Vimentin/cardiolipin complex as a new antigenic target of the Antiphospholipid Syndrome

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Running title: Anti-vimentin/cardiolipin antibodies in APS

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ABSTRACT

Antiphospholipid Syndrome (APS) is an autoimmune disease characterized by arterial and venous thrombosis, recurrent abortions and antiphospholipid antibodies (aPL). However, it is possible to find patients with clinical signs of APS, but persistently negative for aPL tests (“seronegative APS”, SN-APS). Aim of this study was to identify new antigenic target(s) of autoantibodies in APS patients, which may also be recognized in SN-APS.

We tested sera from patients with SN-APS by a proteomic approach, analysing endothelial cell-surface membrane proteins. Sera from SN-APS patients revealed two reactive spots, corresponding to vimentin, a protein which is shown to bind cardiolipin in vitro. Anti-vimentin/cardiolipin antibodies were tested in 29 SN-APS patients, 40 APS patients, 30 with systemic lupus erythematosus, 30 with rheumatoid arthritis, 30 with venous or arterial thrombosis and 32 healthy controls. We observed that not only a large proportion of SN-APS patients, but also almost all the APS patients displayed the presence of anti-vimentin/cardiolipin antibodies. To verify the possible pathogenic role of these autoantibodies, we demonstrated that affinity purified anti-vimentin/cardiolipin antibodies induced IRAK phosphorylation and NF-kB activation in endothelial cells.

Our results prompt to identify vimentin as a “new” cofactor for aPL, which may represent a useful tool mainly in SN-APS patients.
INTRODUCTION

Antiphospholipid Syndrome (APS) is an autoimmune disease characterized by arterial and venous thrombosis, recurrent abortions or foetal loss, associated to circulating antiphospholipid antibodies (aPL). Diagnosis of APS requires the combination of at least one clinical and one laboratory criterion. Anticardiolipin (aCL) and anti-β2 glycoprotein-I (anti-β2GPI) antibodies detected by enzyme linked immunosorbent assay (ELISA) and the lupus anticoagulant (LA), detected by clotting assays, are the recommended tests for the detection of aPL. Indeed, aPL represent a heterogeneous family of antibodies that react with serum phospholipid-binding plasma proteins, among which β2GPI represents the main protein cofactor. In addition, protein S, protein C, prothrombin, annexin V or annexin II have been also demonstrated as antigenic targets for these autoantibodies.

Nevertheless, new antigenic targets for aPL in APS have been proposed. In particular, it has been described that antibodies directed to the lyso(bis)phosphatidic acid (anti-LBPA) may represent a marker of APS. Moreover, we demonstrated the possibility of detecting aPL, by immunostaining on thin layer chromatography (TLC) plates. However, in daily clinical practice it is possible to find patients with clinical signs suggestive of APS who are persistently negative for the routinely used aCL, anti-β2GPI and LA tests. Therefore, it was recently proposed for these cases the term of “seronegative APS” (SN-APS). Although it is known that the routine screening tests (aCL and/or LA) might miss some cases, careful differential diagnosis and repeat testing are mandatory before the diagnosis of SN-APS.

Aim of this study was to identify possible new antigenic target(s) of autoantibodies in these SN-APS patients who presented clinical signs of APS, but were repeatedly negative for the conventional used aCL, anti- β2GPI and LA.

Since aPL are able to trigger a signal transduction pathway in endothelial cells, leading to IRAK phosphorylation and NF-kB activation, our primary aim in this study was to seek and characterize endothelial molecules specifically recognized by serum autoantibodies in patients with APS, and with the so-called SN-APS. Thus, we decided to use an autoantibody-based screening method, a proteomic approach using endothelial cell-surface membrane proteins, which represents a potent tool for the identification of target antigens. By this approach, sera from SN-APS patients revealed two strongly reactive spots that we identified as vimentin, a protein which is shown to bind cardiolipin in vitro. We analyzed by ELISA the presence of serum autoantibodies specific to the complex vimentin/cardioli opin and we observed that not only a large proportion of SN-APS patients, but also almost all the APS patients displayed the presence of these antibodies. In order to verify the
possible pathogenic role of these autoantibodies, we demonstrated that IgG specific for the vimentin/cardiolipin complex, from sera of SN-APS patients induced IRAK phosphorylation and NF-κB activation in endothelial cells.

PATIENTS AND METHODS

Patients
This study included highly selected 29 consecutive patients, 20 attending the Lupus Clinic at Saint Thomas’ Hospital of London (UK), and 9 attending the Rheumatology Unit at “Sapienza” University of Rome. All the patients were women with multiple autoimmune manifestations. They presented clinical features consistent with a diagnosis of APS but tested persistently negative (at least 2 times 12 weeks apart) for conventional aCL, anti-β2GPI and LA tests. All experiments involving patients were approved by the Istituto Superiore di Sanità ethical committee. Clinical manifestations included venous and/or arterial thrombosis, and pregnancy morbidity as stated in the classification criteria to definite APS. All the patients showed normal screening for other causes of thrombophilia, such as antithrombin III, protein C and protein S deficiency, hyperhomocysteinemia, Factor V Leiden and prothrombin mutations. For each patient two serum samples have been studied far apart at least 12 weeks.

In addition, we analysed 40 patients with APS, diagnosed according to the classification criteria to definite APS (positive for aCL), 30 patients with systemic lupus erythematosus (SLE), 30 patients with rheumatoid arthritis (RA) and 30 unselected patients with venous or arterial thrombosis. A group of 32 healthy Caucasian controls matched for age, and gender was also included in the study. After having obtained an informed consent from each patient in accordance with the Declaration of Helsinki, a venous bleeding was performed. Sera were stored at –20°C until the assay.

ELISA for anti-β2GPI, anti-annexin V and anti-prothrombin
aCL and anti-β2GPI ELISA kits were obtained from Inova Diagnostics Inc. (San Diego, CA, USA). ELISA was performed according to manufacturer’s instructions. Anti-annexin V and anti-prothrombin were performed as previously described. A positive control and several normal human sera were run in the same assay to confirm the specificity of the results.
**LA test**

LA was studied in two coagulation systems, a dilute sensitized activated partial thromboplastin time (aPTT) and a dilute Russell’s viper venom time (dRVVT), followed by confirm test, using reagents and instrumentation by Hemoliance Instrumentation Laboratory, Lexington, MA, USA.

**Proteomic assay of endothelial cell-surface membrane proteins**

The immortalized hybridoma cell line EAHy926 was used as endothelial cells. Endothelial cells were grown to 60-70% confluence and seeded at 5 x 10^6 well on glass cover slips. Cell surface membrane proteins were purified from endothelial cells using the Pierce Cell Surface Protein Isolation Kit, according to the manufacturer’s instructions with slight modifications (Pierce, Rockford, IL). Briefly, 1 x 10^7 cells were incubated in 1 ml Sulfo-NHS-SS-Biotin, a cleavable biotinylation reagent. After the biotinylation step, we washed the cells twice with Tris Buffered Saline (TBS). The cells were subjected to sonication and the biotinylated proteins were incubated with Immobilized NeutrAvidin Gel (Pierce). After extensive lavage of the gel (nine times), the proteins were eluted according to the protocol and loaded in two dimensional electrophoresis (2DE). Isoelectrofocusing was performed on 7 cm immobilized pH gradient strips (range: pH 3–10) by using the IPGphor Isoelectric Focusing System (Amersham Pharmacia Biotech Little Chalfont, Buckinghamshire, UK). The second dimension was performed on a 10% SDS/PAGE system after equilibrating the strips for 10 min in SDS Equilibration buffer, containing 50 mM Tris/HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, 2% DTT and 2.5% iodoacetamide. Gels were then stained by colloidal Coomassie blue (Sigma-Aldrich, St Louis, MO, USA) or used for Western blot analysis, incubated with human sera diluted 1:50. Peroxidase-conjugated goat anti-human IgG (Biorad, Richmond, CA, USA) were used as second antibodies and the reactions were developed with 3-3’ dianinobenzidine (Sigma Chem. Co).

Colloidal Coomassie blue-stained spots corresponding to those identified by immunoblotting were excised from gels, and destained by washing twice with 50% acetonitrile (ACN) in 5mM ammonium bicarbonate. Mass spectrometry was performed by Nurex service (Nurex srl, Sassari, Italy). Gel pieces were dried for 30 min in ACN. Dried pieces of gel were subjected to protein digestion by trypsin. Mass spectrometry analysis were performed with a MALDI micro MX (Micromass, Manchester, UK) equipped with a delayed extraction unit, according to the tuning procedures suggested by the manufacturer. Sample was loaded onto MALDI target using 2 μL of the tryptic digest mixed 1:1 with a solution of α-cyano-4-hydroxy-cinnamic acid (10 mg/ml in 40% ACN, 0.1 % v/v trifluoroacetic acid). Peak list was generated with Proteinlynx Data Preparation.
using the following parameters: external calibration with lock mass using mass 2465.1989 Da of
adrenocorticotropic hormone, background subtract type adaptive combining all scans, performing
deisotoping with a threshold of 5%. Peak list created as above described was used in Mascot using
Swiss-Prot database. Search settings allowed one missed cleavage with the trypsin enzyme selected,
oxidation of methionine as potential variable modification, carboxamidomethyl of cysteine as fixed
modifications, peptide tolerance of 100 ppm, taxa Human.

Vimentin/cardiolipin complex
Cardiolipin (50 µg/ml) (Sigma Chem. Co) in methanol was evaporated under nitrogen and then was
resuspended with human recombinant vimentin (5 µg/ml) (R&D System, Minneapolis, MN, USA)
in 0.05 µM NaHCO₃ buffer, pH 9.5. The association between vimentin and cardiolipin in the
complexes was tested by coimmunoprecipitation experiments.

Vimentin/cardiolipin binding assay
Vimentin/cardiolipin complexes were re-suspended in a buffer containing 20 mM Tris-HCl, pH 7.5,
0.15 M NaCl, 1 mM EDTA, 0.02 % NaN₃, 10 mM NaF. The mixtures were incubated with 10 µg of
goose polyclonal anti-vimentin (R&D System) per mg of protein and rocked for 2 h at 4°C. At the
end of the incubation, protein A-sepharose (Sigma Chem. Co) was added and the mixture was
rocked at 4°C for an additional 1 h. As a negative control, immunoprecipitation was performed with
an irrelevant goat IgG (Sigma Chem. Co). A major portion of the immunoprecipitate was subjected
to phospholipid extraction according to the method of Folch²⁴ and separated by high-performance
thin layer chromatography (HPTLC) in a single dimension by using a solvent system of
chloroform/methanol/acetic acid/water (100:75:7:4, v/v/v/v). Phospholipids were stained by
exposure to iodide vapors and also immunostained with the purified human aCL IgG as previously
reported.¹⁷
In parallel experiments, a mixture of cardiolipin (and, as a control, phosphatidylcholine,
phosphatidylserine, or 20/80 phosphatidylserine/phosphatidylcholine), including 1% (w/w) [³H]1-
palmitoyl-2-[11-[4-trifluoromethylazirinyl]undecanoyl]-sn-glycero-3-phosphorylcholine
([³H]PTPC/11) in chloroform, was evaporated under nitrogen, according to Perides et al.²⁵ Briefly,
10 µg of vimentin was incubated with 20 µg of phospholipid vesicles at room temperature for 10
min. The reaction mixture was irradiated with UV light and subjected in SDS-polyacrylamide gel
electrophoresis. Bands were cut out and the gel slices incubated in 1ml of toluene-350 (Packard
Instrument Co., Meriden, CT, USA) for 24 h at 37°C, 10 ml of toluene-based scintillation mixture,
containing 0.005% (w/v) 1.4-bis[2-(5-phenyl)-oxazolyl]benzol and 0.416% (w/v) 2.5-
diphenyloxazol was added. After the mixtures had been incubated another 24 h at 37°C, their radioactivities were measured in a Packard Tri-Carb 460CD liquid scintillation spectrometer.

Detection of anti-vimentin/cardiolipin complex antibodies by ELISA

Anti-vimentin/cardiolipin complex antibodies were detected by a slight modification of ELISA previously reported method. Ninety-six-well polystyrene plates were coated and incubated overnight at 4°C with 100 µl/well of cardiolipin (50 µg/ml) (Sigma Chem. Co), in methanol and then with 100 µl/well of human recombinant vimentin (5 µg/ml) (R&D System) in 0.05 µM NaHCO₃ buffer, pH 9.5. Coated plates were incubated overnight at 4°C and then washed three times with phosphate buffered saline (PBS) containing 0.1% Tween 20 (PBS-T). Plates were blocked for 2h at room temperature with 100 µl of 1% bovine serum albumin (BSA) in PBS. After washing three times with PBS-T the wells were incubated, for 1h at room temperature, with 100 µl of patients sera, diluted 1:100 in the blocking buffer. Each serum was analysed in triplicate. Goat polyclonal anti-vimentin (R&D System) was used as positive control. After three washes with PBS-T, the plates were incubated for 1h at room temperature with HRP-conjugated antibodies, either goat anti-human IgG or rabbit anti-goat IgG (Sigma Chem. Co) were diluted in 1% BSA in PBS. The plates were washed three times with PBS-T, the bound peroxidase was then revealed with 100 µl of O-phenylenediamine dihydrochloride and color development was stopped with H₂SO₄ 0.2 M for 5 min. Absorbance was measured at 492 nm in a microplate reader. Data were presented as the mean optical density (O.D.) corrected for background (wells without coated antigen). Thirty-two normal human sera were also tested and a cut-off value was established at a mean of optical density (O.D.)+3 standard deviations of normal human sera. Parallel experiments were performed in which all the procedure was identical without coated cardiolipin/vimentin complex. Virtually no reactivity was detected in all the samples (data not shown). Anti-vimentin antibodies were also detected using human recombinant vimentin (5 µg/ml) (R&D System) as antigen by ELISA.

Purification of specific autoantibodies from patients’ sera.

The purification of antibodies was performed as previously described. In brief, vimentin/cardiolipin complex or, as a control, vimentin alone (R&D System) were spotted onto a nitrocellulose filter and incubated with the sera from patients with APS. To purify antibodies specific for the vimentin/cardiolipin complex, we used sera from patients resulted IgG positive to the complex, but negative to vimentin in ELISA. After washing with PBS-T the antibodies were eluted with glycine 100 mM, pH 2.5, and mixed for 10 minutes. The eluted antibodies were immediately neutralized with 1M TRIS-HCl, pH 8. Antibodies from a preparation of intravenous
immunoglobulin (IVIG), precipitated by saturated ammonium sulfate solution (SAS), were used as a control.

“In vitro” exposure of endothelial cells to affinity purified anti-vimentin/cardiolipin antibodies from SN-APS patients

For “in vitro” studies, HUVEC, Human Umbilical Vein Endothelial Cells, (PromoCell, Heidelberg, Germany) were maintained in PromoCell Growth Medium containing endothelial cell growth medium kit at 37°C in a humified 5% CO₂ atmosphere. Experiments were performed in cells grown to 60-70% confluence. HUVEC were incubated with affinity purified anti-vimentin/cardiolipin antibodies (200 μg/ml), according to Raschi 20, with affinity purified anti-vimentin antibodies (200 μg/ml), with normal human IgG fractions (NHS-IgG, 200 µg/ml), or with LPS (100 ng/ml) as a positive control. All the materials contained less the 0.00025 ng endotoxin/µg protein, as determined by the Limulus amebocyte lysate test, performed at Associates of Cape Cod (Falmouth, MA).

Preparation of cell extracts

Unstimulated or stimulated HUVEC cells with affinity purified anti-vimentin/cardiolipin antibodies, purified anti-vimentin antibodies, NHS-IgG fractions or LPS were incubated for 45 min at 37°C, in 5% CO₂. After treatment the medium was removed, cells placed on ice, washed once in PBS and scraped in PBS. For the preparation of whole cell extracts cells were resuspended in lysis buffer (20 mM HEPES, pH 7.2, 1% Nonidet P-40, 10% glycerol, 50 mM NaF, 1 mM Na₃VO₄, including protease inhibitors, Sigma Chem. Co). DNA was sheared by brief sonication and soluble proteins were recovered after centrifugation of lysates at 15,000 x g for 15 min at 4°C. Nuclear extracts were prepared as described previously. 28 Briefly cells were resuspended in buffer A (20 mM HEPES, pH 7.9, 20 mM KCl, 3.0 mM MgCl₂, 0.3 mM Na₃VO₄, and freshly added 200 µM leupeptin, 10 µM E64, 300 µM PMSF, 0.5 µg/ml pepstatin, 5 mM DTT, 0.1% Nonidet P-40) and vortexed. After 30 min on ice, cells were centrifuged for 30 min at 10,000 x g at 4 °C. Pellets were resuspended in buffer B (40 mM HEPES, pH 7.9, 0.84 M NaCl, 0.4 mM EDTA, 50% glycerol, 0.3 mM Na₃VO₄, and freshly added 200 µM leupeptin, 10 µM E64, 300 µM PMSF, 0.5 µg/ml pepstatin, 5 mM DTT), and vortexed. After 1h on ice, nuclear extracts were cleared at 10,000 x g for 1 h at 4 °C and supernatants were transferred to new vials. Protein content was determined by Bradford assay using BSA as a standard (Bio-Rad Lab.) and samples were frozen at –80 °C.
Western blot analysis of phospho-IRAK1 and phospho-NF-κB

Equal amounts of whole or nuclear extracts proteins (from unstimulated or stimulated HUVEC with affinity purified anti-vimentin/cardiolipin antibodies, purified anti-vimentin antibodies, NHS-IgG fraction or LPS) were separated in 7.5% SDS-PAGE under unreducing conditions. The proteins were electrophoretically transferred onto nitrocellulose membrane (Bio-Rad Lab.) and then, after blocking with PBS, containing 1% albumin, probed with polyclonal anti-phospho-IRAK1 (Cell Signalling, Inc, Danvers, MA, USA) or polyclonal anti-phospho-NF-κB p65 (Cell Signalling, Inc). Bound antibodies were visualized with HRP-conjugated anti-rabbit IgG (Sigma Chem. Co) and immunoreactivity was assessed by the chemiluminescence reaction using the ECL Western blotting system (Amersham Pharmacia Biotech). As a control for nonspecific reactivity, parallel SDS-PAGE gels were blotted as described, using an anti-rabbit IgG (Sigma Chem. Co). As a control for loading and purity of preparation, phospho-NF-κB p65 blotted membranes were stripped and reprobed with polyclonal anti-histone H1 antibodies (Upstate, Billerica, MA, USA). IRAK1 blotted membranes were stripped and reprobed with polyclonal anti-actin antibodies (Sigma Chem. Co).

RESULTS

Demographic and clinical characteristics of SN-APS patients

All patients included in this group were Caucasian women with a mean age of 47.4 years (range 23-82 years), and a mean disease duration of 9 years (range 1-57 years). Clinical characteristics of patients are summarised in Table 1. The prevalence of clinical manifestations of APS in our cohort of patients was 17/29 for vascular thrombosis (10 venous, 6 arterial and 1 venous and arterial thrombosis) and 16/29 for pregnancy morbidity.

All the patients were previously screened for conventional antiphospholipid tests (aCL by ELISA and LA)⁴, as well as for anti-β₂GPI, anti-annexin V and anti-prothrombin, resulting repeatedly negative.

Vimentin is identified as an endothelial protein cofactor in SN-APS patients

In order to identify a possible cofactor protein in these patients’ sera, endothelial cell-surface membrane proteins separated by 2DE were transferred onto nitrocellulose membrane and analyzed
with serum from two SN-APS patients (Fig. 1A). The two spots identified, which revealed a molecular weight of 54 and 57 kDa, were excised from 2DE gel, digested with trypsin, and then analyzed by MALDI-TOF MS. The detected peptide masses were searched against Swiss-Prot database protein. Results of the database search revealed vimentin as significant candidate protein of the two spots that therefore represented two distinct isoforms of the same protein (Fig. 1B and 1C). The cell-surface expression of vimentin on endothelial cells was confirmed by FACS analysis (Figure S1).

Evidence for vimentin/cardiolipin binding “in vitro”

A strict in vitro association between vimentin and cardiolipin was demonstrated by coimmunoprecipitation experiments. Indeed, it shows that both phospholipid and protein are present in vimentin/cardiolipin complexes. Cardiolipin was detected in the immunoprecipitate by HPTLC analysis (Fig. 2A); vimentin was detected by Western blot after SDS-PAGE gel electrophoresis (Fig. 2B). As a control for specificity of the immunoprecipitation an irrelevant goat IgG was employed. Next, we demonstrated that increasing concentrations of vimentin dose-dependently associate with coated cardiolipin, as revealed by anti-vimentin binding (Fig. 2C). The reactivity of serum antibodies to vimentin/cardiolipin complex was shown to be dose-dependent and was completely prevented by previous adsorption with the complex (Fig. 2D). The analysis of labeling of vimentin with \[^{3}\text{H}]\text{PTPC/11 in the presence of cardiolipin compared with other types of phospholipids vesicles revealed that the radioactivity bound to vimentin was 4310\pm128 cpm for cardiolipin, 460\pm14 cpm for phosphatidylcholine, 2170\pm65 cpm for phosphatidylserine and 2520\pm72 for 20/80 phosphatidylserine/phosphatidylcholine mixture. This finding demonstrates that cardiolipin shows a stronger interaction with vimentin, as compared to phosphatidylcholine and phosphatidylserine.

Detection of antibodies to vimentin/cardiolipin

The analysis of the sera under test showed that 37/40 patients with APS (92.4%), 16/29 with SN-APS (55.2%), 13/30 with SLE (43.3%), 5/30 with RA (16.6%), 2/30 (6.6%) with venous or arterial thrombosis and none of healthy subjects displayed IgG anti-vimentin/cardiolipin antibodies. The analysis of IgM anti-vimentin/cardiolipin antibodies revealed similar results (Table 2). The occurrence of both IgG and IgM antibodies was significantly higher in patients with APS, SN-APS and SLE as compared to healthy donors (p<0.0001). Moreover, the occurrence in APS patients was also significantly higher as compared to SLE, RA and patients with venous or arterial thrombosis.
(p<0.0001). O.D. of the sera under test are reported in Fig. 3. The test performed with a second sample obtained at least 12 weeks from the previous one, confirmed the same result in all APS and SN-APS patients. No significant correlation was found between IgG or IgM anti-vimentin/cardiolipin antibodies and clinical manifestations in SN-APS patients (Table 3). Furthermore, no significant correlation was found between IgG or IgM anti-vimentin/cardiolipin antibodies and thrombotic risk factors (see Table 1).

Of note, only 26% of total APS patients showed IgG and 23.1% IgM to vimentin, with O.D. significantly lower as compared to O.D. detected using vimentin/cardiolipin complex (p<0.001) (Table S1). It suggests a role for vimentin/cardiolipin complex in the “in vitro” binding of the antibodies.

**Affinity purified anti-vimentin/cardiolipin antibodies from SN-APS induce IRAK1 phosphorylation and NF-kB activation in endothelial cells**

Western blot analysis of cell lysates showed that affinity purified anti-vimentin/cardiolipin antibodies, as well as LPS, induced IRAK1 phosphorylation, as revealed by anti-phospho-IRAK1 antibodies reactivity (Fig. 4A). On the contrary, cells stimulated with purified anti-vimentin antibodies or with normal human IgG virtually did not show anti-phospho-IRAK1 reactivity. Cell lysates obtained using non IRAK1-specific IgG yielded no reactivity (data not shown).

Since IRAK phosphorylation leads to NF-kB activation, we investigated the effects of affinity purified anti-vimentin/cardiolipin antibodies on p65 NF-kB. Western blot analysis of nuclear extracts revealed that affinity purified anti-vimentin/cardiolipin antibodies, as well as LPS, induced NF-kB phosphorylation, as revealed by anti-phospho-NF-kB p65 Ser antibodies reactivity (Fig. 4B). On the contrary, cells stimulated with purified anti-vimentin antibodies or with normal human IgG virtually did not shown anti-phospho-NF-kB p65 Ser reactivity. Cell lysates obtained using non NF-kB p65-specific IgG yielded no reactivity (data not shown). These findings indicate that IgG from SN-APS induce IRAK1 phosphorylation, with consequent NF-kB activation in endothelial cells.

**DISCUSSION**

Antiphospholipid Syndrome is a clinical entity characterized by a wide spectrum of aPL, including antibodies directed to cofactor proteins (mainly β2GPI, prothrombin, protein S, protein C,
annexin V, annexin II), phospholipids-protein complexes and/or pure phospholipids. However, a seronegative catastrophic antiphospholipid syndrome has been recently described.

In this study, we analysed new possible antigenic target(s) of the antibodies, which prompted to identify these patients. With this aim, we tested sera from patients with so-called seronegative APS by a proteomic approach, analysing endothelial cell-surface membrane proteins. By this analysis we identified vimentin as strongly immunoreactive autoantigen. Vimentin is a cytoskeleton intermediate filament protein ubiquitously expressed. Surface-expressed forms of vimentin have recently been discovered on several cell types, including apoptotic neutrophils and T cells, activated macrophages, platelets, vascular endothelial cells, brain microvascular endothelial cells, Sezary T cells, and skeletal muscle cells. The mechanism by which vimentin reaches the cell surface, which domains are exposed, and its function at the surface, remain unknown. However, vimentin and cardiolipin can interact at the surface of apoptotic cells to form an immunogenic particle. Anti-vimentin antibodies were detected in patients with SLE, however, a role for these antibodies in the diagnosis of this disease has not been confirmed and the detection of these antibodies is not included in the criteria consensus for the diagnosis. In our study, vimentin molecule was shown to be able to bind cardiolipin “in vitro”. Cardiolipin-vimentin binding may be due to electrostatic interaction between positive charged aminoacids of vimentin and negative charged of cardiolipin. Our findings on phospholipids-vimentin interaction revealed that cardiolipin shows a stronger interaction with vimentin, as compared to phosphatidylcholine and phosphatidylserine. These findings are consistent with previous observations of Perides et al., who analyzed the interaction of vimentin with different phospholipid bilayers.

It prompted us to identify vimentin/cardiolipin complex as a molecular target of the antibodies in patients with SN-APS. Interestingly, we observed that not only a large proportion of SN-APS patients, but also almost all the APS patients under test, displayed the presence of anti-vimentin/cardiolipin complex antibodies. It suggests that vimentin may be considered a “new” antigenic cofactor for aPL in APS. This finding is not completely surprising, since a significant correlation between anti-vimentin and anti-cardiolipin antibodies has been already reported. Moreover, their particular role in the pathogenesis of thrombotic events in autoimmune diseases has been described. In particular, Leong and colleagues demonstrated that anti-vimentin antibodies lead to activation of platelets and leukocytes, as revealed by induced expression of P-selectin, fibrinogen, tissue factor and formation of platelet-leukocyte conjugates via platelet-activating factor. Furthermore, platelet vimentin may regulate fibrinolysis in plasma and thrombus formation by binding platelet-derived v bronectin-plasminogen activator inhibitor complexes.
Recently, it was demonstrated that aPL may exert their pathogenic role by triggering a signal transduction pathway involving Toll-like receptor 4 (TLR-4), IRAK phosphorylation, NF-kB activation and translocation with consequent release of proinflammatory and procoagulant factors by endothelial and/or monocytic cells. In order to verify the possible pathogenic role of autoantibodies in SN-APS patients, we verified whether affinity purified anti-vimentin/cardiolipin antibodies from sera of these patients were able to induce IRAK phosphorylation and NF-kB activation by endothelial cells. Our results confirmed this hypothesis.

How can vimentin/cardiolipin complex become antigenic? At present, this point remains unknown. However, previous studies reported a cell surface expression of intermediate filament proteins, including vimentin, in thrombin-activated platelets as well as in apoptotic cells, suggesting that programmed cell death is a physiopathological mechanism which may expose this antigen on the plasma membrane, as well as cardiolipin. The presence of autoantibodies in certain diseases may be caused by abnormal exposure of the autoantigen on apoptotic cells. In fact, caspase-dependent cleavage of vimentin, with consequent exposure of vimentin on the cell surface, is a necessary requisite for apoptosis.

Taken together, our results, obtained with both a proteomic and an immunological approach, prompt to identify vimentin/cardiolipin as a “new” target of the APS. However, although the presence of these antibodies may be considered highly sensitive in these patients, it is not very specific, since they were also detected in 43.3% of patients with SLE and in 16.6% of patients with RA. Detection of these antibodies may represent a useful tool mainly in those patients with clinical features suggestive of APS in which the classical tests for detection aPL result persistently negative.

AUTHORSHIP CONTRIBUTIONS

Ortona, Capozzi, Colasanti, Conti, Alessandri, Longo and Margutti performed research; Garofalo and Misasi designed research and analyzed data; Khamashta and Hughes selected the patients and performed clinical and laboratory analyses; Ortona, Valesini and Sorice designed research and wrote the paper.

CONFLICT OF INTEREST DISCLOSURES

A patent relating to the content of the manuscript is applying.
REFERENCES


Table 1. Clinical characteristics of SN-APS patients.

<table>
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<th>Characteristics</th>
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<td>Vascular thrombosis</td>
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<td>Venous thrombosis</td>
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<td>Pregnancy morbidity</td>
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<td>Normal fetus deaths</td>
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<tr>
<td>Hypercholesterolemia</td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td>8 (27.6)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>8 (27.6)</td>
</tr>
<tr>
<td>OC/HRT</td>
<td>6 (20.7)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>3 (10.3)</td>
</tr>
<tr>
<td></td>
<td>1 (3.4)</td>
</tr>
</tbody>
</table>
Table 2. Prevalence of antibodies to vimentin/cardiolipin complex.

<table>
<thead>
<tr>
<th>Autoantibodies to vimentin/cardiolipin complex</th>
<th>SN-APS</th>
<th>APS</th>
<th>SLE</th>
<th>RA</th>
<th>THROMBOSIS</th>
<th>HEALTHY SUBJECTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-vimentin/cardiolipin IgG</td>
<td>16/29 (55.2%)</td>
<td>37/40 (92.4%)</td>
<td>13/30 (43.3%)</td>
<td>5/30 (16.6%)</td>
<td>2/30 (6.6%)</td>
<td>0/32 (0%)</td>
</tr>
<tr>
<td>Anti-vimentin/cardiolipin IgM</td>
<td>11/29 (37.9%)</td>
<td>32/40 (80.0%)</td>
<td>13/30 (43.3%)</td>
<td>5/30 (16.6%)</td>
<td>0/30 (0%)</td>
<td>0/32 (0%)</td>
</tr>
</tbody>
</table>
Table 3. Prevalence of antibodies to vimentin/cardiolipin complex in SN-APS patients (n=29) according to the clinical manifestations.

<table>
<thead>
<tr>
<th>Autoantibodies</th>
<th>Total thrombosis N=17</th>
<th>Arterial thrombosis N=7</th>
<th>Venous thrombosis N=11</th>
<th>Recurrent thrombosis N=6</th>
<th>Pregnancy morbidity N=16</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n° (%)</td>
<td>n° (%)</td>
<td>n° (%)</td>
<td>n° (%)</td>
<td>n° (%)</td>
</tr>
<tr>
<td>Anti-vimentin/cardiolipin IgG</td>
<td>9 (52.9)</td>
<td>3 (42.8)</td>
<td>7 (63.6)</td>
<td>2 (33.3)</td>
<td>9 (56.2)</td>
</tr>
<tr>
<td>Anti-vimentin/cardiolipin IgM</td>
<td>3 (17.6)</td>
<td>1 (14.3)</td>
<td>3 (27.3)</td>
<td>1 (16.7)</td>
<td>6 (37.5)</td>
</tr>
<tr>
<td>Anti-vimentin/cardiolipin IgG or IgM</td>
<td>9 (52.9)</td>
<td>3 (42.8)</td>
<td>7 (63.6)</td>
<td>2 (33.3)</td>
<td>10 (62.5)</td>
</tr>
</tbody>
</table>
LEGENDS FOR FIGURES

Figure 1. Identification of vimentin as an endothelial protein cofactor in SN-APS patients. (A) Endothelial cell-surface membrane proteins separated by 2DE were transferred onto nitrocellulose membrane and analyzed by immunoblotting with serum from two “APS seronegative” patients. The two spots, with a molecular weight of 54 and 57 kDa, strongly reactive with serum IgG were identified (circled). (B, C) The two spots identified were excised from 2DE gel, digested with trypsin, and then analyzed by MALDI-TOF MS. MS/MS spectra of trypting peptides matching two isoforms of vimentin. X-axis, m/z; y-axis, relative ion intensity. The matched amino acid sequences are bold underlined.

Figure 2. Evidence for vimentin/cardiolipin binding. (A) Vimentin/cardiolipin complexes were resuspended in a buffer containing 20 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1 mM EDTA, 0.02% NaN₃, 10 mM NaF. The mixtures were immunoprecipitated with polyclonal anti-vimentin. The immunoprecipitates were subjected to phospholipid extraction and analyzed by monodimensional HPTLC analysis and then stained by exposure to iodide vapors. As a negative control, immunoprecipitation was performed with an irrelevant goat IgG. (B) Vimentin/cardiolipin complexes were resuspended in a buffer containing 20 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1 mM EDTA, 0.02% NaN₃, 10 mM NaF. The unextracted precipitates were separated by SDS-PAGE and probed with the anti-vimentin Ab. As a negative control, immunoprecipitation was performed with an irrelevant goat IgG. (C) Scalar doses of vimentin in 0.05 μM NaHCO₃ buffer, pH 9.5, from 10 μg/ml to 1.25 μg/ml, were incubated with 50 μg/ml of cardiolipin in methanol. After washes with PBS-T and blocking in PBS containing 3% BSA, plates were incubated with goat polyclonal antibodies against vimentin. (D) Sera of SN-APS patients with detectable levels of anti-vimentin/cardiolipin antibodies (diluted 1:100, 1:200, 1:400, 1:800, 1:1600) were tested by ELISA with vimentin/cardiolipin complex. As a control of binding specificity sera were previously adsorbed with vimentin/cardiolipin (incubation, 2:1 v:v, with 3 mg/ml cardiolipin micelles, containing 3 μg/ml vimentin for 1h at 37°C and then overnight at 4°C. The mixture was centrifuged at 27,000 x g for 15 min at 4°C). The supernatants were kept as adsorbed sera and tested by ELISA.

Figure 3. Anti-vimentin/cardiolipin complex antibodies in patients and healthy subjects. The sera of patients with APS, SN-APS, SLE, RA, venous or arterial thrombosis and of healthy subjects were analyzed by ELISA for the detection of IgG and IgM anti-vimentin/cardiolipin antibodies.
occurrence of both IgG (A) and IgM (B) antibodies was significantly higher in patients with APS, SN-APS and SLE as compared to healthy donors ($p<0.0001$). Moreover, the occurrence in APS patients was also significantly higher as compared to SLE and RA ($p<0.0001$).

**Figure 4. Affinity purified anti-vimentin/cardiolipin antibodies from SN-APS induce IRAK1 phosphorylation and activates NF-kB.** Endothelial cells were stimulated with affinity purified anti-vimentin/cardiolipin antibodies (200 µg/ml), NHS-IgG fraction (200 µg/ml), LPS (100 ng/ml), affinity purified anti-vimentin antibodies (200 µg/ml) for 45 min at 37°C, in 5% CO$_2$ and cellular extracts were obtained. (A) Phosphorylated levels of IRAK1 (p-IRAK1) were analyzed in whole cell extracts by Western blot with anti-phospho-IRAK1 antibodies; for control, the blotted membranes were stripped and reprobed with anti-actin antibodies. Bound antibodies were visualized with HRP-conjugated IgG and immunoreactivity was assessed by ECL. One example representative of the patients with anti-vimentin/cardiolipin antibodies. (B) NF-kB activation was analyzed in nuclear cell extracts by Western blot with anti-phospho-NF-kB p65 Ser antibodies; for control, the blotted membranes were stripped and reprobed with anti-histone H1 antibodies. Bound antibodies were visualized with HRP-conjugated IgG and immunoreactivity was assessed by ECL. One example representative of the patients with anti-vimentin/cardiolipin antibodies.
Figure 1
Figure 2
Figure 4
Vimentin/cardioplin complex as a new antigenic target of the antiphospholipid syndrome

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