Autoantibodies to Annexin A2 and cerebral thrombosis: Insights from a mouse model

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Abstract
Introduction: Antiphospholipid syndrome (APS) is an autoimmune disorder manifested by thromboembolic events, recurrent spontaneous abortions and elevated titers of circulating antiphospholipid antibodies. In addition, the presence of antiphospholipid antibodies seems to confer a fivefold higher risk for stroke or transient ischemic attack. Although the major antigen of APS is β2 glycoprotein I, it is now well established that antiphospholipid antibodies are heterogeneous and bind to various targets. Recently, antibodies to Annexin A2 (ANXA2) have been reported in APS. This is of special interest since data indicated ANXA2 as a key player in fibrinolysis. Therefore, in the present study we assessed whether anti-ANXA2 antibodies play a pathological role in thrombosis associated disease.

Materials and Methods: Mice were induced to produce anti-ANXA2 antibodies by immunization with ANXA2 (iANXA2) and control mice were immunized with adjuvant only. A middle cerebral artery occlusion stroke model was applied to the mice. The outcome of stroke severity was assessed and compared between the two groups.

Results: Our results indicate that antibodies to ANXA2 lead to a more severe stroke as demonstrated by a significant larger stroke infarct volume (iANXA2 133.9 ± 3.3 mm3 and control 113.7 ± 7.4 mm3; p = 0.017) and a more severe neurological outcome (iANXA2 2.2 ± 0.2, and control 1.5 ± 0.18; p = 0.03).

Conclusions: This study supports the hypothesis that auto-antibodies to ANXA2 are an independent risk factor for cerebral thrombosis. Consequently, we propose screening for anti-ANXA2 antibodies should be more widely used and patients that exhibit the manifestations of APS should be closely monitored by physicians.

Keywords
Annexin, autoimmunity, antiphospholipid syndrome, thrombosis, stroke, MCAo

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Introduction

Antiphospholipid syndrome (APS) is an autoimmune disorder, manifested by thromboembolic events (arterial and venous), recurrent spontaneous abortions and elevated titers of circulating antiphospholipid antibodies (aPL). Similar antibodies are found in up to 50% of patients with systemic lupus erythematosus (SLE). Arterial thrombosis accounts for a third of the thromboembolic events of APS and primarily affects cerebral blood arteries. In 2015 the Antiphospholipid Syndrome Alliance for Clinical Trials and International Networking estimated 13.5% of individuals with stroke are aPL positive. A recent study demonstrates that the presence of aPL seems to confer a five-fold higher risk for stroke or transient ischemic attack (TIA). A recent study demonstrates that the levels of aPL correlate with stroke severity and outcome in young patients with APS. In addition, a wide variety of effects on coagulation proteins, endothelial cells and platelets had been ascribed to aPL. Thus, aPL are not only serological markers for APS, but direct contributors to the development of thrombosis.

The major antigen of aPL is beta-2-glycoprotein I (β2GPI) and anti-β2GPI antibodies are considered to be the primary perpetrators of the APS hypercoagulable state. However, it is now well established that aPL are heterogeneous and bind to various protein targets and interact with diverse cell types, receptors, and enzymes with over 20 autoantigens reported in APS. Auto-antibodies to Annexin A2 (ANXA2) have been reported in APS by 3 different groups in 3 different populations (French, Chinese, and Mexican). The important role of anti-ANXA2 antibodies in thrombosis and fibrinolysis is also consistent with reports of the presence of these antibodies in the sera of patients suffering from pre-eclampsia and cerebral venous thrombosis. Moreover accumulating evidence indicates the protein ANXA2 is related to thrombosis, coagulation and fibrinolysis: In children with sickle cell disease, single nucleotide polymorphisms in the ANXA2 gene are associated with increased risk of stroke, whereas other ANXA2 single nucleotide polymorphisms have been associated with elevated risk of avascular necrosis of bone (osteonecrosis), hypothesized to be caused by reduced blood flow to the bone.

Substantial data indicate that ANXA2 can bind to β2GPI and ANXA2 can also form a heterotetramer with the protein p11 (also known as S100A10) and this complex is a major co-receptor for plasminogen and tissue plasminogen activator (tPA) indicating ANXA2 is a key regulator of the fibrinolytic process. Cesarman-Maus and co-workers have reported human ANXA2 antibodies can promote thrombosis via two mechanisms: First by inhibiting the tPA-dependent plasmin formation and second by inducing endothelial cell activation and tissue factor expression that can promote coagulation and fibrin formation. These results suggest that cell surface ANXA2 represents a prominent autoantibody target associated with a thrombosis.

We have established an animal model by immunizing mice with recombinant highly purified ANXA2. These animals do not develop antibodies to β2GPI and display behavioral changes unlike those found in β2GPI-immunized mice. In the present study, we assessed whether anti-ANXA2 antibodies can play a pathological role in thrombosis associated disease using the middle cerebral artery occlusion (MCAo) stroke model in the ANXA2 immunized mice model compared to adjuvant immunized controls. Our results indicate that the presence of antibodies to ANXA2 leads to a more severe stroke as demonstrated by significantly larger stroke volume and more severe neurological outcome.

Methods

Expression and purification of the recombinant ANXA2 protein

Recombinant human (98% identical to the mouse ANXA2) ANXA2+His-tag was expressed in E. coli cells and purified using a His-Trap column (GE Healthcare) as previously described. Human subjects

Serum samples were collected from 4 patients with primary APS, mean age for patients was 44 ± 5, and from normal anonymous age matched controls as part of a larger study (EASI-ANA #8112-10). The definition of APS was according to the 2006 classification criteria and specifically all patients exhibited high levels of antibodies to cardiolipin/beta2glycoprotein1 or anticoagulant together with clinical vascular involvement.

Animals

Female BALB/c mice obtained from Envigo Laboratories Limited (Israel) were housed under standard conditions (23°C ± 1°C, 12 h light/dark cycle (7 am ± pm) with ad libitum access to food and water. The animal procedures were approved by the Chaim Sheba Medical Center (Tel Hasomer, Israel) Animal Welfare Committee (#1078/16/ANIM).

Immunization protocol

BALB/c mice, 16-week-old females were immunized subcutaneously in the lower flanks with 30μg
recombinant human ANXA2, emulsified in Freund’s Complete Adjuvant (CFA); respective control mice were injected with CFA alone. Four weeks later, a boost injection with the same amount of antigen (diluted in PBS) was administered subcutaneously to the mice; control mice received PBS only.

**Blood coagulation tests**

Three weeks after the boost injection mice were sacrificed. Blood samples were collected from the vena cava to tubes with 3.2% buffered sodium citrate and centrifuged at 1500 g for 10 min. Afterward, 200 µl of the plasma was collected and stored at −20 °C.

To test the effect of IgG on plasma, 30 µl of human citrated plasma from healthy donors (Instrumentation Laboratory) were supplemented with: 1000 µg of immunoglobulin G (IgG) purified from iANXA2 mice, 1000 µg of IgG purified from control mice, 10 µg of commercial affinity purified rabbit polyclonal anti-ANXA2 IgG (Novus biologicals NBPI-31310) or 10 µg of commercial affinity purified rabbit polyclonal anti-Dopamine D2 receptor IgG (Merck Millipore AB5084P) (control antibody).

Plasma samples were sent to American Medical Laboratories LTD (Israel) for prothrombin time (PT) and activated partial thromboplastin time (aPTT) measurements. Briefly, determination of coagulation factors was performed on the Sysmex CA 1500 coagulation analyzer. PT was determined using the Dade Innovin reagent that initiates the clotting via the extrinsic and common pathway. aPTT was determined using the Dade Actin FS reagent that, after the addition of calcium ions, triggers the coagulation process via the intrinsic pathway.

**ROTEM (Rotational thromboelastometry) assay.**

Thromboelastometry is a whole blood assay performed to evaluate the viscoelastic properties during blood clot formation and lysis. The tPA-ROTEM was performed according to published methods,26 within 2 h of obtaining the human blood sample. The EXTEM mode was used by adding tissue factor, CaCl₂ and rtPA to the whole blood. Selected samples of mice sera were added to the tube immediately before the reaction started (control = 4, ANXA2 = 4 animals). The device temperature was set to 37 °C and maximum runtime was 60 min. The following parameters were analyzed: Alpha angle (the speed at which a solid clot forms), CFT (clot formation time), AUC total (area under the curve during all reaction), AR15 (area under the curve after 15 min), AUC total-AR15 (area under the curve during the last phase of the reaction), and LI30 (the percentage of remaining clot stability in relation to the maximum clot firmness value, at 30 min after clot starts to form).

**Western blot analysis for anti-murine ANXA2 antibodies**

**Blood clots:** Blood samples were collected from the mice vena cava to clean tubes and incubated at RT for 1 h. The samples were centrifuged at 1500 g for 10 min. The blood clots were collected and stored at −20 °C.

**GL261 murine glioma cell-line:** Mouse glioma cells were grown in Dulbecco’s modified Eagle's medium (DMEM; Bet Haemek, Biological Industries, Israel) supplemented with 10% fetal bovine serum (Bet Haemek, Biological Industries, Israel) and 1% penicillin and streptomycin in a 5% CO2-humidified atmosphere. Cells were seeded in T-75 flask (1X10⁶ cells/flask). Following 24 hours incubation, cells were transferred to serum-free medium for another 24 hours. The cells were washed and lysed in RIPA buffer (containing in mM: 50 TRIS HCl pH 8, 150 NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS), a protease inhibitor cocktail (Merck Millipore 539134, 1:100), 0.1 mM sodium orthovanadate and 2 mM PMSF. The cells were scraped, collected, and centrifuged (16,000g × 20 min) at 4 °C. The supernatants were collected and stored at −20 °C.

**Western blot:** Protein concentration was determined by means of a bicinchoninic acid (BCA) assay. Samples (30 µg) were separated by SDS-polyacrylamide gel electrophoresis. The proteins were transferred onto nitrocellulose membranes. Membranes were incubated with rabbit-annexinA2 antibody (1:500, NBPI-31310, Novus, known to cross react with mouse ANXA2) or pooled serum of ANXA2 mice (1:100), overnight at 4 °C and washed with Tris-buffered saline and 0.1% Tween 20 (TBST). Membranes were then incubated at RT with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibody (1:10,000, Jackson Immunoresearch Laboratories) and detected by ELC. Protein bands were detected by a peroxidase based ECL method.

**Induction of middle cerebral artery occlusion**

The middle cerebral artery occlusion was performed as described in our previous work.27 Briefly, mice were anesthetized with 2.5% isoflurane mixed in oxygen and delivered through a facemask. Under an operating microscope, the right common carotid artery (CCA) was exposed through a midline neck incision and was carefully dissected free from surrounding nerves and fascia, and from its bifurcation to the base of the skull. The occipital artery branches of the external...
carotid artery (ECA) were then isolated, dissected and coagulated. The CCA and the internal carotid artery (ICA) were temporally ligated, and a knot was placed on the distal part of the ECA with a 6-0 silk suture. Next, a 6-0 silk suture was tied loosely around the ECA close to its bifurcation. A monofilament suture (Doccol Corp, Redlands, CA, USA) was inserted through a small hole in the ECA and the loose knot was tied on the ECA to prevent bleeding through the arteriotomy. The ECA was cut between the knots and the tied section, or stump attached proximal to the CCA junction was straightened to allow the filament to enter the ICA and block the middle cerebral artery (MCA). The filament was carefully advanced up to 11 mm from the carotid artery bifurcation or until resistance was felt, confirming that the filament was not in the pterygopalatine artery. The temporal ligations of the ICA and CCA were removed and the neck was closed using 5-0 silk suture. During surgery, the body temperature of the mice was maintained at 37°C with the aid of a heating blanket. Following surgery, the mice were kept in a heated chamber until recovery. Exclusion criteria include massive bleeding during surgery and/or brain hemorrhage.

**Evaluation of neurological deficits after MCAo**

Twenty four hours after the occlusion, neurological deficits were measured for each animal and scored using a five-point neurologic severity score as described by Longa et al.28 A score of 0 indicated no neurologic deficit, a score of 1 (failure to extend left forepaw fully) indicated a mild focal neurologic deficit, a score of 2 (circling to the left) indicated a moderate focal neurologic deficit, a score of 3 (falling to the left) indicated a severe focal deficit; and mice with a score of 4 (did not walk spontaneously) had a depressed level of consciousness, therefore not included in the study.

**Calculation of infarct volume**

After evaluating the neurological score, the animals were sacrificed using an overdose of 50 mg/kg of sodium pentobarbital injected intraperitoneally. The brain of each animal was immediately removed and placed in a steel brain matrix (1 mm, Coronal, Stoelting, IL, USA). The brains were cut starting at their anterior side (starting at slice 3, 2 mm anterior to the bregma). The slices were placed in a 24-well plate filled with 750 μl 2% triphenyltetrazolium chloride (TTC) solution for a duration of 30 min. Next, the slices were moved to a new plate filled with 750 μl of 4% formaldehyde for fixation. After 30 min of fixation, the slices were mounted on microscope slides and scanned (600 DPI resolution) for infarct area analysis. The area of the lesion was manually traced and calculated using ImageJ (NIH), a Java based image processing program. All calculations were performed blinded with respect to the group assignments and the mice.

**Statistical analysis**

Statistical analyses were conducted using Graphpad Prism v. 6 for Windows (Graphpad, CA, USA). Statistical significance difference between the groups was assessed by one way unpaired Student’s t test for parametric parameters and one way unpaired Mann-Whitney for non-parametric parameters. For multiple comparison 2-way ANOVA with repeated measures followed by the Sidak’s multiple comparison test was used. Data are presented as mean ± standard error of mean (SEM). Statistical significance was determined as P < 0.05.

**Results**

We have established an ELISA method for measuring ANXA2 antibodies in a mouse model.24,25 Currently, we utilized this method to measure such antibodies in humans. As can be seen in Figure 1(a), patients with APS had significantly higher levels of anti-ANXA2 antibodies than age and gender matched controls (0.3635 ± 0.03 and 0.24 ± 0.02 a.u., respectively, p < 0.02 by t test). Figure 1(b) displays representative levels of anti-ANXA2 antibodies in mice induced by immunization with ANXA2 and in adjuvant immunized control mice measured by the same assay used in humans and presented in comparable absorbance units. Levels of the antibodies in these mice are significantly higher than the controls (0.74 ± 0.08 and 0.14 ± 0.013 a.u., respectively, p < 0.0001 by t-test) and more than the human samples (approximately 5.5 versus 1.5 fold increase, respectively). There is a clear overlap between the high anti-ANXA2 human values and the mean levels measured in mice. The specificity of the generated antibody produced in ANXA2 mice was assessed by western blot analysis of the binding to mouse tissues expressing ANXA2. As can be seen in Figure 1(c), the serum antibodies bound to a major band corresponding to the expected molecular weight ANXA2 in the glioma cells and this was confirmed by similar binding of commercial anti-ANXA2 antibody. Interestingly, both sera antibodies and commercial antibody bound a lower MW protein in the mouse clot which probably represents a proteolytic cleavage product of ANXA2 and we consider the definitive identification of this band to have no major implication on the conclusions of the manuscript.29 We thus utilized these mice to examine the effect of such antibodies on coagulation and stroke.
Since the literature reports that anti-ANXA2 may interfere with the coagulation process and can inhibit fibrinolytic process, we assessed blood coagulation characteristics in the in vivo mouse model and in vitro. Mice were immunized with recombinant ANXA2 emulsified in CFA and compared to controls, immunized with CFA alone. Four weeks later a boost injection was given to the mice and, as found in our previous studies, this results in an elevation in anti-ANXA2 antibodies in the mice sera which peaks two weeks after the boost. Next, plasma samples were taken from the mice and coagulation parameters were analyzed. This analysis revealed no difference in the mean coagulation time of the extrinsic and common pathway as measured by PT (iANXA2 9.6 ± 0.64 sec, n = 4 and control 9.1 ± 0.19 sec, n = 5; p = 0.41, Figure 2(a)) nor a difference in the mean coagulation time of the intrinsic pathway as measured by aPTT (iANXA2 24.2 ± 3.2 sec, n = 8 and control 20.7 ± 1.6 sec, n = 8; p = 0.33, Figure 2(b)). However, when the individual levels of the mice are observed, it is evident that the variances of aPTT measurements in the iANXA2 group were significantly higher than in the control group (F(1,14) = 7.6, p = 0.0157, Figure 2(b)) which may indicate indirectly a complex interaction of the antibodies with the coagulation cascade. In order to study the direct and immediate effects of anti-ANXA2 antibodies on coagulation we applied high levels of commercial rabbit polyclonal anti-ANXA2 IgG or IgG purified from iANXA2 mice to human plasma in-vitro. Total IgG derived from rabbit and mice used as controls. Neither of these antibodies affected the coagulation time measured by aPTT (data not shown). In order to study the effects of anti-ANXA2 antibodies on fibrinolysis we measured the direct effect of ANXA2 mice sera on clot formation and lysis by the ROTEM test (representative charts are seen in Figure 2(c) and (d)). Significant difference were found between the measures in the presence of ANXA2 and control mice sera (AUC_{total}: p < 0.05, Figure 2(e), AUC_{total-AR15}: p < 0.04, Figure 2(g), and LI30: p < 0.04, Figure 2(j)) but not in the alpha-angle measure (p > 0.2, Figure 2(h)), the CFT (p > 0.1, Figure 2(i)) and the AR_{15} (p > 0.05, Figure 2(f)), indicating ANXA2 sera components modify the general complex process of clot formation, probably with negligible effect to clot formation phase and most significantly to the fibrinolysis phase (from 15 minutes after clot formation started, when fibrinolysis is most substantial).

Two weeks after the boost injection, when the level of anti-ANXA2 antibody peaked, mice were subjected to MCAo and an examination of neurological deficits was preformed 24 h following MCAo. iANXA2 mice demonstrated a significantly higher neurological score compared to control mice (iANXA2 2.2 ± 0.2, n = 10 and control 1.5 ± 0.18, n = 8; p = 0.03 by Mann-Whitney), indicating that the stroke in iANXA2 mice had a more severe outcome than in control mice (Figure 3).

Next, the brains of the mice were cut coronally into sections (Figure 4(a)) and stained with TTC (Figure 4(b)), as described in Methods to assess the extent of the ischemic damage by calculating the total infarct volume, which demonstrated larger infarct size in the iANXA2 mice compared to control mice (Figure 4(c),

![Graphs](image-url) **Figure 1.** Anti-ANXA2 antibodies levels in serum. (a) Moderate but significant increased serum levels of anti-ANXA2 antibodies in APS patients (n = 4) compared to matched controls (n = 14) (t test, p = 0.0162). (b) Pronounced and significant increased serum levels of anti-ANXA2 antibodies in mice immunized with ANXA2 injection (n = 5) compared to adjuvant immunized control mice (n = 5) (t test, p value < 0.0001). (c) Western blot analysis of anti-ANXA2 mouse serum and commercial anti-ANXA2 antibody to mouse blood clot and glioma derived tissues showing a major band corresponding to ANXA2 at the predicted MW. Values are presented as mean±SEM.
iANXA2 133.9 ± 3.3 mm³, n = 10 and control 113.7 ± 7.4 mm³, n = 8; p = 0.017). Evaluation of the distribution of infarct size, by serial sections of the brain lesion caused by MCAo, revealed that the major difference between groups was at areas outside of the ischemic core (slices No. 3 & 8) (Figure 4(d), iANXA2 39.5 ± 2.2 mm³, n = 10 and control 26.9 ± 2.9 mm³, n = 8; p = 0.015 by two way ANOVA with repeated measures.

Figure 2. Assessment of anti-ANXA2 antibodies effect on blood coagulation. (a) PT (n = 4 for iANXA2 and n = 5 control) and (b) aPTT (n = 8 for each group) analysis of plasma samples from iANXA2 and controls mice. In both tests, no difference in the averages of the groups was observed. However, the variances in each group of the aPTT measurements are significantly different (F test p value = 0.0157). Representative charts of ROTEM test in the presence of (c) control mice sera and (d) iANXA2 mice sera. ROTEM measures analysis of area under the curve (e) during all reaction (AUCtotal), (f) during the initial 15 min of clot formation (AR15) and (g) during the last phase of the reaction (AUCtotal-AR15). ROTEM kinetics measures analysis of (h) the speed at which a solid clot forms (alpha-angle) and (i) clot formation time (CFT). (j) The percentage of remaining clot stability in relation to the maximum clot firmness value, at 30 min after clot starts to form (lysis index, LI30). *p = 0.05.
with Sidak’s correction). Interestingly, this area is outside the ischemic core (slices No. 5-6) and may represent an area of potentially reversible damage.

Discussion

The main findings of the present study indicate that mice with high levels of anti-ANXA2 antibodies display a larger lesion area induced by MCAo than adjuvant immunized controls. This is consistent with the observation that iANXA2 mice have a significantly more severe neurological deficit. These results are in agreement with the known association of APS with stroke and suggests that anti-ANXA2 antibodies specifically are involved in this increased risk in patients with APS and SLE.

The model induced in the present study represents antibodies specific to ANXA2 with no cross-reactivity with β2GPI. Therefore, it represents a unique opportunity to evaluate the specific contributions of these antibodies to ischemic stroke in contrast to the more heterogeneous and complex model induced by β2GPI immunization. The aPTT results of iANXA2 mice model are different from the effects on aPTT described in mice immunized with β2GPI. This seems to indicate that the effects of anti-ANXA2 antibodies on thrombosis and fibrinolysis differ from those associated with anti-β2GPI antibodies. This is in line with the suggestion that the auto-antibodies to ANXA2 alone may represent a significant risk factor for cerebral thrombosis, independent of the other criteria needed to establish the diagnosis of APS. This theory can also explain how some patients exhibit the manifestations of APS but are seronegative for anti-β2GPI antibodies such as in the case of a recently published study.

The present results are immediately relevant to the role of anti-ANXA2 antibodies that have been described in APS and SLE and confirmed in the present study. These antibodies probably affect

![Figure 3. iANXA2 mice demonstrate a more severe neurological score after stroke. Neurological function was evaluated 24 hours following MCAo. The scores in the iANXA2 group (n = 10) were significantly higher than the control group (n = 8). Values are presented as mean ± SEM. *p = 0.05 by Mann-Whitney test.](image-url)
fibrinolysis and therefore have a negative effect on the outcome of ischemic stroke in these patients. Such an effect may also be relevant in patients with such antibodies in the absence of clear APS or SLE. There is a need therefore to establish standardized and widely available laboratory test kits for testing APS, SLE and stroke patients in general for levels of anti-ANXA2 antibodies in order to verify the clinical relevance of these antibodies.

Conclusions
Our results support the hypothesis that auto-antibodies to ANXA2 in patients with APS and SLE are an independent risk factor for cerebral thrombosis. If this association is indeed significant, it raises the possibility of treating the risk of thrombosis by lowering levels of anti-ANXA2 antibodies with immnosuppressant drugs, plasma exchange or B-cell depleting drugs. Consequently, we propose screening for anti-ANXA2 antibodies should be more widely used and that positive patients should be closely monitored by physicians.

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