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TNF-alpha and annexin A2: inflammation in thrombotic primary antiphospholipid syndrome

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Abstract Antiphospholipid syndrome (APS) is characterized by thromboses and/or pregnancy losses. Laboratory criterion for the diagnosis of APS is the presence of antiphospholipid antibodies (anticardiolipin, anti-beta2-glycoprotein I (aβ2gpI) and lupus anticoagulant). On the one hand, the latest classification criteria for the diagnosis of APS emphasized that thrombotic manifestations of the syndrome should be without any signs of an inflammatory process, while on the other hand, some recent reports have suggested that APS is a "pro-inflammatory state." This article is focused on the importance of TNF-alpha and annexin A2 (anxA2) for patients with vascular (thrombotic) manifestations of the primary APS. The classic antithrombotic and antiplatelet therapy does not protect APS patients from the development of recurrent thrombosis. Therefore, an urgent need for the introduction of new therapeutic approaches in the treatment of APS patients is obvious. This review provides a rationale for the necessity for the use of immunomodulatory medications that could interfere with β2gpI binding to its receptor(s), such as anxA2, and/or inhibit TNF-alpha activity.

Keywords Annexin A2 · Antiphospholipid antibodies · Primary antiphospholipid syndrome · Tumor necrosis factor alpha

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Antiphospholipid syndrome

"Antiphospholipid syndrome (APS) can be primary (PAPS) when it is not associated with another disease, secondary (SAPS) when it is associated with other autoimmune disease (most often systemic lupus erythematosus, SLE) or life-threatening catastrophic (CAPS) syndrome that it is characterized by multiple thromboses of various vital organs" [1–3].

Antiphospholipid syndrome is an autoimmune disease that is characterized by arterial and/or venous thrombosis and/or recurrent pregnancy loss (RPL) [2]. Obstetric APS (OAPS) is considered as a distinct entity from vascular APS (VAPS) [4] due to the facts that some APS patients can display only thrombosis (without RPL) or vice versa and that simultaneous presence of both thrombosis and RPL was observed in small fraction of APS pregnancies [5, 6].

Laboratory criterion for the diagnosis of APS is the presence of antiphospholipid antibodies (aPL Abs: anticardiolipin (aCL), anti-beta2 glycoprotein I (aβ2gpI) and lupus anticoagulant (LA)). The group of Abs against PLs is increasing, and new studies are revealing the importance of Abs against various annexins [7, 8].

Due to the fact that thrombotic events occur only occasionally despite the persistent presence of aPL Abs, a "two-hit hypothesis" has been introduced. According to this hypothesis, presence of aPL Abs represents a "first hit" that increases the thrombophilic risk and the clotting occurs in the presence of another factor ("second hit"). It was suggested that inflammation represents a "second hit" for aPL Abs-mediated thrombosis and RPL. However, results from experimental study, where rats received lipopolysaccharide (LPS) before infusion of the IgG anti-β2gpI Abs and develop thrombosis [9], provide a rationale for vice versa conclusion, i.e., that inflammatory response may act as a "first hit," while anti-β2gpI Abs might represent

a "second hit" [10]. Although the exact nature of "first hit event" is not elucidated yet, there are reports that emphasized the essential roles of the innate immunity receptors, co-receptors and accessory molecules in the response of monocytes (MOs), platelets and endothelial cells (ECs) to aPL Abs [10]. A recent studies suggested that annexin A2 (anxA2) is a receptor for β2gpI [11–13] on certain cell types and that the treatment of MOs with β2gpI/anti-β2gpI Abs complexes induced tumor necrosis factor alpha (TNF-alpha) expression [12]. The importance of TNF-alpha as a cause of placental thrombosis and spontaneous abortions in OAPS has been reported in several studies [14–16]. In vivo murine models have revealed that injection of soluble receptors (that block TNF-alpha activity) had protective effect against the induction of abortions by aPL Abs [17].

A murine model of experimental APS (expAPS) has revealed brain inflammation, thrombosis and increase in TNF-alpha levels [18, 19]. Thrombosis and inflammation are interlinked in many clinical conditions [12], but the role of inflammation in vascular (thrombotic) APS is not elucidated yet.

On the one hand, the latest classification criteria for the diagnosis of APS [2] emphasized that thrombotic manifestations of the syndrome should be without any signs of an inflammatory process [1, 2], while on the other hand, some recent reports [20, 21] have suggested that APS is a "pro-inflammatory state." Above-mentioned studies suggested the importance of pro-inflammatory cytokines in OAPS [4, 14–16]. In addition, although experimental animal models of APS provided some evidence for the importance of pro-inflammatory responses in the pathogenesis of aPL Abs-mediated thrombotic events, studies that included patients are confusing (overlapping groups of patients with SAPS, SLE and PAPS, etc.). Therefore, the analysis of TNF-alpha and anxA2 importance only for patients with vascular (thrombotic) manifestations of the primary APS is the main focus of the article. Due to the fact that PAPS patients frequently suffer from recurrent thrombotic episodes (despite lifelong anticoagulant or antiplatelet therapy), there is an urging need for the introduction of new therapeutic approaches that would complement previously mentioned classic treatment of the disease.

Tumor necrosis factor alpha: general information

Tumor necrosis factor alpha is regarded as "the most pleiotropic of all cytokines" [22–24]. The main cell sources of TNF-alpha are macrophages, T lymphocytes, natural killer (NK) cells, dendritic and other cell types [22, 24].

Tumor necrosis factor alpha is a 26-kDa transmembrane protein, while TNF receptor I (TNF-RI) and II (TNF-RII) are two distinct TNF receptors expressed as trimmers in the plasma membranes of most cell types [22]. The recruitment of an adaptor protein that activates caspases and elicits apoptosis is observed when cytokine binds to TNF-RI. The induction of gene expression and/or cell death can be elicited by different members of TNF receptor family [24].

Tumor necrosis factor alpha has multiple effects on ECs and leukocytes which together enhance the local delivery of cells that are engaged in fighting the microorganisms or in tissue repair. The increase in the expression of the ligands for leukocyte integrins (such as intracellular adhesion molecule 1, ICAM-1, and vascular cell adhesion molecule 1, VCAM-1) and induction of E-selectin expression on post-capillary venules are the consequences of TNF-alpha activation of transcription factors (including NF-kB) [24]. The chemokine secretion by various cell types is also induced by TNF-alpha. Functions of TNF-alpha on endothelium and on leukocytes have fundamental role for local inflammatory response to microorganisms [24]. Tumor necrosis factor alpha is also involved in inflammation observed in autoimmune diseases, in which case neutrophils and macrophages are activated secondarily to adaptive immune system stimulation by self-(auto-)antigens [24]. Different functions of TNF-alpha are presented in Table 1.

Table 1 Functions of TNF-alpha [22–24]

1	Necrosis of tumors (as a consequence of tumor blood vessels thrombosis)
2	Apoptosis
3	Intravascular thrombosis (due to the impairment of normal anticoagulant properties of endothelium)
4	Stimulates endothelial cell expression of tissue factor
5	Stimulates monocyte and neutrophil adhesion to endothelium
6	Inhibits protein C system
7	Inhibits expression of thrombomodulin (it is an inhibitor of coagulation)
8	Inhibits myocardial contractility and vascular smooth muscle tone (consequence of that is decrease in blood pressure or shock)
9	Increases prostaglandins synthesis in hypothalamic cells (leads to fever, hence the name "endogenous pyrogen")
10	Cachexia

Annexin A2: general information

Annexins (anx) are a group of structurally similar proteins [7, 8, 25–27] that must express two major features: the presence of "annexin repeat" or "annexin fold" (composed of a fourfold repeat of approximately 70 amino acid residues) and the ability to bind to negatively charged phospholipids (in a Ca^{2+}-dependent manner) [8, 25–27]. The members of anx family are classified into groups designated as A, B, C, D, E, and group A is further subdivided into A1–A12 and A13. The term "annexinopathies" was first used by Rand et al. for the description of human diseases that were related to dysregulations in annexins activity and/or their expression [8, 25, 26].

Annexin A2 (anxA2) is also known as annexin II, p36, lipocortin II [28]. It is a 36-kDa protein that belongs to the annexin superfamily [29]. The 30-kDa core structure of anxA2 is composed of four Ca^{2+}-binding "annexin repeats" (each is composed of five alpha-helices that are connected by short loops and are highly homologous to other annexin family members) [29–32]. The 3-kDa N-terminal tail of anxA2 is unique and is composed of approximately 33 amino acid residues. The N-terminal domain of anxA2 has several functions. It was suggested that it contains a binding sites for p11 and that includes tyrosine and serine phosphorylation sites [30]. However, N-terminal domain of anxA2 is not a target antigen of anti-annexin A2 Abs. Therefore, for now, the nature of immunodominant epitope of anxA2 is not elucidated and further studies are needed to investigate whether immunodominant epitope is located on C-terminal domain of anxA2 [30].

Annexin A2 is expressed in various tissues: intestine, lungs, spleen, brain, kidney, brush-border membrane of placental syncytiotrophoblast and by different cell types including MOs, ECs, smooth muscle cells, keratinocytes and variety of tumor cells [26–31].

Annexin A2 exists in several forms: monomer, heterodimer and heterotetramer. Two molecules of anxA2 and two molecules of p11 (S100A10) form heterotetramer $(anxA2*p11)_2$, which exists in outer leaflet of the cell membrane. Annexin A2 monomer can be predominately located in the cytoplasm, but it can also be found in the nucleus [32].

Annexin A2 functions as a co-receptor for tissue plasminogen activator (tPA) and plasminogen (PlG) [31]. It was suggested that both PlG and tPA interact with anxA2 from $(anxA2*p11)_2$ heterotetramer on ECs surface [29]. However, other studies suggested that anxA2 binds tPA through N-terminal domain, while PlG binds to lysine from C-terminal domain of anxA2 or from the p11 subunit of $(anxA2*p11)_2$ heterotetramer [33, 34]. Kwon et al. [35] have reported that p11 is a binding site for tPA and PlG, while anxA2 function as an "anchor for p11 to the plasma membrane" [29]. Therefore, although the exact binding site(s) of anxA2 for tPA and PlG remains inconclusive, it was suggested that anxA2 functions as a cofactor for "plasmin generation and for localization of fibrinolytic activity to the cell surface" [36]. The presence of anxA2 would provide a predisposition toward a thrombolytic environment, hence the name "fibrinolytic receptor" [33]. Fan et al. [37] have reported that exogenous, recombinant anxA2 might be useful as an "enhancer of tPA-mediated thrombolysis."

Alterations in ECs that lead to reduction in the anxA2 expression (or chemical modifications of anxA2) would initiate a predisposition toward cardiovascular diseases [25], and it was reported that the anxA2 levels in cardiomyocytes were increased during the end-stage heart failure [25].

In addition, it was reported that anxA2 is a fundamental cellular target for the glutathiolation (as a response to oxidative stress provoked by TNF-alpha or hydrogen peroxide) [25]. Table 2 presents some functions of anxA2.

Table 2 Functions of annexin A2 [27–32, 48, 49]

1	Co- receptor for tissue plasminogen activator (tPA) and plasminogen (promotes fibrinolytic activity, hence the name "fibrinolytic receptor")
2	Potential adjunct to conventional thrombolytic therapy (preclinical studies)
3	Different cell functions (endocytosis, exocytosis, DNA synthesis, RNA binding, F-actin reorganization, cell proliferation, adhesion and migration)
4	Angiogenesis and tumor metastasis, apoptosis; inflammation
5	Placental transport of immunoglobulins
6	Down-regulation of anxA2 (and increase in anti-anxA2 Abs) was observed in placentas from patients with preeclampsia
7	Receptor for β2gpI on certain cell types (mediates the binding of β2GPI to ECs; anxA2 is involved in ECs activation by anti-β2GPI Abs
8	Target for autoantibodies (anti-annexin A2 Abs) in patients with APS (anxA2 is involved in the pathogenesis of APS-associated thrombosis)
9	Increased anxA2 expression in patients with acute promyelocytic leukemia (promotes myeloma cell growth, reduces apoptosis in myeloma cell lines, increases osteoclast formation)
10	High anxA2 levels observed in idiopathic pulmonary fibrosis and immune liver fibrosis

The importance of TNF-alpha and anxA2 in PAPS

Dunoyer-Geindre et al. [38] incubated the ECs with anti-β2gpI Abs, and this elicited the slow redistribution of NF-kB from the cytoplasm to the nucleus (with a delay in several hours) and in increased tissue factor (TF) expression and increased expression of leukocyte adhesion molecules. However, incubation of ECs with TNF-alpha provoked fast redistribution of NF-kB (within 30 min) and more pronounced expression of adhesion molecules and TF. The authors have concluded that NF-kB response to anti-β2gpI Abs is indirect due to slower response compared to response induced by TNF-alpha [38]. Vega-Ostertag [39] reported that mechanism for aPL Abs-induced TF expression is p38 mitogen-activated protein kinase (p38MAPK) phosphorylation in EC and platelets.

It was suggested that aCL Abs act synergistically with TNF-alpha in the induction of pro-coagulant (pro-thrombotic, pro-inflammatory) phenotype of EC that is characterized by increased expression of E-selectin, VCAM-1, ICAM-1, chemokines, pro-inflammatory cytokines (such as TNF-alpha), TF and fibrinolysis inhibitor (PAI-1) [11, 12, 40]. A correlation has been found between thrombosis and pro-inflammatory cytokines. Patients with PAPS had significantly increased TNF-alpha levels in comparison with control subjects [19]. In addition, higher levels of TNF-alpha have been observed in APS patients with thrombosis in comparison with patients without [20].

Bertolaccini et al. [41] have reported that TNF-alpha levels were increased in APS patients (no difference between PAPS and SAPS patients in this regard was obtained) in comparison with healthy control subjects. It was suggested that TNF-alpha levels might be "rather associated with the pathogenesis of thrombosis (this feature is typical for both PAPS and SAPS) than with the inflammation (this feature is very limited in PAPS)" [41, 42].

The authors of the study [42] have measured the TNF-alpha concentrations in 147 SLE patients, 21 SLE-like syndrome (SLE-LS) patients, 36 SAPS and only 20 PAPS patients and in healthy control subjects, and the authors have reported that all the above-mentioned patient groups had higher TNF-alpha concentrations in comparison with control subjects. In addition, the highest TNF-alpha concentrations were observed in PAPS patients and this was followed by values for TNF-alpha concentrations obtained in a group of SAPS patients [42].

The authors of the study [42] have reported that patients with LA or with increased levels of the IgG isotype of aCL and/or anti-β2gpI Abs had higher TNF-alpha levels in comparison with the patients without any type of aPL Abs. Therefore, the authors [42] concluded that the presence of aPL Abs was associated with higher TNF-alpha levels in the mixed group of patients (SLE, SLE-LS, SAPS, PAPS), but this finding is inconclusive regarding the above-mentioned groups of patients separately. In addition, the authors [42] have found a weak, but statistically significant correlation between aCL IgG Abs and TNF-alpha concentrations. However, the authors did not find such an association for the IgM isotype of aPL Abs. Interestingly, an highly significant association of both the IgG and IgM isotypes of aCL, anti-β2gpI, anti-anxA5 and anti-oxLDL Abs with TNF-alpha concentrations was obtained in the setting of non-autoimmune disease where both TNF-alpha and aPL Abs were present in low concentrations [43].

The authors of the study [42] did not find any significant differences in TNF-alpha levels between patients with clinical features of APS and those patients without it in mixed group of analyzed subjects [42]. In disagreement, a study [44] that included well-formed group of 44 PAPS patients that were subdivided according to their clinical features has found that PAPS patients with cerebrovascular insults (which are one of the main complications of arterial thrombosis in PAPS patients) showed a positive correlation between TNF-alpha and the IgG isotype of aCL Abs. However, PAPS patients with myocardial infarctions and patients with peripheral arterial thrombosis showed no association between TNF-alpha concentrations and any of the analyzed aPL Abs [44]. Only PAPS patients with pulmonary emboli (which are the main complication of venous thrombosis in PAPS) had a positive correlation between concentrations of TNF-alpha and the IgM isotype of anti-annexin A5 Abs [44]. However, studies that analyze the association between TNF-alpha and anxA2 (or anti-annexin A2 Abs) in PAPS are lacking.

A study [21] that investigated the difference between female and male PAPS patients in regard to pro-inflammatory proteins has revealed that although TNF-alpha and aPL Abs were equally distributed between female and male PAPS patients, a positive correlation between TNF-alpha concentrations and the IgG isotype of anti-β2gpI Abs was only observed in male PAPS patients [21].

The importance of complement activation in APS was first observed in animal models of aPL Abs-induced pregnancy losses due to the fact that C4a, C3a and C5a (anaphylatoxins) increased vascular permeability and enhanced TNF-alpha secretion (from monocytes), which further increased coagulation and inflammation [45, 46]. Oku et al. [47] have reported the existence of hypocomplementemia (lower C3 and C4 values in comparison to healthy subjects and in patients with non-SLE connective tissue diseases) in vascular (thrombotic) PAPS. The authors [47] suggested that observed hypocomplementemia in PAPS was a consequence of complement pathway activation and complement protein consumption due to the fact that significant negative

(inverse) correlation between low C3 and C4 and increased C3a and C4a levels was observed. Therefore, the authors suggested that aPL Abs provoked MOs and Mfs activation via above-mentioned anaphylatoxins [47].

It was reported that anxA2 is autoantigen in some rheumatic diseases, such as SLE and APS [40]. Alterations in anxA2 levels were associated with various clinical conditions [48, 49], but the focus of this article is on the importance of this protein for clinical and/or serological features of APS. It was reported that anxA2 mediates the pathogenic effects of aPL Abs in vivo and in vitro APS [11]. Annexin A2 mediates EC activation by aPL/aβ2gpI Abs [28]. In addition, it was reported that anxA2 was involved in the activation of MOs by anti-β2gpI Abs/β2gpI complexes [50]. It was suggested that effects of aPL Abs are not mediated via Fc receptors, and it was hypothesized that anxA2 might be receptor for β2gpI on ECs. Anti-β2gpI Abs bind to domain I (DI) of β2gpI molecule, while domain V (DV) of β2gpI serves to anchor β2gpI to anxA2 on ECs [51].

Zhang et al. [28] have reported that anti-β2gpI Abs activate EC in the presence of β2gpI and that anti-anxA2 Abs can "provoke" EC activation in a "similar magnitude and time course." In addition, bivalent anti-anxA2 $F(ab)_2'$ fragments also elicited EC activation, but monomeric Fab fragments did not cause activation (actually these fragments blocked activation induced by anti-anxA2 and $F(ab)_2'$ fragments). Binding of β2gpI to an EC receptor, with receptor cross-linking or clustering occurring as a result of the binding of anti-β2gpI Abs to receptor-bound β2gpI may lead to activation of EC signaling responses and cellular activation. Unstimulated EC binds β2gpI through a high-affinity interaction with anxA2 expressed on EC. However, anxA2 is a non-transmembrane protein. Therefore, it was suggested that anxA2 requires "unknown adaptor protein" for signal transduction to the cells and it was proposed that toll-like receptors (TLRs) might represent "structure" responsible for signal transduction. Toll-like receptors are a component of innate immunity, and these receptors can recognize various products of microorganisms [10, 24, 52]. In addition, TLRs are involved in the pathogenesis of autoimmune diseases [10]. The TLR family members belong to type I transmembrane proteins composed of intracellular signaling domain (TIR) and extracellular leucine-rich repeats (LRR). These receptors are expressed in lymphoid and non-lymphoid tissues [10, 24, 27, 52].

It was demonstrated that TLR4 is included in activation of ECs and MOs by aPL Abs [52]. In addition, Sorice et al. [53] have reported that anti- β2gpI Abs reacted with β2gpI in association with anxA2 and TLR4 (in lipid rafts of the MOs' plasma membrane) and in this way a pro-thrombotic, pro-inflammatory phenotype was obtained [53]. Recently, it was published that aPL Abs-mediated effects in an arterial model of thrombosis were dependent on TLR4 [54]. In addition to importance of TLR4 and TLR2, a study by Doring et al. [55] has revealed the relevance of TLR8 in APS. The authors of the study [55] observed that aPL Abs increased TNF-alpha secretion from MOs by up-regulation of TLR8 mRNA and protein expression levels.

Heterotetrameric complex (anxA2*p11)2 is located in lipid rafts of neuronal dendrites [56], and it was reported that anxA2 was associated with neurological manifestations observed in APS patients [57]. Higher anti-anxA2 Abs titers were associated with behavioral changes in experimental mouse model, and in addition, the authors observed the alterations in the levels of components of (anxA2*p11)2 heterotetramer complex [57]. Ceserman-Maus et al. [36] have reported that anti-anxA2 Abs were more prevalent in patients with cerebral venous thrombosis than in healthy control subjects (12.5 vs. 2.1 %, $P < 0.01$) [36].

Anti-anxA2 Abs blocked endothelial surface tPA-dependent generation of plasmin and induced EC to express elevated levels of TF, and therefore, it was suggested that anti-anxA2 Abs have multiple pathogenic effects in the setting of autoimmune diseases [29, 58].

Ceserman-Maus et al. [59] have reported that anti-anxA2 Abs were more frequent in APS patients with thrombosis than in healthy subjects (22.6 vs. 2.1 %, $P < 0.000$). In addition, in this group of APS patients, anti-anxA2 Abs were more prevalent in comparison with patients with non-autoimmune thrombosis (22.6 vs. 0 %, $P = 0.017$) or SLE patients without thrombosis (22.6 vs. 6.3 %, $P < 0.001$) [56].

Salle et al. [60] have analyzed the prevalence of anti-annexin A2 Abs in 53 SLE patients, 71 patients with primary Sjogren syndrome, 17 with systemic sclerosis, 18 with systemic vasculitis, 119 with rheumatoid arthritis, 99 healthy control subjects and only 16 patients with PAPS. The authors concluded that anti-anxA2 Abs were present in 14.8 % of PAPS patients and in 2 % of control subjects. In addition, the authors observed an inverse correlation between anti-anxA2 and aPL Abs (aCL and anti-β2gpI Abs).

Ao et al. [61] have analyzed the anti-anxA2 Abs in 101 APS patients (only 19 had PAPS), 41 patients with SLE and thrombosis, 124 SLE patients without thrombosis and 120 healthy control subjects. They have reported that frequency of the IgG isotype (the IgM isotype was not analyzed) of anti-anxA2 Abs was 21.8 % in APS patients (but the authors did not differentiate between PAPS and SAPS patients), 26.8 % in SLE patients with thrombosis, 6.5 % in SLE without thrombosis and 4.2 % in healthy control subjects. In a mixed group of 266 patients with APS and SLE, 142 had a history of thrombosis and/or RPL while 124 did not have such history. The authors have observed that 33 of 142 (23.24 %) patients with history of thrombosis and/or

RPL had positive titers of anti-anxA2 Abs while 8 of 124 (6.45 %) patients without history of thrombosis and/or RPL also had elevated anti-anxA2 Abs levels. Therefore, the authors observed that the IgG anti-anxA2 Abs were more prevalent in mixed group of patients with APS and SLE with thrombosis than in healthy subjects and SLE patients without thrombosis, and authors concluded that the presence of the IgG anti-anxA2 Abs was associated with APS clinical features. In addition, they suggested that determination of anti-anxA2 Abs would be beneficial in seronegative APS patients (i.e., they have clinical features of the syndrome, but are negative for laboratory criteria for recommended aPL Abs).

Conclusion

The classic antithrombotic and antiplatelet therapy in the treatment of APS patients does not protect these patients from the development of recurrent thrombotic episodes. Therefore, an urgent need for the introduction of new therapeutic approaches in the treatment of APS patients is obvious and this review provides a rationale for the necessity for the use of immunomodulatory medications that could interfere with β2gpI binding to its receptor(s), such as anxA2, and/or inhibit TNF-alpha activity.

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Compliance with Ethical Standards

Conflict of interest Author Mirjana Bećarević declares that she has no conflict of interest related to this manuscript.

Ethical approval This article does not contain any studies with human participants performed by any of the authors.

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