Pathogenesis of antiphospholipid antibodies; Impairment of fibrinolysis and monocyte activation via the p38 mitogen-activated protein kinase pathway

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Abstract

Antiphospholipid syndrome (APS) is characterized by recurrent thrombosis or pregnancy morbidity associated with antiphospholipid antibodies (aPL). Impaired fibrinolysis is a contributing factor for the development of thrombosis, and the effect of aPL in the fibrinolytic system has been investigated. Impaired release of tPA and enhanced release of PAI-1 after endothel activation is reported in patients with APS. Elevated Lipoprotein (a) levels have been found in APS, which results in inhibition of fibrinolytic activity. Phospholipid-bound β_2-glycoprotein I (β_2GPI) is a major autoantigen for aPLs. β2GPI exerts both anti-coagulant and pro-coagulant properties mainly by interacting with other phospholipid-binding proteins such as coagulation factors and protein C. Dramatic increase in the affinity of β_2GPI to the cell surface is induced by binding of pathogenic anti-β_2GPI antibodies, which may modify the physiological function of β_2GPI and may affect the coagulation/fibrinolysis balance on the cell surface. Using chromogenic assays for measuring fibrinolytic activity, we demonstrated that addition of monoclonal anticardiolipin antibody (aCL) decreases the activity of extrinsic/intrinsic fibrinolysis. Significantly lower activity of intrinsic fibrinolysis was also demonstrated in the euglobulin fractions from APS patients.

Endothelial cells and monocytes are activated by aPLs *in vitro*, resulting in production of tissue factor (TF), a major initiator of the coagulation system. Recently, aPLs are reported to induce thrombocytes to produce thromboxane. The importance of apoE receptor 2 on platelets for the binding of artificially-dimerized β_2GPI was suggested. By investigating aPL-inducible genes in peripheral blood mononuclear cells, we found that mitogen-activated protein kinase (MAPK) pathway was up-regulated. Using monocyte cell line, phosphorylation of p38 MAPK, NF-κB

translocation to the nuclear fraction, and up-regulated TF mRNA expression were demonstrated after treatment with monoclonal aCL. These phenomena were observed only in the presence of β_2GPI. Moreover, a specific p38 MAPK inihibitor SB203580 decreased aCL/β_2GPI-induced TF mRNA expression.

Thus, aCL/β_2GPI plays dual roles in the pathogenesis of APS, firstly by deranging fibrinolytic system and secondly by activating monocytes, endothelial cells and thrombocytes to produce tissue factor or thromboxane.

Introduction

Antiphospholipid syndrome (APS) is a clinical condition characterized by recurrent arterial/venous thrombosis or pregnancy morbidity associated with the antiphospholipid antibodies (aPL). These autoantibodies are not only markers of APS, but also believed to play pathogenic roles in the development of symptoms in patients with APS (Pierangeli et al.,1996;Tsutsumi et al.,1996). The mechanism of aPL-induced thrombosis is not fully understood, although many discoveries have been made in these two decades. Impairment of fibrinolysis or acceleration of coagulation system induced by aPL has been studied as "classic" mechanism. Then, importance of premature atherosclerosis accelerated by aPL has been demonstrated *in vitro* and *in vivo* (George et al., 2000; Matsuura et al., 2002). Recently, activation of endothelial cells or other cell types has been focused by many investigators. In addition, importance of the complement system activation in pregnancy morbidity is also reported (Girardi et al., 2003). Thus, aPLs presumably induce thrombosis or pregnancy morbodity via multiple mechanisms. In this review, we focus on impairment of fibrinolysis and cell activation which are induced by aPL.

Impaired Fibrinolysis

Impaired fibrinolysis is a contributing factor for the development of thrombosis, and the effect of aPL in the fibrinolytic system has been investigated. In patients with connective tissue diseases including APS, plasminogen activator inhibitor-1 (PAI-1) release after endothelial activation was greatly enhanced compared with healthy controls, but no difference was found in tissue plasminogen activator (tPA) release (Jurado et al., 1992). Impaired

release of tPA and enhanced release of PAI-1 after endothel activation suggests that tPA/PAI-1 balance is important in the development of thrombosis in APS (Ames et al., 1996). Elevated levels of lipoprotein (a) [Lp(a)] have been reported in APS patients (Atsumi et al., 1998). Lp(a) obtains different numbers of kringle domains that interact with fibrinogen and inhibits fibrinolytic activity by inhibiting tPA unconpetitively. Lp(a) also increases PAI-1 expression in endothelial cells.

In patients with APS, pathogenic aPL are not directed against phospholipids itself, but against phospholipid-binding proteins, such as β_2GPI, prothrombin, annexin V, protein C or protein S. Among these, phospholipid-bound β_2-glycoprotein I (β_2GPI) is one of the major target antigens for aPLs present in patients with APS (McNeil et al., 1990; Galli et al., 1990; Matsuura et al., 1990). β2GPI, also known as apolipoprotein H, is a 50-kDa phospholipid-binding protein present in plasma at an approximate concentration of 200μg/ml, and has been recognized as a natural anti-coagulant because β2GPI inhibits prothrombinase and tenase function, factor XII activation and ADP-dependent activation of platelets (Nimpf et al., 1986; Schousboe et al., 1995; Nimpf et al., 1985). Recently, β2GPI has been shown to bind directly to factor XI and attenuate its activation (Shi et al., 2004). However, individuals with β_2GPI deficiency do not have a thrombotic tendency, thus aCL/β_2GPI associated thrombosis cannot be merely explained by "β_2GPI insufficiency" (Yasuda et al., 2000; Takeuchi et al., 2000). β2GPI also exerts pro-coagulant activities mainly by inhibition of protein C pathway (Mori et al., 1996). Binding of pathogenic anti-β_2GPI antibodies increases the affinity of β_2GPI to the cell surface (Takeya et al., 1997). Increased affinity of β_2GPI to the membrane may modify physiological function of β_2GPI and may affect the coagulation/fibrinolysis balance on the

cell surface by interacting with other phospholipids-binding proteins such as coagulation factors and protein C. Using a chromogenic assay for measurement of extrinsic fibrinolysis, we demonstrated that addition of monoclonal anticardiolipin antibody (aCL) decreases the activity of extrinsic fibrinolysis in the presence of tPA, plasminogen, fibrin and β_2GPI (Ieko et al., 2000). We also demonstrated that addition of monoclonal aCL in the presence of β_2GPI decreased fibrinolytic activity by newly-developed chromogenic assay for measuring intrinsic fibrinolysis. In this system, in the presence of phospholipid and plasminogen, kaolin was added as a stimulator and plasmin generation was measured using plasmin specific substrate S-2251. When we measured intrinsic fibrinolysis activity of euglobulin fractions from APS patients and healthy controls, significantly lower activity of intrinsic fibrinolysis was evident in the patient group (Takeuchi et al., 2002) (Fig.1). These data suggest that impairment of intrinsic and extrinsic fibrinolysis induced by pathogenic anti-β_2GPI antibodies is one of the mechanisms for thrombosis in patients with APS.

Cell-activation Mechanisms – contribution of p38MAPK pathway

Recently, cell-mediated mechanisms have been reported in the formation of thrombosis in patients with APS. Endothelium is one of the major organs that cover the inner surface of blood vessels, and its perturbation or damage has been reported in many disorders. Inappropriately-activated endothelial cells alter their properties from “antithrombotic” to “pro-thrombotic”, by producing pro-coagulant substances and allies of adhesion molecules such as VCAM-1, ICAM-1, E-selectin, or endothelin–1. Actually, aPL induce tissue factor expression on endothelial cells *in vitro*, which results in the initiation of the

extrinsic coagulation system (Amengual et al., 1998; Branch et al., 1993; Kornberg et al., 1994; Conti et al., 2003). Tissue factor is a cofactor for factor VIIa that activates factor IX and factor X. Then the factors IXa/Xa complex activates prothrombin to thrombin. Using endothelial cells, Raschi et al (Rashi et al., 2003) reported the importance of TRAF6 and MyD88 in NF-κB activation induced by monoclonal aCL in the presence of β_2GPI. They proposed that aCL reacts with β_2GPI likely associated to a member of toll-like receptor/IL-1 receptor family. Annexin A2, alternatively named as annexin II, also mediate the binding of aCL/β_2GPI to the surface of endothelial cells (Ma et al., 2000; Zhang et al., 2005). Annexin A2 is expressed on the endothelial cell surface and binds to β_2GPI with high affinity. They suggested that cross-linking of the cell-surface annexin A2 via aCL/β_2GPI stimulates activation of endothelial cells. However, how annexin A2 cross-linking mediate signal transduction remains unknown. Toll-like receptor is a candidate as a member of a multiprotein signaling complex on the cell surface. Procoagulant activity of monocytes was reported to be increased in patients with systemic lupus erythematosus, although correlation with positive lupus anticoagulant was not found (de Prost et al., 1990). Such procoagulant activities/tissue factor expression in normal monocytes were induced by purified IgG from APS patients or aPL (Martini et al., 1996). We and others demonstrated the up-regulation of TF pathway in patients with APS (Atsumi et al., 1997; Cuadrado et al., 1997). Autoantibodies against tissue factor pathway inhibitor (TFPI), which is a Kunitz-type protease inhibitor that inhibits tissue factor activity by forming a complex with tissue factor, factor VIIa and Xa, have been detected in APS patients (Forastiero et al., 2003). Thus, cell-mediated tissue factor up-regulation and antibody-mediated TFPI down-regulation work coordinately toward the hyper-coagulable state.

Recently, aPL have been reported to induce thrombocytes to produce thromboxane in the presence of subactivating amount of thrombin. Platelets sensitized by aCL/β_2GPI showed increased deposition on collagen-containing surface. In this study, interaction between dimmerized β_2GPI and apolipoprotein E receptor 2 (ApoER2) on platelets was reported (Lutters et al., 2003). These findings partly explain the pathophysiology in this syndrome.

In order to address the question how the binding of aPL/cofactor to these cell surfaces cause production of pro-coagulant molecules, we investigated aPL-inducible genes in peripheral blood mononuclear cell using cDNA array system. Two-hours after exposure to EY2C9, a monoclonal IgM class aCL established from APS patient, mRNAs related to MAPK pathway, such as p38 regulated/activated protein kinase (PRAK), Sp-1, TRAF6 (TNF receptor associated factor 6) and SAPK4 (p38δ), were increased more than two fold. Tissue factor and inflammatory cytokines such as TNF-a and IL-1 expression were also confirmed using real-time PCR (Bohgaki et al., 2004). Using monocyte cell line RAW264.7, phosphorylation of p38 MAPK, translocation of NF-κB to the nuclear fraction, and expression of TF mRNA were demonstrated after treatment with monoclonal aCL. These phenomena were observed only in the presence of β_2GPI. Moreover, a specific p38 MAPK inihibitor SB203580 decreased aCL/β_2GPI-induced TF mRNA expression.

Almost simultaneously, Vega-Ostertag et al. (Vega-Ostertag et al., 2004) treated platelets with aPL and demonstrated increased phosphorylation of p38 MAPK and production of thromboxane B2, which was abrogated by SB203580. F(ab') fragments of purified IgG from patients effectively increased the phosphorylation of p38 MAPK and calcium-dependent cytosolic phospholipae A_2, but not that of ERK-1/2 MAPKs. Recently, the same group investigated human

umbilical endothelial cells (HUVECs) in the similar system and have demonstrated the involvement of p38 MAPK in the up-regulation of tissue factor (Vega-Ostertag et al., 2005). This up-regulation was again inhibited by SB203580, and also by MG132, a specific inhibitor for the downstream NF-κB. They also found that aPL induced HUVECs to express IL-6, IL-8, and inducible nitric oxide synthase, and that these processes involve p38 MAPK activation. Thus, p38 MAPK pathway plays an important role in the aPL-mediated activation of endothelial cells, monocytes, and platelets, providing a posible therapeutic target in APS. P38 MAPK isoforms are activated by environmental stress such as oxidative stress, UV irradiation, hypoxia, ischemia, gram-negative bacteria-derived LPS, or inflammatory cytokines such as TNF-α, IL-1β, and IL-18. Activation of p38 MAPK induces proinflammatory cytokines, such as TNF-α and IL-1β, resulting in enhancement of inflammatory reaction. Following p38 MAPK phosphorylation, transcriptional factors such as activating transcriptional factor-2 (ATF2) are activated, which forms a herterodimer with Jun family transcriptional factors and associates with the activator protein-1 (AP-1) binding site. NH_2-termini of histone H3 undergoes structural alteration in a p38-dependent pathway after LPS stimulation, which results in enhancement of accessibility of the cryptic NF-κB binding sites (Saccani et al., 2002). The promoter region of the tissue factor gene contains two AP-1 binding sites and one NF-κB binding site, and these transcription factors are proven required for maximal induction of TF gene transcription. Proposed mechanisms of cell activation induced by aPL are illustrated in Figure 2.

Thus, it would be reasonable as a choice for the treatment of APS, to suppress p38 MAPK activation using its inhibitors. In murine models, administration of SB203580 was beneficial for endotoxin-induced shock and collagen-induced arthritis. Several other inhibitors for p38 MAPK

have been developed and some of these inhibitors were tested in clinical trials. For example, BIRB796 inhibited LPS-induced coagulation activation, as measured by plasma concentrations of the prothrombin fragment F1+2, during human endotoxemia (Branger et al., 2003). RWJ67657 inhibited TNF-α, IL-8, and IL-6 in human without significant adverse effects (Faas et al., 2002). Although expression of p38 MAPK is relatively ubiquitous and p38 MAPK also activates anti-inflammatory IL-10 and tumor-suppressive p53, p38 MAPK suppression is an attractive choice of treatment in the future. At the same time, hunt for more specific treatment target would be favorable. It remains to be determined how aCL/β_2GPI binds to the cell surface and how signal transduction events occur upstream of p38 MAPK in monocytes, which are major producer of TF, although apoER2 on platelets and toll-like receptor or annexin II on endothelial cells are proposed as "ligands" for β_2GPI.

Conclusion

aCL/β_2GPI plays multiple roles in the pathogenesis of thromboses found in APS, firstly by deranging fibrinolytic system, secondly by accelerating atherosclerosis, and lastly by activating monocytes, endothelial cells and thrombocytes to produce tissue factor or thromboxane. To date, anti-platelet agents or anticoagulants are utilized to prevent the recurrence of thrombosis in patients with APS. However, because of the difference of bioavailability among patients or of adverse effects, more specific therapy would be desirable. Understanding the interaction between aPL and cell surface and following signaling events will provide tools for developing novel therapies in patients with APS.

Figure legend

Figure 1.

Activity of intrinsic fibrinolysis in APS patients.

The intrinsic fibrinolytic activities of euglobulin fractions from APS patients and healthy controls were measured in the presence of β_2GPI, using kaolin was used as an activator.

Figure 2.

Proposed function of antiphospholipid antibodies on endothelial cells, monocytes, or platelets. β_2GPI; β_2-glycoprotein I, aCL; anticardiolipin antibody, p; phosphorylation, EC; endothelial cell, TF; tissue factor, apoE2R; apolipoprotein E receptor 2, $cPLA_2$; calcium-dependent cytosolic phospholipae A_2, TXB_2; thromboxane B_2

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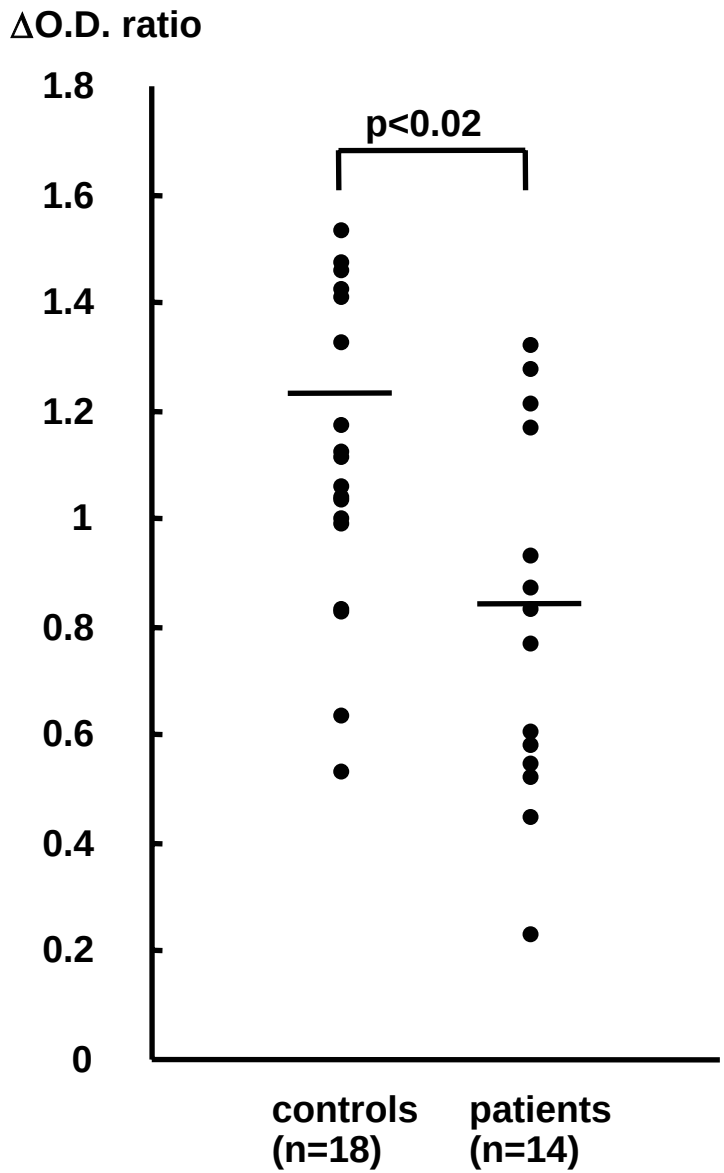
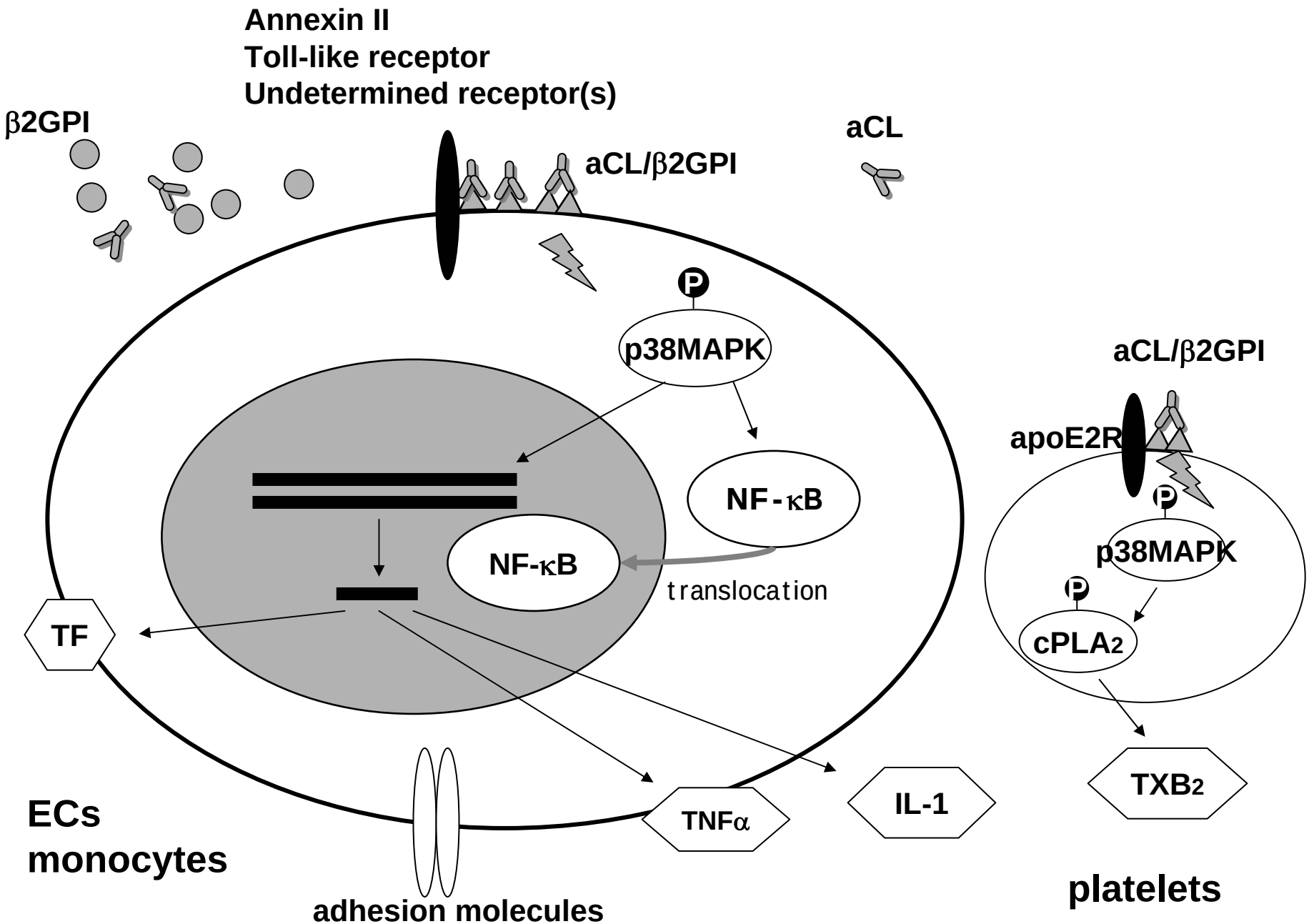


euglobulin fraction
+ phospholipid
+ plasminogen

↓ kaolin

S-2251
(plasmin-specific
substrate)


Annexin II
Toll-like receptor
Undetermined receptor(s)
β2GPI
aCL
aCL/β2GPI
P
p38MAPK
NF-κB
NF-κB
translocation
TF
aCL/β2GPI
apoE2R
P
p38MAPK
P
cPLA2
TXB2
TNFα
IL-1
ECs
monocytes
adhesion molecules
platelets