APOPTOSIS, ANNEXIN A5 AND ANTI-ANNEXIN A5 ANTIBODIES IN THE ANTIPHOSPHOLIPID SYNDROME

APOPTOZA, ANEKSIN A5 I ANTITELA ANEKSINA A5 U ANTIFOSFOLIPIDNOM SINDROMU

Mirjana Bećarević[1,2], Svetlana Ignjatović[2,3], Nada Majkić-Singh[2,3]

[1]*Medical Faculty, University of Novi Sad, Serbia*
[2]*Clinical Center of Serbia, Belgrade, Serbia*
[3]*Pharmaceutical Faculty, University of Belgrade, Serbia*

Summary

It has been proposed that apoptosis is one of the mechanisms involved in the generation of antiphospholipid antibodies. The presence of antiphospholipid antibodies is the main laboratory criterion for a definite diagnosis of the antiphospholipid syndrome. Annexinopathies are disorders characterized by deregulation of annexins expression levels and function. Annexin A5 has been used as an agent for molecular imaging techniques (visualization of phosphatidylserine-expressing apoptotic cells) *in vitro* and *in vivo* in animal models and in patients (injection of human recombinant anxA5 into the patient's circulation). Although the determination of titers of anti-annexin A5 antibodies is not mandatory for the diagnosis of the antiphospholipid syndrome, it was reported that patients with primary antiphospholipid syndrome with a history of recurrent abortions had elevated titers of anti-annexin A5 antibodies, while the presence of thromboses was not associated with elevated levels of these antibodies.

Keywords: apoptosis, annexin A5, anti-annexin A5 antibodies, antiphospholipid syndrome

Kratak sadržaj

Apoptoza je predložena kao jedan od mehanizama uključenih u generisanje antifosfolipidnih antitela. Prisustvo antifosfolipidnih antitela glavni je laboratorijski kriterijum za dijagnozu definitivnog antifosfolipidnog sindroma. Aneksinopatije su poremećaji koji se karakterišu deregulacijom nivoa ekspresije aneksina i njihove funkcije. Aneksin A5 se koristio kao agens za tehnike molekularnog snimanja (vizuelizacija apoptotičnih ćelija koje ispoljavaju fosfatidilserin) *in vitro* i *in vivo* u animalnim modelima i kod pacijenata (injektovanje humanog rekombinantnog aneksina A5 u cirkulaciju). Iako određivanje titra antitela anti-aneksina A5 nije obavezno za dijagnozu antifosfolipidnog sindroma, prijavljeni su slučajevi pacijenata sa primarnim antifosfolipidnim sindromom i istorijom spontanih pobačaja koji su imali povišene titre antitela anti-aneksina A5, dok prisustvo tromboza nije bilo povezano sa povišenim nivoima ovih antitela.

Ključne reči: apoptoza, aneksin A5, antitela anti-aneksina A5, antifosfolipidni sindrom

Antiphospholipid Syndrome

Antiphospholipid syndrome (APS) is an autoimmune disease that is characterized by thromboses (arterial and/or venous) and/or the presence of recurrent miscarriages associated with the presence of antiphospholipid antibodies (aPL) (1). It may be primary (PAPS) or secondary antiphospholipid syndrome, when it is associated with another disease. According to the revised and updated criteria for the diagnosis of the APS, the presence of at least one clinical and one laboratory criterion is necessary for the diagnosis of definite APS (2). Clinical criteria are the presence of thromboses (arterial and/or venous) and/or the presence of pathological pregnancies. Laboratory criteria are: the presence of IgG and/or IgM anticardiolipin antibodies in medium and high titers, anti-β2glycoprotein I (β2gpI) antibodies of IgG and/or IgM class and/or the presence of lupus anticoagulant (2).

Address for correspondence:

Mirjana Bećarević, PhD
Institute of Medical Biochemistry, Clinical Center of Serbia
Višegradska 26, Belgrade, Serbia
e-mail: mb58303@gmail.com

Antiphospholipid antibodies are a heterogenous group of antibodies, and originally it was supposed that these antibodies were directed against negatively charged phospholipids. Also, it was thought that these phospholipids are a specific subset of lipids that play a crucial role in the coagulation cascade (3, 4).

Previously, it was proposed that apoptosis (programmed cell death) is one of the mechanisms responsible for the generation of antiphospholipid antibodies (5).

Apoptosis is characterized by DNA cleavage, nuclear condensation and fragmentation, changes in membrane lipid distribution, detachment of cells from the extracellular matrix, and these transformations result in the phagocytosis of cells. This type of cell death is in contrast with necrosis (plasma membrane integrity is impaired and cellular contents are enzymatically degraded and released, which results in pathological inflammation) (6).

Exposure of phospholipids during apoptosis provides an antigenic stimulus for the production of antiphospholipid autoantibodies. In addition, the phospholipid–protein complexes formed during apoptosis are targeted by pathogenic antiphospholipid antibodies (7).

Phospholipids – Components of Cell Membranes

The lipid composition of the inner and the outer leaflet of the plasma membrane is different.

The inner, cytoplasmatic leaflet is mainly composed of aminophospholipids (phosphatidylserine and phosphatidylethanolamine), while cholinephospholipids (sphingomyelin and phosphatidylcholine) comprise the outer leaflet of the plasma membrane.

The maintenance of the membrane asymmetric distribution of lipids is crucial for normal cell functions (8). The lipid asymmetry of the plasma membrane is maintained by an energy-dependent lipid transport mechanism. Translocase activity is ATP-dependent and involves inward movement of phosphatidylserine and phosphatidylethanolamine to the inner side of the plasma membrane, while floppase activity is also ATP-dependent and promotes outward movement of both aminophospholipids and cholinephospholipids (9). During apoptosis (10), phosphatidylserine moves from its physiological location in the inner leaflet of the plasma membrane to the outer leaflet (11).

Modification of the plasma membrane phospholipid distribution is an event that provides a signal related to different mechanisms occurring in various pathophysiological processes, such as the activation of the coagulation cascade, recognition and removal of the apoptotic cells. Exposure of phosphatidylserine on apoptotic cell membranes has two functional pro-

perties. Firstly, it contributes to the activation of the coagulation factors (IX, VIII, X, V, II) and the generation of thrombin. Secondly, it acts as a recognition signal for macrophages which are involved in the clearance of apoptotic cells (12). The following antigen processing and presentation by antigen presenting cells provide an antigenic stimulus to specific clones of T-lymphocytes and B-lymphocytes which leads to production of antiphospholipid antibodies. These antibodies enhance the immune response to phospholipid–protein complexes by promoting an antibody-mediated phagocytosis (7).

Cardiolipin is a phospholipid that is located in the inner mitochondrial membrane (13). During death receptor-mediated apoptosis, cardiolipin and its metabolites shift from mitochondria to other organelles and to cell surface (14). Changes in the distribution of cardiolipin occur before or during membrane exposure of phosphatidylserine (15).

Cardiolipin is the most highly unsaturated lipid in the human body (16). It is composed of two phosphate groups and four fatty acid chains (16). Cardiolipin metabolites generated by rapid deacylation (monolysocardiolipin and dilysocardiolipin) are transported to endoplasmic reticulum for reacylation. The number of acyl chains in cardiolipin derivatives is important for the binding of β2gpl to phospholipids and for the generation of epitopes of anticardiolipin antibodies for specific cardiolipin metabolites.

Hydroperoxidation of cardiolipin is essential for enhancing its binding with antiphospholipid antibodies (17). Apoptosis can lead to cell surface exposure of cardiolipin and monolysocardiolipin which might become targets for antiphospholipid antibodies. The clinical features of the APS may be related to a dearrangement of cardiolipin metabolism.

Endothelial cells and monocytes are cellular targets for antiphospholipid antibodies. Treatment of these cells with TNF-alpha seems to enhance the binding of antibodies directed to endothelial epitopes (18).

Interaction of antiphospholipid antibodies with endothelial cells can trigger an inflammatory response (19). Stimuli capable of triggering apoptosis can also generate reactive oxygen species (ROS). Cells exposed to ROS precursor can undergo death with apoptotic morphology (20).

Antiphospholipid antibodies are directed to neoepitopes of oxidized phospholipids. Also, antiphospholipid antibodies are directed against neoepitopes generated during the formation of adduct products between oxidized phospholipids and associated proteins (17).

It was proposed that *in vivo* β2gpl-phospholipid interaction induces the formation of highly immunogenic phospholipid or protein epitopes. The different

classes of antiphospholipid antibodies are directed against oxidation-dependent and oxidation-independent neoepitopes. Some antiphospholipid antibodies recognized oxLDL and this potential cross-reactivity could be explained by the observation that oxLDL represent a source of both phospholipid and protein oxidized derivatives (21).

Annexins

Annexins are a group of proteins that can bind to negatively charged phospholipids in a calcium-dependent and reversible manner (22). Their homology is located in a conserved core domain including four homologous repeat sequences (65 to 70 amino-acids long) (23). Annexins have predominantly intracellular localization (suggested by the absence of classical signal sequences in their polypeptide chains).

Annexin A5

Annexin A5 is present in cells exposed to blood (platelets, trophoblasts and endothelial cells) (24, 25). It is an anticoagulant of 35.7 kDa (26).

The tertiary structure of anxA5 consists of a core of four domains that are arranged in a cyclic array. This arrangement gives the molecule a slightly curved shape with a convex and a concave face. The domains within the anxA5 molecule interact with each other via non-covalent interactions. The interdomain interactions result in the formation of two associated modules consisting of domains IV and I, and domains II and III. The interactions between domain I and IV are mediated in a non-covalent manner by the amino-terminal tail. Domains II and III are covalently linked via a short inter-helical turn (27). Domain III is the most distant from the membrane surface, while domain II is the closest (28, 29). All four domains contain Ca^{2+}- dependent binding motifs (28).

The Ca^{2+} and phosphatidylserine binding sites are located at the convex, membrane-facing side of the protein (30, 31). A short aminoterminal tail is located at the concave side of the molecule. N-terminal tail is recognized as the binding site for various ligands (phospholipase A2, heparin, etc.).

In solution, anxA5 molecules are monomers, but upon binding to phosphatidylserine-expressing membranes, they form trimers. This is followed by the formation of a two-dimensional crystal lattice. Membrane-bound annexin A5 assembles into a trimer which is organized in such a way that domain II of each individual molecule is located in the centre of a trimer (32).

The capacity of annexin A5 to bind to phosphatidylserine has led to a proposal that annexin A5 has antithrombotic features (33, 34). Annexin A5 competes with Va, Xa and prothrombin for binding to phosphatidylserine and prevents formation of the prothrombinase complex and consequently formation of thrombin (35). Also, it was proposed that annexin A5 forms a twodimensional lattice on the phosphatidylserine expressing surface and prevents formation of thrombin (36). Anticoagulant properties of annexin A5 *in vitro* have been described (37, 38), but the physiological function of a low concentration of circulating levels of annexin A5 is not clear yet (25).

Annexin A5 can inhibit phospholipase A2 (PLA2) in cytosolic and also in soluble forms (39). Phospholipase A2 is an enzyme important in prostaglandin synthesis and in the generation of inflammatory phospholipids (40, 41). This enzyme is present in atherosclerotic lesions (42, 43).

Inhibition of annexin A5 binding may increase PLA2 activity, and, as a consequence, this leads to raised production of inflammatory lipids (44). In addition, annexin A5 binds to proteoglycans in the arterial wall and this might be an important early step in atherogenesis (45).

In a rabbit carotid artery injury model, it was demonstrated that arterial thromboses could be inhibited by recombinant annexin A5 (46). It was suggested that annexin A5 might be used as a therapeutic agent that reduces the risk of plaque rupture and atherothrombosis by covering exposed procoagulant surfaces in the plaque (44).

Annexin A5 binds to apical surfaces of placental syntitiotrophoblasts and could therefore be important for the maintenance of blood flow through the placenta (47). Annexin A5 is deficient in placentas from patients with APS. Also, it is deficient in cultured trophoblasts and endothelial cells exposed to antiphospholipid antibodies. It was reported that anxA5 forms an antithrombotic shield around procoagulant anionic phospholipids (which blocks their participation in phospholipid-dependent coagulation reactions), but in APS patients formation of this antithrombotic shield is disrupted by antibodies with specificities against phospholipid-binding proteins.

Annexin A5 was used as an agent for molecular imaging techniques (visualization of phosphatidylserine-expressing apoptotic cells) *in vitro* and *in vivo* in animal models and in patients (injection of human recombinant anxA5 into a patient's circulation.

Anti-Annexin A5 Antibodies

Annexinopathies are disorders characterized by deregulation of annexin expression levels and function (48). It was suggested that anti-annexin A5 antibodies might be the cause of thrombotic events interfering with the functions of anxA5 (49, 50).

Anti-annexin A5 antibodies cause placental thrombosis and fetal absorption in mice (51), while some authors reported no association between anti-annexin A5 antibodies and a history of thrombosis (52). Weak, but statistically significant correlation between the IgM isotype of anti-annexin A5 antibodies and adiponectin concentrations was found in type II diabetes mellitus patients ($r = 0.285$, $p = 0.011$) (53).

Anti-annexin A5 antibodies have been detected in patients with some systemic autoimmune diseases (systemic lupus erythematosus, rheumatoid arthritis) and this observation was associated with higher incidences of intrauterine fetal loss, preeclampsia and arterial and venous thromboses (54–57). Although elevated anti-annexin A5 antibody levels were found in patients with different autoimmune diseases, the role of anti-annexin A5 antibodies in PAPS has not been explained yet. Previously, in a study which included 192 patients with SLE and only six patients with PAPS, it was found that detection of anti-annexin A5 antibodies of the IgG and IgM isotype is not relevant for identification of patients at risk for thrombosis (58).

In agreement with the abovementioned investigation, in a study (59) which included 44 PAPS patients, no correlation between anti-annexin A5 antibodies and arterial or venous thromboses was found. Positive correlation was found between concentrations of the IgM isotype of anticardiolipin antibodies, anti-β2gpI antibodies and anti-annexin A5 antibodies of the same isotype were found in PAPS patients (59).

Antiphospholipid antibodies can decrease the binding of annexin A5 to the endothelium thereby promoting atherothrombosis and its clinical manifestations (myocardial infarctions, stroke) (60). However, another study found no association between anti-annexin A5 antibodies and myocardial infarctions in patients with primary antiphospholipid syndrome (59).

PAPS patients with pulmonary emboli showed a positive correlation between IgM isotype of anti-annexin A5 antibodies and TNF-alpha ($r = 0.894$, $p = 0.041$) (61). In PAPS with cerebrovascular insults a positive correlation was noticed between TNF-alpha and the IgG isotype of anti-annexin A5 Abs ($r = 0.768$, $p = 0.006$) (61). Isotype analysis of antiphospholipid and anti-annexin A5 Abs and investigation of their association with TNF-alpha is important for differentiation of PAPS patients that are prone to further deterioration of arterial and venous thromboses (61).

During placental development trophoblasts fuse to form syncytiotrophoblast (62) which is accompanied by surface expression of phosphatidylserine making syntitium a potential site for activation of coagulation processes (63, 47). Annexin A5 is abundantly present on the syncytial surface and forms a twodimensional lattice, thereby preventing the coagulation process (64, 65). Antiphospholipid antibodies can disrupt the organization of the annexin A5 twodimensional lattice (66).

It was reported that patients with APS had significantly reduced annexin A5 levels on placental villi (64). Also, it was reported that antiphospholipid antibodies decreased levels of annexin A5 on the surfaces of cultured trophoblasts and placental villi (67). These findings suggest that reduced expression of annexin A5 in placentas leads to a hypercoagulable state in the intervillous space (57).

Exposure of anionic phospholipids is a substrate for cationic annexin A5 binding which neutralizes procoagulant phospholipids and increases antigen density that is suitable for anti-annexin A5 antibodies (57). Also, anti-annexin A5 antibodies have a role in pregnancy-associated complications, because 36% of patients with habitual fetal loss had anti-annexin A5 antibodies. These antibodies were found in 20% of patients with preeclampsia (68). The association between anti-annexin A5 antibodies and abortions in the general female population is controversial (69).

Only PAPS patients with a history of repeated miscarriages have significantly elevated IgG anti-annexin A5 antibody concentrations (OR = 4.788, $p = 0.036$, 95% CI: 1.104–20.762). This finding must be viewed with caution because it reflects a small number of patients with recurrent abortions (n=6) that were included in the study (59). Therefore, it is possible that the IgG isotype of anti-annexin A5 antibodies is a predictor of recurrent abortions in PAPS. Also, these antibodies might occur after one or more abortions, and thus represent an immunological epiphenomenon of abortions (70).

Previously, it was reported that annexin A5 is involved in lupus anticoagulant-induced apoptosis because the IgG isotype of anti-annexin A5 antibodies induced apoptosis of endothelial cells (71).

Monoclonal anti-annexin A5 antibodies reacting with annexin A5 induced syncytiotrophoblast apoptosis in the primary trophoblast culture (57).

Although the determination of titers of anti-annexin A5 antibodies is not mandatory for the diagnosis of the antiphospholipid syndrome, it was reported that patients with primary antiphospholipid syndrome with a history of recurrent abortions had elevated titers of anti-annexin A5 antibodies, while the presence of thromboses was not associated with elevated levels of these antibodies.

Conflict of Interest Statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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