoglobulin superfamily CAM includes ICAM-1 (intracellular adhesion molecule-1, CD54)),ICAM-2 and VCAM-1 (vascular cell adhesion molecule-1), which are expressed by many cell types including endothelial (constitutively) and smooth musle cells (SMC). In response to vascular injury these cells exhibit upregulated expression of ICAM-1 and VCAM-1 (8), accounted for leukocyte adhesion.

Adhesion protein VWF is a marker of endothelial injury. It is synthesized in endothelial cells, in platelet precursor megakaryocytes, and is deposited in Weibel–Palade bodies of endothelial cells and α-granules of platelets (34). The structure of VWF is a dimer of two subunits joined by the C-ends, which form a block for building the large multimer. The VWF molecule contains domains responsible for binding blood coagulation factor VIII and platelet integrins such as glycoprotein transmembrane complexes GP Ib/IX/V and integrin αIIb3 (GPIIb/IIIa), and also collagen. VWF binds subendothelial collagens and after immobilization attaches to platelet via receptor –integrin GP Ib (34). VWF may be a factor of pathogenesis of acute thrombotic occlusion of stenosed arteries (26). P-Selectin could serve as an anchor site for the ultra large VWF multimers on the surface of activated endothelium, to facilitate their cleavage by the disintegrin and metalloproteinase with thrombospondin motif-13 (ADAMTS-13) (35,36).

3.2. Pletelet glycoproteins for cell adhesion, aggreration and inflammation

Platelet adhesion to activated endothelium and leukocytes one of the key events of thrombogenesis and inflammation (13). Platelets not only aghere at site of vascular injury, aggregate and form the procoagulant surface for blood coagulation, but also they release substances that promote inflammation and tissue repair (13,37,38). GP Ib –integrin, 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 (30). GP Ib/V/IX is a multifunctional receptor that able to interact with VWF, P-selectin, the leukocytic integrin Mac-1 (α M/β2, CD11b/CD18), a high-molecular-weight kininogen, and also thrombin , the factors XI and XII. 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 (30).GP Ibα is similar to PSGL-1 in structure and specifically binds P-selectin through the domain of sulfated tyrosines (39). GP Ib α is a receptor for β_2-integrin of leukocytes,Mac-1 (CD11b/CD18), and is responsible for platelet binding to neutrophils (40). The N-terminal domain of GP Ibα is responsible for the binding I (insert) Mac-1 domain that homologous to the A1 domain of VWF. The cytoplasmic domain of GP Ib α 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 domain of GP Ibα by P-selectin, VWF, and Mac-1 is responsible for platelet binding to endothelium, leukocytes, matrix, and to one another (30).

VWF initiates platelet adhesion to the subendothelial matrix at the sites of vascular injury even under high flow conditions through interaction with GP Ib and activates cells. However, the mechanism of the signal transmission mediated through GP Ib remains unclear. According to some data, the signal is transmitted via the cytoplasmic domain of GP Ib α bound to the actin-binding protein and adaptor proteins 14-3-3 ζ (30) and results in increase in the concentration of intracellular calcium ($[Ca^{2+}]_i$), release of a platelet aggregation agonist, ADP and generation of another platelet activator, thromboxane A_2 (TXA_2) ((41, 42). According to other data, the complex GP Ib/IX/V is associated with two ITAM (immunoreceptor tyrosine-based activating motifs)-containing proteins (FcγRIIa and FcR γ), and the binding with the agonist leads to phosphorylation of the ITAM tyrosine with the family of Syk kinases, activation of phospholipase C γ (PLC), protein kinase C (PKC), and increase in$[Ca^{2+}]_i$ (30,43,44). These reactions result in the activation and exposition on the cell surface of another VWF receptor– integrin αIIb/β3 .

Integrins as αIIb/β3 and the immunoglobulin-like GPVI- collagen receptor, account for rapid stabilization of adhesive platelets and following its activation for promotion of thrombus formation (13). Integrin α IIb/ β 3 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 (44). Integrin αIIb/β3 ensures the binding of cells with fibrinogen VWF and other adhesive proteins: fibronectin, thrombospondin, vitronectin, laminin, collagen, etc. The majority of αIIb/β3 integrins on activate platelets are occupied by fibrinogen. The interaction of αIIb/β3 with adhesive proteins promote platelet activation and as a consequence promote a shift to a high -affinity state of the major platelet integrin αIIb/β3 which mediates platelet aggregation (13). Platelet activation with inducers released, exposed, or produced in the area of vascular injury (ADP, thrombin, collagen, platelet activating factor (PAF), etc.) also lead to activation of integrin α IIb/ β 3 to a high - affinity state. Conformation of the integrin αIIb/β3 extracellular domain is controlled by interaction of the β3-

chain cytoplasmic domain with intracellular mediators and cytoskeleton proteins (44). Integrins are receptors of adhesive molecules, and their agonists regulate cell functions. Although mechanisms of increase in the concentration of intracellular calcium on activation of integrin α IIb/ 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/FcRγ-chain) in this process (45). It has been shown that Src kinase Fyn, which phosphorylates ITAM tyrosine, also phosphorylates the C-end of the β3 -chain cytoplasmic domain of α IIb/ β3 (46). 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 α IIb/ β3 . 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) (45).

The platelet adhesion through GP Ib or α IIb/ 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 (45). Separate calcium spikes arising on activation of GP Ib induce a reversible activation of the platelet, exposition of integrin α IIb/ β3 onto the surface, and an unstable adhesion of the cell to the substrate. The subsequent resistant calcium oscillations associated with activation of α IIb/ β3 provide stable adhesion. Collagen binding to receptors, GP VI and integrin α2β1, activates the cells promoting their stable adhesion, activation, and the thrombus growth (45). GP VI plays the crucial role in platelet binding to collagen to the injury site of a blood vessel. Monoclonal antibodies to GP VI decreased 89% the platelet adhesion to subendothelium of denuded carotid artery in mice (47). The adhesion and aggregation of platelets were decreased in GP VI-deficient mice. The shift of α2β1 and α IIb/β3 integrins from the low-affinity to high-affinity state after GP VI binding result in the platelet activation and stable cell adhesion to the damaged vascular wall (45). Binding of collagen on 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 (47). 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 (47). 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 (48).

During adhesion to endothelium platelets are activating and release proinflammatory cytokine CD40-ligand (CD40L) (CD154, gp39) that can stimulate the endothelium, stabilize of platelet aggregates, their binding to blood cells and vascular wall cells and promote the stable thrombus formation. CD40L is expressed on activated platelets and also on immune system cells activated during inflammation (activated CD4+ T cells, basophiles, and mast cells) (49). This factor is transmembrane protein of the II type related to tumor necrosis factor (TNFα). 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) (50). The CD40/CD40L interaction plays an important role in inflammation and atherothrombosis, which depend on platelet activation. Binding to the ligand, CD40 induces the inflammatory response independently of cytokines. 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 (49-51). 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 activating factor (PAF), which induces platelet aggregation with leukocytes and also contributes to remodeling of vessels, stimulating neoangiogenesis (52).

Platelet activation is associated with split of a soluble three-dimensional fragment of the CD40-ligand (sCD40L) (52). 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. The interaction of sCD40L with endothelial cells stimulates the cell proliferation by a VEGF-dependent mechanism. sCD40L has been shown to promote blood coagulation by two mechanisms: induction (similarly to sP-selectin) of the tissue factor expression on monocytes and activation of platelets as agonist of integrin αIIb/β3. In the CD40L structure the specific for adhesive proteins motif Lys-Gly-Asp (KGD) has been detected. The binding of the 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 (53).The sCD40L binding to integrin αIIb/β3 activates platelets at high shear stress and stabilizes arterial thrombi (49). By means of mutation in the KGD sequence (replacement D117E) of the sCD40L structure, a crucial role of Asp117 was shown in the activation of integrin αIIb/β3 (53). An antagonist of αIIb/β3 – integrilin, inhibited the ligand binding to the receptor and the platelet aggregation. Levels of CD40L on platelets and sCD40L in the blood flow were increased in patients with myocardial infarction, angina pectoris, and other cardiovascular diseases (50,54).

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. During acute and chronic inflammation platelets expose the molecules with proinflammatory functions and interact with leukocytes and endothelium.

Platelet activation during inflammation and expression of adhesive proteins, P-selectin and integrins, leads to their aggregation with leukocytes and release of contents of intracellular granules . P-Selectin expressed by activated platelets interacts with its ligand PSGL-1 on both monocytes and microparticles released from monocytes (55). 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 (56,55).

Activated platelets also express and secrete the chemokines: RANTES (CCL5) (regulated upon activation normal T-cell expressed and secreted) that forms bridges between monocytes and endothelium activated during inflammation, platelet factor 4 (PF4, CXCL4) that accelerates macrophage differentiation, and MIP1α (macrophage inflammatory protein-1α, CCL3) is a powerful mediator of virus-induced inflammation (56). 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 (54,57).

For acute inflammation (in myocardial reperfusion) proinflammatory mediators 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 . 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.

4. TISSUE FACTOR AS TRIGGER OF BLOOD COAGULATION

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. 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). Formation of arterial thrombi are mainly contributed by platelets, which adhere to the damaged vascular wall or 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 (see Part 3). Platelet adhesion to activated endothelial cells and leukocytes is one of the first events in thrombogenesis, inflammation, and atherogenesis.

4.1. TF structure and functions

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/VIIa and triggers on 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 (CD142,TF) belongs to the class II cytokine receptor superfamily, which are significantly homologous to interferon and IL-10 receptors (58). TF 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 TF on the cell membrane is essential for high affinity binding of factors VII/VIIa, maintaining proteolytic activity of FVIIa and initiating blood coagulation. TF expression levels vary in different organs.Brain, lung, heart, kidney, uterus, testis, skin, and placenta express high levels of TF, whereas liver, spleen, skeletal muscle and thymus express low levels of TF (59). Low TF mice (containing the transgene as the only source of TF ($mTF^{-/-}$ /hTF^{+})) exhibited reduced survival compared with wild-type mice and excessive bleeding in the event of vascular injury (60). Autopsies revealed spontaneous hemorrhages in the lung, brain and intestine that indicated on impaired haemostasis (59). Tissue factor has been found in blood vessel adventitium, in the lipid cover of atherosclerotic plaque, disruption of which results in exposition of tissue factor into blood. TF is constitutively expressed by a variety of perivascular (and epithelial) cells, including fibroblasts and SMC.TF also is expressed by leukocytes and endothelial cells in response to inflammatory stimuli (61). Inflammatory mediators directly induce rapid up regulation of TF in cells of the innate immune system (the first of all monocytes), whereas endothelial cell expression of TF appears to be late, dependent on secondary mediators and restricted to selective vascular beds (2).

TF was found in circulating blood on microparticles, released upon cell activation, and inactive and/or in a very low concentration in α-granules of platelets (62,63). 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 (64). 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 TF) with neutrophils and monocytes are responsible for the function of TF in blood as a stimulator of thrombogenesis, especially in myocardial infarction, acute stroke, pulmonary artery thromboembolism, and sepsis (62). Some unfavorable clinical results of attempts to use integrin αIIb/β3 antagonists seem to be caused by increased expression of TF (and also of P-selectin and CD40L manifesting proinflammatory features) (65,66).

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 (67). The soluble TF (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.TF acts as a cofactor and the high affinity receptor for serine proteinase (factor VIIa) and proenzyme (factor VII). FVIIa complexed with TF (TF/VIIa) triggers the cascade of proenzyme activation into active proteinases (Figure 1). TF/VIIa activates receptors PAR on fibroblasts and on epithelial and endothelial cells (see below) (58,68,69).

4.2. Generation 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 (58). The domain structure of the protein molecule and organization of FVII gene are like those of other vitamin K-dependent factors—FIX, FX, protein C (PC), and protein S (PS). In human blood there is about 1% of FVIIa with an extremely weak activity, which increases by six orders of magnitude upon binding to TF. FVII 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 (sTF,deprived of the transmembrane and cytoplasmic domains) with FVIIa (the 3D-structure of TF/FVIIa complex) inhibited at the active site has revealed that the enzyme bound to the membrane through the Gla-domain has elongated contacts with TF (58). All four domains of FVIIa (Gla,EGF1,EGF2 and protease) are in contact with TF and FVII protease domain bonds the same epitope on TF as shown with sTF mutants (70). TF is liked to the anchor that had been incorporated into phospholipid vesicles of the membrane, and FVIIa molecule turn off around it. The main sites of FVIIa contact with tissue factor are located in the first EGF domain and in the protease 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. TF factor changes the location and orientation of FVIIa active site relatively to the membrane: the distance from the enzyme active site to the membrane decreases from 83 to 75 Å, that contribute to an effective cleavage of peptide bonds of the substrates (first of all of FX) that are also bound to the membrane through Gla-domains (58). TF is the allosterical activator of FVIIa, changing its catalytic properties.

The TF/VIIa complex binds FX and converts it into FXa. FXa molecule retains the association with the complex, and this triple complex activates PAR1 and PAR2 on the cells (68). The attachment of FXa to the TF/VIIa complex intensified fivefold the cell response as compared to the effect of FXa alone (71). The modeling of the TF/VIIa/Xa complex structure by docking (analysis of complementarity of tertiary structures) and site-directed mutagenesis has revealed that FXa has an elongated conformation similar to that of FVIIa structure in the TF/VIIa complex (72). The interaction of FXa with TF/VIIa involves all domains of FXa, 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 FXa, as well as the N-end of this domain, which is oriented to provide both activation by FXa of the TF-bound factor VII and activation by FVIIa of the proenzyme, factor X (72). But the EGF1 domains of FXa is the most important for the activation of FVII-TF (73).

The extrinsic pathway of blood coagulation induced by TF has now been shown to be the major mechanism of thrombinogenesis in hemostasis and thrombosis (73,74). The TF/VIIa complex has the narrow substrate specificity and activates by limited proteolysis only factors X and IX. TF/VIIa forms with FX a TF/VIIa/X ternary complex and cleaves it at the rate $2 \cdot 10^7$-fold higher than free FVIIa (74). Generation of trace picomolar concentrations of FXa in the early stage of initiation of blood coagulation (phase 1) stimulates the production of picomolar concentrations of thrombin which partially activates the platelets, binds thrombomodulin (TM), and cleaves the coagulation cofactors factors V and VIII into active cofactors - Va and VIIIa (Figure 1). During this phase fento- to picomolar amounts of FVIIa, factor IXa and factor XIa are also generated. Activation of cofactors 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) and to promote 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 FXa and of complex TF/VIIa.

During the propagation phase, TF/VIIa activates factor IX on the membrane of activated platelets, microparticles, and endothelium and generates tennase, which activates factor X (Figure 1). 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 FX with

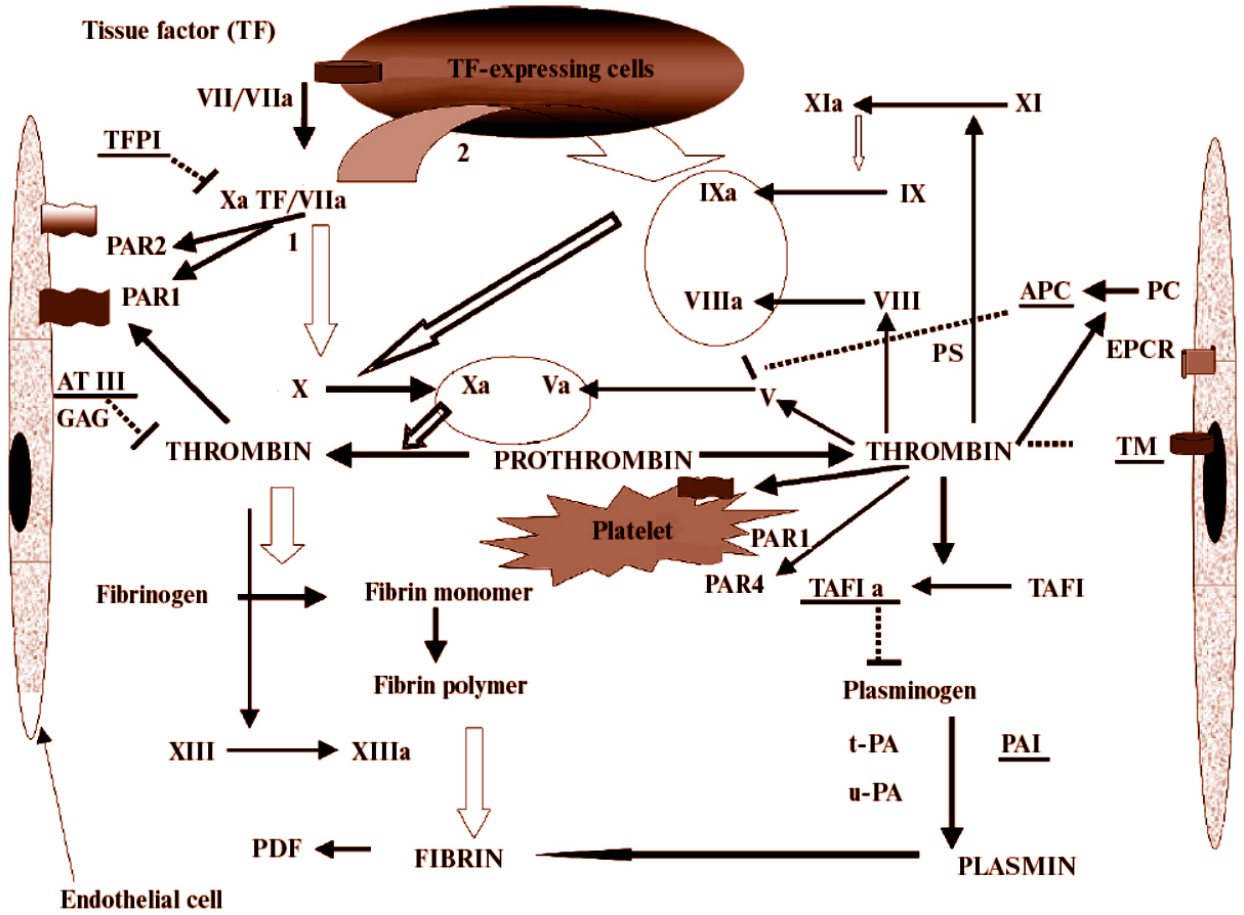

Figure 1. Generation and functions of serine proteinases of the blood coagulation system. Tissue factor (TF) is a cofactor and receptor of serine proteinase (factor VIIa) and proenzyme (factor VII). Factor VIIa in complex with tissue factor (TF/VIIa) triggers the cascade of proenzyme activation into 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 (factor Xase, 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 (12).

tennase is 50-100 times more effective than its activation with complex TF/VIIa (75).

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-light 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, responsible for binding of thrombin to GP Ibα, heparin, and glycosaminoglycans, are produced (16,76,77). Thrombin 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 (12) (Figure 1).

4.3. Regulation of blood coagulation and inflammation by TFPI

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 (10, 62, 78). Mainly, 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.The concentration of TFPI is increased in the blood plasma of patients with myocardial infarction. TFPI has been suggested to play a role in heart defense during inflammation (78,79).

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 heparan sulfate proteoglycans, such as syndecans and glypicans, is located. This full-length form of TFPI (TFPIα) also is located within lipid raft/caveole microdomains on the surface of endothelium through a glycosyl phosphatidylinositol (GPI) anchor. Other truncated form of TFPI (TFPIβ) is directly attached to GPI-anchor (79-81).Thrombin induces the release and the redistribution of TFPI on the endothelial cell surface. TFPI expression is upregulated by heparin, grows factors, shear stress and downregulated by lysophosphatidylcholine (79). TFPI specifically binds to thrombospondin-1 of platelets. When TF appears on the surface of activated cells, TFPI inhibits the blood coagulation cascade as follows. TFPI first complexes (through the second domain) with and inactivates factor Xa and binds (through the C-end of the third domain) to negatively charged proteoglycans of the cell membrane. The resulting complex, TFPI-FXa , inactivates TF/VIIa (79,80). The EGF1 domains of FXa are important for the formation of TF /FVIIa- TFPI -FXa- complex (73). TFPI inhibits the ability of TF/VIIa for activating factor X and also ,possibly, cells through the proteinase -activated receptor 2 (PAR2) (Figure 1). Degradation of TFPI may enhance tissue factor activity. During inflammation, matrix metalloproteinases (MMP) are released from pathogen-activated leukocytes. MMP are zinc-dependent endopeptidases , that hydrolyzes components of the extracellular matrix and can cleave TFPI (79,80). MMP 7 (matrilysin) and MMP 9 (gelatinase) can hydrolyze only TFPI, not affecting TF/VIIa and Xa (82). Serine proteinases, such as thrombin, plasmin, and factor Xa, can cleave TFPI to partially degraded form with a decreased affinity for endothelial proteoglycans (79). Disorders in the structure of TFPI and decrease in its activity promote an uncontrolled activation of the blood coagulation cascade.

Thrombin generation on the surface of monocytes activated during inflammation may be mediated by an additional mechanism that is independent of TF and FVII. The activating monocytes express the αMβ2 integrin (CD11b/CD18), which has a binding site for FX. The activation of adhered FX by cathepsin G (that is released from activated leukocytes) into FXa is responsible for the conversion of prothrombin to thrombin (83,84). The alternative, independent of TF pathway of FVII activation by means of a novel plasma serine protease named as FVII activating protease (FSAP) was found (85,86). FSAP was relealed to be both a potent pro-coagulant and pro-fibrinilytic agent, as activator of single-chain plasminogen activator-pro-urokinase (scuPA) (87). Moreover FSAP inhibits PDGF (platelet-derived growth factor) BB- mediated cell proliferation and migration of vascular smooth muscle cells (88).

Blood coagulation leads to appearance of proteinases, such as FVIIa and FXa and thrombin, that not only interact with coagulation protein zymogens but also with specific cell receptors to induce signaling pathways which mediate inflammatory responses (1,4,12, 14- 18, 21).

5. FUNCTIONS OF HEMOSTATIC SERINE PROTEINASES IN INFLAMMATION

5.1. Thrombin signalling pathways in inflammation

There is increasing evidence that thrombin contributes to the inflammatory and reparative processes after tissue injury,is involved not only in coagulation but also in activation of many cell types, including endothelial cells, platelets, monocytes and leukocytes, SMC, fibroblasts, mast cells etc. (4,12,14-18,21,22,89-91). Thrombin mediates inflammation by activating endothelial cells and adhesion of monocytes, increasing the permeability of the endothelial monolayer. Thrombin induces expression on the surface of activated endothelium of growth factors, adhesion proteins, and selectins that are responsible for blood cell attachment to endothelium. Thrombin is involved in cell migration, aggregation, proliferation, and in stimulation of mediators (histamine, cytokines, PAF,NO etc) release by mast cells (14,89-93). Evidences were accumulated for an essential role of thrombin not only in thrombogenesis and inflammation,

but also in the central and peripheral nervous systems (CNS and PNS) injury, neuroinflammation (4,14,15,18).

Thrombin as well as other proteinases can act as signal molecules that specifically regulate cells by cleaving and activating members of a novel family of seven-domain, transmembrane G - protein-coupled receptors named as proteinase-activated receptors (PARs) on cells (22,94-96). Four members of the PAR family have been cloned and identified: PAR1, PAR3 and PAR4 as the thrombin (as primary activating protease) receptor, PAR2 as receptor of trypsin, mast cell tryptase, TF/FVIIa, FXa and also MT-SP1 (membrane-type serine protease1), proteinase-3, acrosien and other.The secondary activating proteases for PAR1 can be FXa, APC, granzyme A, trypsin and plasmin (18).Activation is initiated by cleavage of the N terminus of the receptor by a serine protease resulting in the generation of new so-called the tethered ligand that interacts with the receptor within extracellular loop2 (95,96).

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 (ABE1) in the thrombin molecule (95). Recognition of this site by thrombin accerelates the cleavage of the N-terminal peptide ESKATNATLDPR^{41}SFLLRN of PAR1 and liberates a new N-terminal peptide SFLLRN, the tethered ligand, which activates the receptor. A negatively charged sequence capable of complementary interaction with the ABE1 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 cleave 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 (90). The absence of the key proline residue in the position P_2 of the cleave 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 (17). Nonspecific proteinases, such as trypsin, cathepsin G, plasmin,elastase, proteinase-3 seem to act as agonists or antagonists of PARs (18). Activation of PARs with their ligands stimulates signal transduction into the cell and its activation (14,17,18,22,96,97). 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- and βγ-subunits. 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. Thus, thrombin signaling in the endothelium that induced the alteration in vascular permeability, vasomotor tone, cell shape, leukocyte trafficking, migration, DNA synthesis, angiogenesis and connective tissue remodelling, was mediated via the predominant thrombin receptor PAR1 that couped to a α-subunits of the G-proteins ($G_{\alpha i,}$ $G_{\alpha o,}$ $G_{\alpha q,}$ $G_{\alpha 12/13}$) (89,90, 98). A number of signal intermediates, that include mitogen-activated protein kinase (MAPK),protein kinase C (PKC), phosphatidyl inositol 3-kinase (PI3K) and Akt, activated by these G-proteins.

Thrombin signaling results to posttranscriptional events that include calcium influx, cytoskelet reorganization, mobilization platelet activating factor, matrix metalloproteinases (MMP) and also exocytosis of Weibel-Palade bodies and release of presynthesized P-selectin (CD62P) and VWF,that account for promotion of leukocyte trafficking and platelet adhesion (see higher) (96,98,99). Through PAR1 activation , thrombin also stimulates the synthesis and release of various cytokines (IL-6, IL-8), growth factors (platelet-derived growth factors (PDGF)-A,-B), angiopoietin-2 and induction of ICAM-1, VCAM-1,E-selectin, monocyte chemoattractant protein-1 (MCP-1), cyclooxygenase –2 (COX-2),decay accelerating factor (DAF), early growth-response factor (Egr)-1 and plasminogen activator inhibitor-1 (PAI-1), TF, EPCR (Endothelial protein C receptor),etc (96-101).

Thrombin-mediated expression these factors in endothelial cells was shown to involve the transcriptional networks from next factors:NF- κB,AP1,Egr-1,SRF, GATA-2 and DbpB (Y-box binding transcription factor).The last is implicated in thrombin-mediated regulation of next genes: PDGF-B, TF and EPCR. The effect of thrombin
on GATA-2 transcriptional activity is mediated by P13K-, PKC-ζ –dependent signaling and lead to induction of VCAM-1 (98). Thrombin can also induce the expression Egr-1, a member of the immediate-early gene family that induces c-fos,c-jun and early growth-response genes. Egr-1 has been shown to induce a number of downstream genes, including TF, PDGF-A, PDGF-B, TGF-β, TNF-α, u-PA,MMP.

Nuclear factor NF- κB is also a proinflammatory, rapidly inducible transcription factor that upregulated several target genes, including ICAM-1 and VCAM-1. NFκB includes a protein family with a predominant heterodimer, consisting of subunits p65 (RelA) and p50 (102). In unstimulated cells, inactive NFκB dimers are bound to the inhibitor IκB (p105, p100, Bcl-3, etc.) and located in the cytoplasm. On stimulation with proinflammatory mediators (lipopolysaccharide, cytokines (TNF-α, IL-1β, IL6), IκB proteins are phosphorylated with IκB kinases, ubiquitinated, and degraded with 26S proteasomes. Viruses, bacteria and stress can also release NFκB proteins from the inactive cytoplasmic complex (103). 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. NFκB-dependent pathway participates in thrombin-induced MIF (macrophage migration inhibitory factor) expression via PAR1 in human dermal microvascular endothelial cells and umbilical vein endothelial cells. MIF, a proinflammatory cytokine, that is expressed in various cells, including macrophages, and is thought to be involved in cell proliferation and initiation of wound-healing (104).

The binding of PAR1 to $G_{12/13}$ activates the minor G-protein Rho and is responsible for permeability and migration of endothelial cells and for changes in the shape of platelets (96). 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 (96). 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 G βγ 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 (96).

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 (105). This seems to explain the inefficiency of peptide analogs of the agonist PAR3 in activation of the receptor (14,96).

On human platelets, GP Ibα constitutively expressed by the cells acts as a cofactor of PAR1 (96). The major thrombin-binding site, which contains sulfated tyrosines, is located in the N-terminal ligand-binding domain of GP Ib, 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 (76,77). On binding to GP Ibα by two sites of its recognition center (16), thrombin acts as a bridge between GP Ibα receptors of the same platelet or adjacent cells. Some models have been proposed to explain the contribution of GP Ib/IX/V in thrombin-induced platelet activation. One model proposes that thrombin activates platelets through PAR1 and promotes the platelet aggregation inducing multimerization of GP Ibα receptors and increasing the efficiency of the PAR1 cleavage (44). Other model suggests that binding of thrombin to GP Ib/IX/V directly initiates signaling for platelet activation through activation of Rho kinase p160ROCK and phosphorylation of MEK1. This pathway was found to occur in thrombin-stimulated platelets upon desensitization of both PAR1 and PAR4 (106). Recently next pathway was found that selective stimulation of either PAR1 or PAR4, but not GP Ib/IX/V, by thrombin generates signal for RAP1B activation (107). The small GTPase RAP1 is involved in the regulation of integrin activation and cell adhesion. By contrast, in PAR-1- and PAR-4-desensitized platelets, thrombin failed to activate Rap1B. However, in contrast to thrombin, PAR1-AP and PAR4-AP –promoted activation of Rap1B require the support of secreted ADP. But the ability of thrombin to stimulate of Rap1B in an ADP-independent manner is conferred by co-stimulation of PAR1 and PAR4 as well as by binding to GP Ib/IX/V (107).

Thus, thrombin activates human platelets through three different membrane receptors, the protease-activated receptors PAR-1 and PAR-4 and the glycoprotein Ib (GPIb)-IX-V complex. Moreover, the tethered ligand of PAR1 has been shown can activate the neighboring PAR2 through an intramolecular mechanism, when direct PAR1 signaling was blocked with a small molecular antagonist (108).

There is increasing evidence that thrombin in low concentrations can regulate inflammation, play a protective role in cell survival and be essential for vascular integrity and angiogenesis (98). Thrombin was shown to release from endothelial cells nitric oxide (NO), which inhibits the adhesion of monocytes to endothelium and platelet aggregation (16,21). Peptide agonist of the thrombin receptor PAR1 (PAR1-AP) that mimic thrombin effects, was reported to inhibit the release of inflammatory mediators from activated mast cells due to stimulation of NO production (16,92,109). Thrombin can promote the production and secretion of extracellular matrix proteins and positively influences remodeling processes (16,24). PAR1-AP, encapsulated to polymer composites or microparticls, in low concentrations stimulated experimental wound healing in rats and mice via accelerating reparative processes caused by increased migration, cells proliferation, and angiogenesis (neovascularization) in the granular tissue (16,109). Thrombin also can protect cell against death through PAR1 – induced expression of the antiapoptosis genes BCL2-related protein A1 and inhibitor of apoptosis 1 in HUVECs, and a variety of negative regulators of proinflammatory pathways (98,110). Moreover, thrombin via PAR1 induces MCP-1 expression by endothelial cells and monocytes (110). MCP-1 acts not only as a chemoattractant but also as a negative regulator of inflammatory response of

monocytes amd macrophages.The regulatory effect of MCP-1 involves a polarization of the T-cell response toward immunosuppresion, with short-term survival benefits in systemic inflammation (110).

The potential physiological role of PARs is determined by their location: PAR1 is expressed on platelets, endothelium, leukocytes (monocytes), SMC, cardiomyocytes, fibroblasts, neurons, glial cells, mast cells and epithelial cells; PAR2 is expressed on the same cells, except human 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 (4,14,18). 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. Through receptors on the cells (PARs, thrombomodulin, GP Ib), thrombin connects the proteolytic systems, involved in inflammation.

5.2. TF-FVIIa-Xa induced inflammation

The tissue factor/factor VIIa complex (TF/FVIIa) plays the crucial role not only in the initiation of haemostasis and thrombosis. TF binds FVIIa and this complex stimulates the signal transmission into the vascular cells that led to production of proinflamatory mediators, expression of leukocyte adhesion molecules and regulation of cell migration presumably via a coagulation-independent pathway involving PAR2 signaling (2, 11,69). Activation of cells with TF/VIIa complex can precede the development of the coagulation cascade. PAR2, which is a receptor of the nonspecific proteinase – trypsin, mast cells tryptase, the membrane type serine proteinase-1, is considered as the receptor of factors TF/VIIa and FXa. In response to infection TF is expressed by monocytes, that are central to innate immunity.Monocytes also express PAR1, and PAR2 expression is induced during differentiation of monocytes to macrophages (2).PAR2 expression is controlled by IL-4. Differentiation into dendritic cells leads to PAR2 down-regulation, but dendritic cells have been shown to express TF (2,110). PAR2 can be an important receptor in inflammation especially for some of the earliest inflammatory responses (111) TF acts as a cofactor in the activation of PAR2 receptors of endothelial cells because very low (picomolar) concentrations of FVIIa in the presence of FX trigger activation of the cells that express both the TF and PAR2 (68). About 10 pM FVIIa can circulate in blood plasma, and this concentration is quite sufficient to initiate in the presence of TF and FX generation of FXa 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 FVIIa 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 (112).

Complex of TF/VIIa binds FXa and stimulates cell activity.This ternary complex increases the concentration of intracellular Ca^{2+}, stimulates phosphorylation of mitogen-activated protein kinases (MAP kinases) (including p38, extracellular signal-regulated kinase 1/2 ((Erk-1/2, p44/42), c-Jun N-terminal kinase (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 gene), 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 (IL-8,IL-6 and IL-1β, macrophage inflammatory protein (MIP)-2α), growth factors (CTGF, hbEGF, amphiregulin, FGF-5), proteins,involve in cellular reorganization and migration (u-PAR,collagenase1 and 3,RhoE),PAI-2, cyclophilin, PGE2 receptor etc. (113-117). Genetically modified mice that expressed low levels of tissue factor, exhibited reduced inflammation , including IL-6 expression, and increased survival in a mouse model of endotoxemia compared with control mice (118,119).

TF/VIIa and FXa have been shown can activate cells not only through PAR2 but also via PAR1 (68,110) In experiments on PAR-deficient mice was shown that PAR2 is the main endogenous FXa receptor of endothelial cell and PAR2 and PAR1 (together) appear to account for approximately 90% of endothelial Xa signaling. By contrast, in fibroblasts, PAR1 by itself accounted for more part of FXa-induced signalling (phosphoinositide hydrolysis) (68, 117).

Receintly it was shown that PAR-1 or PAR-2 deficiency or hirudin-induced inhibition of thrombin did not affect IL6 expression or mortality in a mouse model of endotoxemia (118,119). However, combining hirudin treatment to inhibit thrombin signaling through PAR-1 and PAR-4 with PAR-2 deficiency reduced lipopolysaccharide-induced IL-6 expression and increased survival. Activation of multiple PARs by haemostatic proteases was suggested to enhance inflammation.

But, the ternary TF-VIIa-Xa complex was demonstrated similarly to activate PAR1 or PAR2 based on efficiency of PAR internalization and ERK phosphorylation, but only PAR2 signaling leads to TF phosphorylation via it's a chort cytoplasmic domain (that like to interferon receptor) with three serine residues (that are potential phosphorylation sites) (120). TF ternary complex signaling (in the case of both PAR1 and PAR2 signaling) is rapid, leading to PKCα membrane recruitment within 1 min. However, TF cytoplasmic domain phosphorylation is a delayed response and this domain played apparently no role in early responses of TF-VIIa signaling (120).

The some other mechanisms may be realized. Thus, TF/VIIa profoundly increased the chemotactic response towards platelet-derived growth factor (PDGF-BB) in human vascular smooth muscle cells, fibroblasts and in porcine aorta endothelial cells (121,122). This hyperchemotaxis towards low concentrations of PDGF-

BB is depended in part on phosphorylation of the TF cytoplasmic domain because deletion of this domain completely abolished the TF-dependent chemotactic response to PDGF-BB (122). TF cytoplasmic domain phosphorylation is dependent on PKCα activation. The phosphorylation of the PKC consensus Ser 253 is necessary for subsequent Ser 258 phosphorylation, that is recognized by the phosphorylation-specific antibody to TF (123). The cytoplasmic domain of TF contributes to leukocyte recruitment and activation as shown in experiments with injection of endotoxin in TF cytoplasmic domain mutant mice (124) The some molecular targets of TF/FVIIa pathway leading to cell migration may be of potential benefit in number of diseases and processes where cell migration is affected such as inflammation, tissue repair , angiogenesis and cancer cell invasion (122). TF cytoplasmic domain phosphorylation appears to be more effecient by ternary TF-VIIa-Xa complex in comparison with TF-VIIa stimulation. Thus PAR2 signaling in endothelial cells (but not PAR1 signaling) specifically targets the TF cytoplasmic domain by inducing TF phosphorylation and TF cytoplasmic domain plays a regulatory role in signaling associated with activation of coagulation.

Tissue factor besides initiating inflammation and blood coagulation, is believed to play an important role in embryonic development, tissue repair, angiogenesis and tumor metastasis (115,125,126). TF/VIIa complex, independent of triggering coagulation, can promote tumor cell migration, invasion and developmental angiogenesis through PAR-2 signaling. The specific antibodies against TF, PAR-2 and chemokine IL-8 that plays a critical role in these processes, inhibited TF-FVIIa-induced cell migration. Expression of IL-8 mRNA in a breast carcinoma cell line, MDA-MB-231 (a cell line that constitutively expresses abundant TF) was markedly up-regulated by FVII/ FVIIa (10 nM) but not thrombin or other hemostatic proteases (126). In this context, the TF cytoplasmic domain can negatively regulate PAR-2 signaling. Mice from which the TF cytoplasmic domain has been deleted (TF –δCT/–δCT) show enhanced PAR2-dependent angiogenesis, in synergy with PDGF-BB (127). Ocular tissue from diabetic patients shows PAR-2 colocalization with phosphorylated TF specifically on neovasculature, suggesting that phosphorylation of the TF cytoplasmic domain releases its negative regulatory control of PAR-2 signaling in angiogenesis.Targeting the TF-VIIa signaling pathway may thus enhance the efficacy of angiostatic treatments for cancer and neovascular eye diseases (127).

Not only TF cytoplasmic domain but also TF extracellular domain is included to TF-induced cell migration via specifically regulation of α 3β1 integrin - dependent migration on laminin 5. Expression of TF suppresses α3β1-dependent migration, but only when the TF cytoplasmic domain is not phosphorylated. Suppression of migration was reversed by FVIIa- induced phosphorylation of TF via PAR-2 activation (128).

TF influences on integrin-mediated cell migration through cooperative intra- and extracellular interactions and phosphorylation regulates function of TF in cell motility.

PAR2 cannot be activated with thrombin, as in contrast to PAR1, PAR3, and PAR4 (14,17,22), but FXa ,like to thrombin (but not FVIIa), induces PAR1 activation and the expression of cytokines (IL8, IL6) and MCP-1 (chemotactic protein 1 of monocytes) in skin fibroblasts (112). The effector cell proteinase receptor (EPR-1) can be a cofactor of PAR2 on endothelial cells and leukocytes during their activation with Fxa (129). Desensitization of PAR2 receptors with trypsin (or PAR2AP) inhibited the cell response induced by FXa. The catalytically inactive FXa retained the ability for binding to EPR-1 of the cells, but did not activate them. However, EPR-1 binding by FXa was not required for activating PAR2 on isolated rat aorta (130). Agonist peptide PAR2AP stimulate the rolling of leukocytes and their adhesion and transmigration in post-capillary venules of rat mesentery, and in PAR2-deficient mice inflammation was suppressed (131).

Factor Xa like as thrombin induced MIF (macrophage migration inhibitory factor, proinflammatory cytokine, involved in cell proliferation and initiation of wound-healing) expression in human dermal microvascular endothelial cells and umbilical vein endothelial cells (104). The effects of FXa were blocked by antithrombin III, but not by hirudin, indicating that FXa might directly enhance MIF production. The PAR2 AP (SLIGRL) induced MIF mRNA expression, showing that PAR-2 mediated MIF expression in response to FXa. Upregulation of MIF by rFXa is regulated by p44/p42 mitogen-activated protein kinase (MAPK)-dependent and NF-κB-dependent pathways. PAR-1 and PAR-2 mRNA expression in endothelial cells was enhanced by MIF. Thus MIF contributes to the inflammatory phase of the wound healing process in concert with thrombin and FXa via PAR-1 and PAR-2 (104).

5.3.Termination of PAR signaling

The mechanism of termination signaling by PARs is similar to the classical pathway of desensitization of many GPCRs (G protein-coupled receptors) (132). Ligand occupation of the PARs induces the translocation of members of the family of G protein receptor kinases (GRKs) from the cytosol to the activated receptor at the cell surface (18).GRKs are serine-threonine kinases that phosphorylate activated PARs, usually within the C-terminus or third intracellular loop. Phosphorylation triggers the membrane translocation of β-arrestins, cofactors for GRKs, which interact with GRK-phosphorylated PAR at the cell surface , disrupt their association with heterotrimeric G proteins, and thereby terminate signal transduction.Thrombin-induced signaling is markedly attenuated by overexpression of GRK3 in the myocardium, but GRK5 mediates PAR1 desensitization in endothelium. (133) β-Arrestins mediate desensitization of PAR1,but not internalization or down-regulation of PAR1, which proceeds with normal kinetics in fibroblasts lacking β-arrestin-1 and -2, whereas agonist-induced endocytosis of the β2-adrenergic receptor is inhibited in this system (134). Different adaptor proteins, such as AP2, may participate in endocytosis of PAR1.Activated PAR1 is endocytosed by a

clathrin-mediated process and the GTPase dynamin mediates detachment of clathrin-coated pits with accumulated PAR1.Whereas activated PAR1 is mostly destined for lysosomal degradation, constitutively endocytosed PAR1 recycles to the plasma membrane. Thus there is a tonic endocytosis and recycling of PAR1 that maintains receptor at the cell surface and in an intracellular pool (18). Activated PAR2 like PAR1 is endocytosed by a clathrin-mediated process, and trafficked to lysosomes.

6. CONTROL OF INFLAMMATION BY NATURAL ANTICOAGULANT PATHWAYS

6.1. Antiinflammatory properties of the protein C system

Anticoagulant functions of the protein C system are discussed in some reviews (3,10, 12, 135). Protein C is activated by thrombin bound to endothelial thrombomodulin (TM) and activated protein C (APC) provides a coagulation feedback loop and inhibits thrombin generation. Thrombomodulin (TM) not only increases protein C activation by thrombin, but also prevents thrombin from binding to PAR1.Thrombin binding to TM involves the specific site on thrombin molecule –ABE1 (anion binding exosite 1) that also accounted for complementary binding with a negatively charged hirudin-like sequence of PAR1.TM, binding with thrombin through ABE1, inhibits the ability of enzyme to cleave specific substrates (fibrinogen, factor V) and to activate PAR1 due to suppression of the procoagulant and proinflammatory properties of thrombin (16).

In addition, thrombin bound to TM activates a plasma procarbopeptidase into the enzyme carboxypeptidase B, the thrombin-activated fibrinolysis inhibitor (TAFIa) which inhibits fibrinolysis by splitting the C-terminal lysine residues from fibrin and thus makes it resistant to fibrinolysis (136) (Figure 1) Moreover, TAFIa regulates inflammation because it is a very potent inhibitor of bradykinin, and anaphylotoxin C5a of the complement system (137). These TAFIa properties probably helps to prevent serve drop in blood pressure, microvascular injury and subsequent edema in animals with severe acute inflammation (138).

TM can regulate inflammation independently of thrombin and protein C. In mice, with a mutant thrombomodulin without N-terminal lectin domain, the ability for generating APC and TAFIa was unchanged, but the antiinflammatory activity was inhibited (139). In these mutant mice, the synthesis of proinflammatory cytokines, adhesion molecules, the ICAM-1-mediated adhesion of leukocytes and mortality in endotoxin-induced sepsis were increased. These inflammatory reactions were inhibited by infusion of the N-terminal lectin domain. Moreover, this domain protected cultured endothelial cells subjected to apoptosis in medium depleted of serum by modulating the NFκB pathway (139).

The binding of thrombin with TM dramatically increases the rate of its neutralization by plasma inhibitors, especially by antithrombin III, which interacts with the carbohydrate (chondroitin sulfate) chain of TM . 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 (140). TM expression on endothelium is decreased in diseases associated with local inflammation (in the region of atherosclerotic injury), such as coronary atherosclerosis (141) or systemic inflammation in sepsis (142). The recovery of anticoagulant and antiinflammatory functions of TM by injection of the recombinant soluble TM seems promising as a therapeutic measure in these situations.

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 promoting 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 (142). 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 (142). TNFα is known to play a crucial role in development of inflammation, disseminated intravascular coagulation (DIC), and other disorders associated with sepsis (143). The injection of recombinant APC (Drotrecogin-α) significantly decreased the mortality of patients with severe sepsis (144).

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) (145). NFκB is a crucial factor in regulation of production of proinflammatory cytokines TNFα and IL-1β by monocytes. 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) (146). 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 (145).

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 (147). 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 (147). APC inhibits the immunologic and inflammatory responses induced by Th2 cytokines in a mouse model of asthma and may represent a novel anti-inflammatory treatment (148). Inhalation of APC significantly inhibited the expression of Th2 cytokines, immunoglobulin E (IgE), eosinophilic inflammation, and hyperresponsiveness. Activator of

transcription 6 (STAT6) and NFκ B oligonucleotides to lung nuclear proteins was significantly reduced in mice treated with APC.

6.2. Endothelial protein C receptor as regulator of coagulation and inflammation

The specific endothelial protein C receptor (EPCR) seems to play a significant role in the anticoagulant and antiinflammatory effect of APC . The rate of protein C activation with TM- 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 (3,147). 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 (149). On binding to EPCR, APC manifests antiinflammatory features. In endotoxin-induced inflammation, APC/EPCR inhibits the release of inflammatory cytokines fist of all TNFα (147). 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 (150). A soluble form of EPCR (sEPCR) is released from the activated endothelium by inducible metalloproteinase and can be detected in the plasma (151). Similarly to activated protein C, sEPCR 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 (147).sEPCR also can bind to selectively activated neutrophils and that the binding appears to involve interaction with the elastase-like proteinase 3 bound to the adhesive integrin Mac-1 (CD11b/CD18) of the surface of activated leukocytes and thus prevents their binding to endothelium and infiltration into tissues (152). Appearance of free receptor APC seems to be a component of protective antiinflammatory mechanisms of protein C system.

Moreover, the release of sEPCR induces 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 (147). 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 (147).

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 α 2 domains of CD1 proteins of the MHP family, most of which are involved in inflammation (153).The EPCR structure is a model for how CD1d binds lipids and further suggests additional potential functions for EPCR in immune regulation, possibly including the anti-phospholipid syndrome (153). The expression of EPCR by leukocytes has been recently shown (145). 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 (145,147,154). 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 (68, 155-157). PAR1 and EPCR are suggested to be located in cooperation in functional microdomains on endothelial cells (68). 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 (156). The EPCR-dependent activation of PAR1 with activated protein C seems to cause the enzyme ability to decrease the lethality of sepsis (156). 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 (158,159).

Activated protein C prevents the hypoxia-caused apoptosis of cultured endothelial cells of brain vessels (158). 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 (158,160). 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/ EPCR and the lectin

domain of thrombomodulin, determines the defense of endothelium against damage in inflammation.

6.3. Antiinflammatory properties of antithrombin

Antithrombin (AT, antithrombinIII,serpin) - is the physiological inhibitor of thrombin, factor Xa and other serine proteinases. There is a growing body of evidence that AT is not only inhibitor of blood coagulation, but also it can reduce the inflammatory responses of endothelial and other cells (161-164). Thus, AT was shown to reduce the mortality of patients with severe sepsis in recent clinical trial (162). AT-induced attenuation of inflammatory responses might be account for promotion of endothelial prodution of prostacyclin and inhibition of leukocyte and endothelial cell expression of pro-inflammatory mediators via suppression of NF-κB activation (161,163-165).

7. THE NEW APPROACH TO CORRECTION OF TF/VIIA/XA-DEPENDENTED THROMBOSIS AND CONJUGATED PROSSESES

Recent evidence suggests that inhibition of TF, factors FVIIa, FXa, their complex and thrombin has the potential to further improve outcomes in atherothrombosis, venous thrombosis, thromboembolism, and many other pathological conditions (as sepsis, diabetes, cancers, DIC (disseminated intravascular coagulation)) associated with hypercoagulability and inflammation (166-168).

Now, anticoagulant therapies involving heparin or coumarin derivatives are generally associated with side effects such as bleeding and thrombocytopenia. In this context, the inhibition of the tissue factor pathway componens by efficient antithrombotic drugs represents a new strategy to reduce this bleeding and to prevent thrombosis events. New anticoagulants that are under evaluation in clinical trials include: inhibitors of the TF/ VIIa pathway (including TFPI, recombinant nematode anticoagulant protein c2 (rNAPc2) and active site-blocked FVIIa (ASIS, FFR-rFVIIa or FVIIai)) ; FXa inhibitors, both indirect and direct; activated protein C and soluble thrombomodulin; and direct thrombin inhibitors (168-171).Although most of these are parenteral agents, several of the direct inhibitors of factor Xa and thrombin are orally active. Clinical development of these therapies often starts with studies in the prevention of venous thrombosis before evaluation for other indications, such as prevention of cardioembolism in patients with atrial fibrillation or prosthetic heart valves.

At present, the greatest clinical need is for an oral anticoagulant to replace warfarin for long-term prevention and treatment of patients with venous and arterial thrombosis. Ximelagatran, an oral peptidomimetic direct thrombin inhibitor, is the first of a series of promising new agents (as bivalirudin, argatroban, dabigatran etc.) that might fulfill this need (172). The active form of ximelagatran is melagatran, which binds noncovalently and reversibly to both fibrin-bound and freely circulating thrombin. In humans, oral ximelagatran exhibits anticoagulant, antiplatelet, and profibrinolytic effects, with only minor prolongation of the capillary bleeding time. Clinical trials (total n>30,000) have evaluated oral ximelagatran in four indications: the prevention of venous thromboembolism (VTE, comprising deep venous thrombosis with or without and pulmonary embolism) after elective hip- or knee-replacement surgery ; treatment and long-term secondary prevention of VTE; the prevention of stroke and other systemic embolic events associated with nonvalvular atrial fibrillation; and the prevention of cardiovascular events after an acute myocardial infarction. The results of these trials suggest that the benefit-risk profile of oral ximelagatran therapy, administered at a fixed-dose without coagulation monitoring, compares favorably with that of currently approved standard therapy (172). Large phase 3 trials evaluating ximelagatran for the secondary prevention of venous thromboembolism and prevention of cardioembolic events in patients with atrial fibrillation have been completed (166,173).

The emerging role of TF in the pathogenesis of diseases such as sepsis, atherosclerosis, certain cancers and diseases characterized by pathological fibrin deposition such as disseminated intravascular coagulation and thrombosis, has directed attention to the development of novel inhibitors of tissue factor for use as antithrombotic drugs. The main advantage of inhibitors of the TF/FVIIa pathway is that such inhibitors have the potential of inhibiting the coagulation cascade at its earliest stage. Thus, such therapeutics exert minimal disturbance of systemic hemostasis since they act locally at the site of vascular injury (174). These approaches have resulted in inhibitors directed specifically towards either FVIIa or TF. Antagonists include active site inhibited FVIIa, TF mutants, anti-TF antibodies, anti-FVII/FVIIa antibodies, naturally-occurring protein inhibitors, peptide exosite inhibitors, and protein and small molecule active site inhibitors. These antagonists can inhibit catalysis directly at the active site as well as impair function by binding to exosites that may interfere with substrate, membrane, or cofactor binding (175).

Active site-inactivated recombinant rFVIIa (rFVIIai) was shown to have a faster association to and a slower dissociation from TF than rFVIIa, resulting in a lower Kd of rFVIIai compared with rFVIIa. In various animal models rFVIIai has been demonstrated to prevent or diminish immediate thrombus formation at the site of vessel wall injury (athroplasty or other forms of mechanical injury) as well as the development of long-term intima thickening. The inflammatory response following endotoxin-induced sepsis was shown to decrease after administration of rFVIIai. Also, survival increased in the rFVIIai-treated animals in this study. In addition, ischemia-reperfusion injury was mitigated by rFVIIai. In a limited number of patients undergoing percutaneous transluminal coronary angioplasty (PTCA), rFVIIai was observed to allow PTCA to be performed at lower doses of heparin than what has been reported previously (176).

Recombinant NAPc2 like other inhibitors of TF may have a promising role in the prevention and treatment of venous and arterial thrombosis, as well as potential

efficacy in the management of disseminated intravascular coagulopathies because of their potent and selective inhibition of TF/FVIIa. rNAPc2 (85-amino acid protein) is a potent (K_i =10 pM) inhibitor of TF/ FVIIa complex. The mechanism of its action requires preliminary binding to circulating FXa or FX to form a binary complex prior to its interaction and inhibition of membrane-bound TF/ FVIIa (170). The binding of rNAPc2 to FX results in an prolonged elimination half-life of longer than 50 h (following either subcutaneous or intravenous administration) (171). This data supports the continued development of this novel anticoagulant for the prevention and treatment of acute thrombotic disorders.Moreover, the administration of rNAPc2 completely blocked the endotoxin-induced thrombin generation in healthy male volunteers and attenuated the endotoxin-induced rise in interleukin (IL)-10, without affecting the rise in other cytokines (177). Thus, the inhibition of TF, FVIIa and their complex by efficient antithrombotic drugs represents a new strategy to reduce this bleeding and to prevent thrombosis events. Moreover, TF/FVIIa inhibition is shown to be useful in the treatment of biological processes independent of the clotting cascade such as angiogenesis and cancer. Among the natural and genetically engineered TF/FVIIa inhibitors, injections of the recombinant protein rNAPc2 show clinical improvements, such as reduced bleeding and thromboembolism, over classical drugs used in the therapy of coronary angioplasty and hip or knee replacement surgery. The knowledge of the 3D-structure of TF/FVIIa complex and examination of co-crystal data of some drugs bound to this complex led to the design and synthesis of numerous TF/FVIIa inhibitors. Among them, pyrimidinones (the p-amidinophenylurea 18 , the pyrimidinones (PHA-927) and the pyridinone 37) are highly potent inhibitors of the TF/FVIIa complex, but not thrombin and factor Xa . These TF/FVIIa inhibitors prevent arterial thrombosis in non-human primate models of thrombosis and represent a safe approach to antithrombotic therapy in patients with cardiovascular risk factors (169).

But, in connection with increasing evidence that very low thrombin concentrations can regulate inflammation, play a protective role in cell survival , be essential for vascular integrity and angiogenesis (98) and, certainly, be principal activator of protein C system (4) with anticoagulant and anti-inflammatory properties, using of direct thrombin inhibitors may cause paradoxical responses. As direct thrombin inhibition seems to unmusk the lethal inflammatory component of coagulation activation in parpicular by increase TF-dependent inflammatory PAR2 signaling (119). But blocking proinflammatory effects of thrombin, thrombin inhibitors attenuate the activation of protein C and its ptotective effects (110).While protein C system attenuates inflammatory pathways that involve PAR2 (110).

Now a potential therapeutic targets in thrombosis diseases may be proteinase-activated receptors (PARs), the first of all receptor of TF/FVIIa/Xa - PAR-2 and PAR-1. The modulation of proteinase's interaction with PAR via antagonists (or agonists) may prove to be an effective approach to control the diseases associated with inflammation and tissue injury.

8. SUMMARY AND PERSPECTIVE

In recent times the traditional view on haemostatic serine proteinases (thrombin, factors VIIa, Xa), as enzymes that regulate only blood coagulation by limited proteolysis of proteins, has significantly changed. These proteinases were shown to act as the signal, hormole-like molecules, controlling cell functions by triggering a novel family of seven domain, G-protein-coupling membrane receptors, termed proteinase activated receptors (PARs). Activation of cells with some of these proteinases can precede the development of the coagulation cascade and led to production of proinflamatory mediators, expression of leukocyte and platelet adhesion molecules and regulation of cell migration presumably via a coagulation-independent pathway.

The generation of active proteinases is initiated by tissue factor that is expressed by cells of the innate immune system and vessel cells after tissue damage induced by trauma, infection and other cell injury. Coagulation and inflammation have some of the same cellular and molecular players participate in both processes. After injury endothelium expresses the adhesive proteins,inductors and receptors, involved in both coagulation and inflammation. The adhesion molecules have been implicated in recruitment of platelets which after activation express or release proteins with procoagulant and proinflammatory properties. Activated platelet mediate additional platelet and leukocyte rectuitment, aggregation and provide the surface for the binding of leukocyte-derived microparticles containing tissue factor for induction of coagulation. Platelets are also aggregated with leukocyte, released microparticles, that mediate cell- cell interaction. These aggregates form a surface, providing activation of the blood coagulation and inflammation and release inducers of these processes and microparticles carrying these inducers. 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 that can induce the expression of genes and synthesis of adhesion molecules, selectins, chemokines, growth factors, inflammatory mediators 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 sheded off from the surface of activated cells. 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, and also thrombin, which binds with PAR1 (Figure 1). The PARs signaling with proteinases stimulates the activation

of some transcription factors as NF- κB,AP1,Egr-1,SRF, GATA-2 and DbpB (by thrombin) and as AP-1/β-Zip, Sp1, and *Egr-1* (by TF/VIIa and Xa) and synthesis of proinflammatory and procoagulant factors.

But thrombin also can protect cell against death through PAR1 - induced expression of the antiapoptosis genes BCL2-related protein A1 and inhibitor of apoptosis 1 and a variety of negative regulators of proinflammatory pathways.Moreover, thrombin via PAR1 induces expression of a negative regulator of inflammatory response -MCP-1 by endothelial cells and monocytes. In addition, on interacting with TM thrombin converts protein C into anticoagulant APC with antiinflammatory and antiapoptotic properties. Thrombomodulin also exhibits antiinflammatory and antiapoptotic effects.

These data suggest that multiple protease-activated receptors mediate a reciprocal crosstalk between coagulation and inflammation. Studies of preparations providing selective activation of anticoagulant, antiinflammatory, and cytoprotective functions of components of protein C pathway are promising also as selective supression of procoagulant and proinflammatory the pathways of tissue factor/factor VIIa/Xa and high thrombin concentrations. Studies of preparations that inhibit platelet–platelet and platelet–leukocyte aggregation dependent on integrins and selectins also are promising in connection with not only procoagulant but also proinflammatory functions of platelets.

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