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Pathogenic Role of Antiphospholipid Antibodies

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Introduction

The antiphospholipid antibody syndrome (APS) is characterized by recurrent arterial and venous thrombosis and/or pregnancy complications (miscarriage and fetal death, preeclampsia, placental insufficiency, and fetal growth restriction) in association with antiphospholipid (aPL) antibodies. Antiphospholipid antibodies are not directed against phospholipids, as the name of the syndrome would suggest, but against plasma proteins with affinity for anionic phospholipids. Although antibodies are directed against many different plasma proteins, the general consensus is that autoantibodies directed against β_2-glycoprotein I (β2GPI) are the clinically relevant antibodies. This important observation on the specificity of the autoantibodies, however, does not help to understand how the presence of these autoantibodies leads to the observed clinical symptoms, as β2GPI is a plasma protein without a known function. The pathogenic mechanisms in APS that lead to *in vivo* injury are incompletely understood. There are many *in vitro* and some *in vivo* indications that antibodies directed against β2GPI can influence both the regulation of haemostasis and of complement. We will discuss the current knowledge on how aPL antibodies can disturb the regulation of haemostasis and thereby lead to an increased thrombotic tendency.

Recent experimental observations suggest that altered regulation of complement, an ancient component of the innate immune system, can cause and may perpetuate complications of pregnancy (1,2). We will present evidence that a means by which aPL antibodies mediate pregnancy complications is through activation of the complement cascade (2,3). Similarly, complement might contribute to aPL antibody-induced thrombosis, and coagulation factors can activate the complement cascade (4). Thus, targeting this pathway holds the promise of new, safer and better treatments.

Haemostasis

Haemostasis is our defense system against loss of blood after trauma. Haemostasis involves a delicately balanced system requiring the interplay between platelets, coagulation, fibrinolysis, monocytes and endothelial cells. Under normal conditions coagulation is prevented, and blood is maintained in a fluid state, but after injury a clot rapidly forms. Platelets continuously examine the vessel wall for leakages, and when they detect damage to the endothelium, they immediately respond by adhering to the exposed subendothelial structures. After the adherence of “sentinel” platelets, newly arriving platelets interact with the activated, subendothelium-bound platelets and successive platelet-platelet interactions result in formation of a platelet plug. The platelet plug can temporarily stop blood loss, but a plug consisting of only platelets is very unstable. To prevent re-bleeding, the platelet plug must be stabilized by a fibrin network. Fibrin formation occurs when tissue factor, present within the vessel wall, becomes exposed to the circulating blood. Factor VIIa, an inactive enzyme present in the circulation, binds to

tissue factor which is an essential cofactor for factor VIIa activation. Tissue factor-VIIa binding allows factor VIIa to become an active enzyme that in turn activates factors IX and X. Factor IXa converts factor X into factor Xa with the help of factor VIIIa. Subsequently, factor Xa with the help of factor Va, converts prothrombin into thrombin. Thrombin is the central enzyme of haemostasis and one of its activities is to convert fibrinogen into fibrin.

The coagulation system, however, cannot distinguish between a ruptured vessel and endothelial cell activation precipitated by other causes, such as inflammatory cytokines. Initiation of the coagulation cascade by activated endothelium, expressing a “prothrombotic phenotype”, will result in thrombus formation within an intact blood vessel and a loss of perfusion to vital organs. These events can result in arterial and venous thrombosis manifested in conditions such as stroke, myocardial infarction and phlebitis. Tight regulation of haemostatic reactions is therefore essential for normal physiology. To this end, endothelial cells synthesize potent antagonists of platelet activation and plasma contains multiple inhibitors of coagulation along with fibrinolytic factors to dissolve thrombi and limit their propagation.

A hypercoagulable state arises from an imbalance between procoagulant and anticoagulant forces. A striking feature of most genetic hypercoagulable states is that each is characterized by thrombotic complications in specific vascular beds. For example, protein C deficiency is associated with deep venous thrombosis and pulmonary embolism only and not with arterial thromboses (5). Functional deficiency of thrombomodulin in mice causes selective fibrin deposition in the lung, heart and spleen, but not in other organs (6). The basis for tissue-specific or vessel-specific haemostatic imbalance, rather than diffuse thrombotic diathesis is not well understood (7). It has been suggested that endothelial cells and local rheology are important regulators of haemostasis. Indeed, there are considerable functional differences among endothelial cells in different parts of the vascular tree. Such heterogeneity, different vessels in different organs expressing distinct phenotypes, is likely a consequence of the local environmental factors to which they are exposed and to which they must adapt (8).

The pathophysiology of APS is strikingly different from other known hypercoagulable states. In APS, thrombotic complications can occur in almost every vessel, arteries and veins, large vessels and microcirculation (9). The hypercoagulable state in APS is clearly not vascular bed-specific. Rather, the presence of aPL antibodies results in a diffuse thrombotic diathesis suggesting global and general dysregulation of the haemostatic balance. In fact, aPL antibodies have been implicated in reactions that interfere with almost all known haemostatic and endothelial cell reactions (Table 1). It is possible that the generalized thrombotic manifestations in APS reflect the multiple effects of aPL antibodies, but an alternative interpretation of the clinical phenotype is that aPL antibodies cause thrombosis by a distinct and novel mechanism.

Antiphospholipid antibodies and cell activation

The generalized thrombotic manifestations of patients with APS suggest a unique mechanism by which aPL antibodies cause thrombotic complications. APL autoantibodies are directed against plasma proteins with affinity for anionic phospholipids, including β2GPI, rather than against anionic phospholipids, which their name would suggest. Because humans and mice deficient in β2GPI do not develop a phenotype, it is unlikely that inhibition of a functional activity of β2GPI explains the clinical manifestations of APS (10,11). Rather, anti-β2GPI antibodies may be considered gain-of-function antibodies. After aPL antibody binding, β2GPI dimers form; dimerization strongly increases affinity of β2GPI for negatively charged phospholipids such that competition is created between β2GPI dimers and clotting factors for binding to anionic phospholipids. This is illustrated a clinically relevant assay for the detection of aPL antibodies in patient plasma, a phospholipid-dependent prolongation of clotting assays (Lupus anticoagulants, LAC) (12). The strong correlation between antibodies that prolong

clotting times and thrombosis suggests that the increased affinity of β2GPI-anti-β2GPI complexes for anionic surfaces could provide a clue to understand the pathophysiology of these antibodies.

Another important finding is that the β2GPI dimers that form as consequence of aPL binding, not only have an increased affinity for phospholipids but also bind strongly to cell surfaces (13). In addition, when these β2GPI dimers cross-linked by aPL antibodies interact with endothelial cells, monocytes and platelets, the cells are activated. Here we will discuss our studies of the effects of anti-β2GPI antibodies on platelet function.

Plasma β_2GPI does not bind to platelets, however, β2GPI in complex with anti-β_2GPI-antibodies does bind to platelets and induces platelet activation that is independent of platelet Fc-receptors (14). Simply binding of β2GPI-antibody complexes to phospholipids in the platelet membrane is unlikely to activate platelets. Rather, we hypothesized that a cell surface receptor transduces the activating signal from the environment and began to search for platelet membrane receptors that bind β_2GPI or β_2GPI-anti-β_2GPI antibody complexes. We discovered that receptor associated protein (RAP), a universal inhibitor of ligand binding to members of the LDL-receptor family, was able to inhibit the platelet activation induced by β_2GPI-antibody complexes (15).

The LDL-receptor family consists of nine family members. A truncated splice variant of LRP-8 (also known as ApoER2) is the only member of the LDL-receptor family present on platelets, thus, our interest was focused on this receptor protein (16). We have recently shown that β_2GPI-antibody complexes bind to LRP-8 and that binding activates platelets and induces thomboxane A2 synthesis (17). Our *in vitro* experiments were confirmed in a mouse model of APS in which we found that a soluble fragment of the LRP-8 receptor was able to completely inhibit thrombus formation (18). Given these findings and clinical experience showing that APS is associated with neurological, cardiovascular and pregnancy morbidity, it is particularly interesting that polymorphisms in LRP-8 have been linked to Alzheimer disease, cardiovascular diseases and pregnancy complications (19-21).

LRP-8 is not the only receptor involved in the interaction of β_2GPI-antibody complexes with platelets. Shi et al. and our own group have simultaneously identified glycoprotein Ibα as a second receptor for β_2GPI-antibody complexes (22,23). Why two receptors are necessary for the activation of platelets by β_2GPI-antibody complexes is unclear. That both LRP-8 and GPIbα are multi-ligand receptors raises the possibility that the requirement for two receptors introduces certain specificity in cellular responses after binding of ligands to multiligand receptors, but further studies are needed to examine these issues.

Toll-like receptor (TLR) 4, a lipopolysaccharide receptor that is highly homologous to GPIbα has been suggested to mediate the effects of β_2GPI-antibody complexes on endothelial cell activation (24). TLR4 is also present on platelets, but there are no indications that TLR4 is involved in platelet activation (25). Direct interaction between β_2GPI-antibody complexes and TLR4 has also never been shown. Further studies are required to determine if indeed there is a role for TLR4 in aPL antibody-mediated platelet activation. Our work presents a new concept for aPL antibody-induced injury: triggering a prothrombotic phenotype by cross-linking platelet cell surface receptors with β2GPI-aPL antibody complexes. Our next challenge is to determine which other cells, perhaps trophoblasts, endothelial cells, or monocytes, are activated in this manner and contribute to the different clinical manifestations observed in APS patients.

Complement activation and tissue injury

The specific antigenic reactivity of aPL antibodies is crucial to their effects. Antigen binding can directly stimulate target cells; it also serves to localize pathogenic antibodies which, via their Fc domains, can activate complement and/or crosslink Fc receptors of effector cells. Because aPL antibodies exert thrombophilia and miscarriage in mice lacking stimulatory Fc receptors (2,26), it has been concluded that Fc receptors are not required for tissue injury, although it is clear that ligation of Fc receptors may amplify damage. Rather, the complement system has been identified as critical for the pathogenic effects of aPL antibodies. Findings from animal models of aPL antibody-induced pregnancy loss and injury-induced thrombosis argue that complement components C3 and C5 are essential proximal mediators of tissue injury (2,3). In addition, results of these studies support the importance of inflammation as a cause of clinical manifestations of APS.

The complement cascade, composed of over 30 proteins that act in concert to protect the host against invading organisms, initiates inflammation and tissue injury by recruitment and activation of inflammatory cells (27,28). The classical pathway is activated when antibodies bind to antigen and unleash potent effectors associated with humoral responses in immune-mediated tissue damage. The mannose-binding lectin (MBL) pathway is activated by carbohydrates (often on infectious agents). Alternative pathway activation mechanisms differ in that they are initiated by the binding of spontaneously activated complement components to the surface of pathogens. Alternative pathway can also serve as an amplification system for the classical and lectin pathways. By means of these recognition and activation mechanisms the complement system identifies and responds to “dangerous” situations presented by foreign antigens, pathogens, tissue injury, ischemia, apoptosis and necrosis (29).

The three complement activation pathways converge on the C3 protein leading to a common pathway of effector functions. The initial step is generation of the fragments C3a and C3b. C3a, an anaphylatoxin that binds to receptors on leukocytes and other cells, causes activation and release of inflammatory mediators (30). C3b attaches covalently to targets, followed by the assembly of C5 convertase with subsequent cleavage of C5 to C5a and C5b. C5a is a potent soluble inflammatory, anaphylatoxic and chemotactic molecule that promotes recruitment and activation of neutrophils and monocytes and mediates endothelial cell activation through its receptor. Binding of C5b to the target initiates the non-enzymatic assembly of the C5b-9 membrane attack complex (MAC) which inserts into cell membranes causing lysis through changes in intracellular osmolarity or damage of nucleated cells primarily by activating specific proinflammatory signaling pathways (31,32).

Soluble and membrane-bound complement regulatory proteins protect against bystander injury at sites of inflammation and serve to limit spontaneous alternative pathway activation. Indeed, defective function of complement regulators is associated with inflammatory and thrombotic injury associated with hemolytic uremic syndrome and glomerulonephritis (33,34). Intact complement regulation is essential for maintenance of normal pregnancies, because in pregnant mice deficient in cell-bound regulators of complement activation fetuses die *in utero* surrounded by inflammatory cells and complement split products; breeding mice that lack complement inhibitors on a complement-deficient background rescues pregnancies (1,35).

Complement activation as mediator of fetal damage induced by aPL antibodies

During trophoblast differentiation, phosphatidylserine is externalized on the trophoblast outer leaflet where it provides a target for aPL antibodies (36,37) which can activate complement via the classical pathway generating split products that mediate placental injury and cause fetal

loss and growth restriction. The resultant exaggerated complement activation could overwhelm the inhibitory capacity of local complement regulatory proteins allowing the complement cascade to proceed. Using a murine model of APS induced by passive transfer of human aPL antibodies, we have shown that complement activation plays an essential and causative role in pregnancy loss and fetal growth restriction (2,3) (Figure 1).

Blockade of the complement cascade *in vivo* with a C3 convertase inhibitor (Crry-Ig) or deficiency of complement C3 prevented fetal loss and growth restriction in pregnant mice that were treated with human IgG containing aPL antibodies. To define the initiating pathways and critical effectors of aPL-induced pregnancy injury, mice deficient in complement elements (C4, factor B, C5, C5a receptor) and inhibitors of complement activation (anti-C5 mAb, anti-factor B mAb, C5a receptor antagonist peptide) were studied in our mouse model of APS. We identified complement component C5, and particularly its cleavage product C5a, as key mediators of fetal injury and showed that antibodies or peptides that block C5a-C5a receptor interactions prevent pregnancy complications. Furthermore, our results indicated that both classical and alternative complement pathway activation contribute to damage. Mice deficient in alternative and classical pathway complement components (factor B, C4, C3 and C5) were resistant to fetal injury induced by aPL antibodies (2,3).

Given that the primary treatment for APS patients is anticoagulation throughout pregnancy, usually with sub-anticoagulant doses of heparin, and evidence that heparin inhibits complement activation *in vitro*, we considered the possibility that heparin prevents pregnancy loss by inhibiting complement activation on trophoblasts and that anticoagulation, in and of itself, is not sufficient to prevent pregnancy complications in APS. We found that treatment with unfractionated heparin or low molecular weight heparin protected pregnancies from aPL-induced damage even at doses that did not cause detectable interference with coagulation. In contrast, treatment with hirudin or fondaparinux (anticoagulants without anti-complement effects) was not protective demonstrating that anticoagulation is insufficient therapy for APS-associated miscarriage (38). Furthermore, heparins inhibited both aPL antibody-induced elevations in circulating C3a and increased C3b deposition in decidual tissues (neither was altered by the other anticoagulants) and blocked C3 cleavage *in vitro*. Thus, heparin may prevent pregnancy complications by limiting complement activation and the ensuing inflammatory response at the maternal-fetal interface, rather than by inhibiting thrombosis.

There are multiple effectors of fetal injury downstream of complement activation (Figure 1). TNF-α is one mediator that links complement activation and pathogenic aPL antibodies to fetal damage. APL antibodies, specifically targeted to decidual tissue, cause a rapid increase in decidual and systemic TNF-α levels, which is absent in C5-deficient mice. That TNF-α is pathogenic is suggested by studies showing that miscarriage induced by aPL antibodies is less frequent in mice deficient in TNF-α or treated with TNF-α blockade (39).

Another means by which complement activation, particularly C5a, triggers fetal damage is through induction of tissue factor expression. Treatment with aPL antibodies increases tissue factor (TF) in decidual tissue without an increase in fibrin deposits or thrombi, and blockade of TF with a monoclonal antibody in wild-type mice or reduced TF through gene targeting prevented aPL antibody-induced inflammation and pregnancy loss (40). TF in myeloid cells, but not fetal-derived trophoblasts, was associated with fetal injury suggesting that neutrophils are the site for pathologic TF. Binding of C5a to C5a receptors on neutrophils has been shown to induce TF expression (40,41) which enhances oxidative burst providing a mechanism for trophoblast injury and pregnancy loss triggered by aPL antibodies (40).

Finally, complement activation products may cause an imbalance of angiogenic factors required for normal pregnancy. Satisfactory development of the fetomaternal vasculature is

required for successful embryonic growth, and insufficient placental vascularization has been associated with early embryonic mortality, preeclampsia, and intrauterine growth restriction (IUGR) (42). Normal placental development requires coordinated expression of angiogenic growth factors, vascular endothelial growth factor (VEGF) and placental growth factor (PlGF), as well as expression of their respective receptors on invasive trophoblasts (43). VEGF promotes placental development and invasiveness primarily through interaction with VEGF receptor-1 (VEGFR-1; also know as fms-like tyrosine kinase-1, Flt-1) and VEGFR-2 (44). Alternative splicing of VEGFR-1 results in production of the secreted protein, soluble VEGFR-1 (also known as sFlt-1), which lacks the cytoplasmic and transmembrane domains but retains the ligand-binding domain (45). Excess sVEGFR-1 has been shown to inhibit placental cytotrophoblast differentiation and invasion (46) and is thought to play a direct role in the pathogenesis of abnormal placentation associated with preeclampsia and IUGR (43,47, 48). We have shown that C5a-C5a receptor interactions directly trigger release of sVEGFR-1 from monocytes which can alter the balance of angiogenic factors in pregnancy and lead to the pregnancy complications associated with APS (49).

Complement activation as mediator of thrombophilia induced by aPL antibodies

Complement activation may also be a contributor to thrombophilia characteristic of APS. APL antibodies have been shown to induce a proinflammatory, proadhesive, procoagulant phenotype in endothelial cells, monocytes and platelets. It is well established that activated complement fragments themselves have the capacity to induce these states either directly through C5b-9 MAC or through C5a receptor-mediated effects (50-53). Using a model of surgically induced thrombus formation, Pierangeli, in collaboration with our group, has demonstrated that complement activation plays an important role in the increased thrombosis and adhesion of leukocytes to endothelial cells caused by treatment with aPL antibodies. Mice deficient in complement components C3, C5 or C5a receptors were resistant to aPL antibody-induced enhanced thrombophilia and endothelial cell activation (3,54,55). In addition, inhibition of C5 activation by anti-C5 monoclonal antibodies also prevented these effects. Although it is clear from the discussion above that there are multiple pathways by which aPL antibodies lead to a prothrombotic state, complement activation certainly contributes to this pathology.

Conclusions

Over the last 25 years numerous studies have established the correlation between the presence of antibodies against anionic phospholipids and the occurrence of thromboembolic manifestations and pregnancy complications but how the presence of these antibodies in the circulation could cause thrombosis and fetal loss was completely unclear for a long time. We have now reached a fascinating period in our attempts to understand the antiphospholipid syndrome. Over the last years, it has become clear that aPL antibodies activate cells via several different pathways to initiate clinical events, and there are likely to be interactions of these pathways. We have focused this review on the influence of aPL antibodies on the activation of platelets and the complement system. There is extensive evidence for links between the coagulation and complement cascades, including the observations that thrombin can cleave C5 (4) and C5a can trigger expression of TF (40). Future research undoubtedly will reveal more interactions in these and other pathways. Indeed, we expect that studies of aPL antibodies will extend our understanding of the importance of complement for thrombotic diathesis and the role of complement inhibition to prevent such complications in normal pregnancies.

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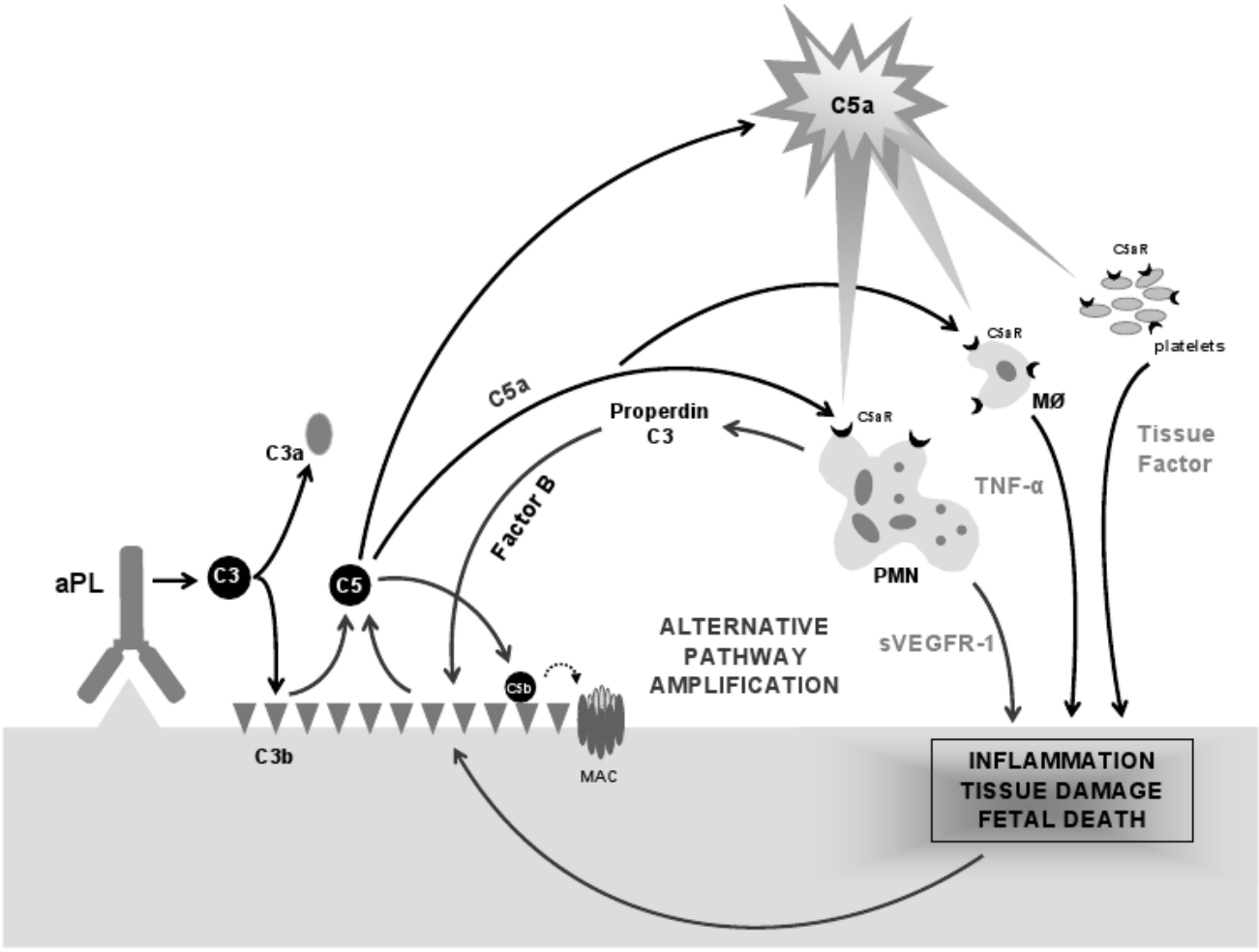

Figure 1. Proposed mechanism for the pathogenic effects of aPL antibodies on fetal injury

Based on the results of our mouse studies, we proposed a mechanism for pregnancy complications associated with aPL antibodies: aPL antibodies preferentially targeted at decidua and placenta activate complement via the classical pathway (Fc- and C4-dependent), leading to the generation of potent anaphylatoxins (C3a and C5a) and mediators of effector cell activation, including TF and TNF-α. Recruitment of inflammatory cells accelerates local alternative pathway activation and creates a proinflammatory amplification loop that enhances C3 activation and deposition, generates additional C3a and C5a, and results in further influx of inflammatory cells into the placenta and increased generation of TF, oxidants, TNF-α, and anti-angiogenic factor sVEGFR-1. Depending on the extent of damage, either death in utero or fetal growth restriction ensues.

Adapted from Girardi *et al.* Complement C5a receptors and neutrophils mediate fetal injury in the antiphospholipid syndrome. J Clin Invest 2003;112(11):1644-54. With kind permission from the American Society for Clinical Investigation.

Table 1
Coagulation Processes Disturbed by Antiphospholipid Antibodies

Inhibition of protein C activity (acquired protein C resistance)
Inhibition of protein S cofactor activity
Inhibition of antithrombin activity
Induction of tissue factor in endothelial cells and monocytes
Inhibition of tissue factor pathway inhibitor
Inhibition of endothelial cell prostacyclin synthesis
Increased deposition of prothrombin leading to increased thrombin formation
Deposition of immune complexes
Inhibition of tPA activity
Inhibition of fibrinolysis via interaction with antiplasmin
Activation of factor XI
Induction of platelet aggregation
Disturbance of a protecting annexin A5 shield
Induction of microparticle formation
Induction of endothelial cell adhesion receptors
Complement activation