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Anti-beta2glycoprotein I (anti-beta2GPI) antibodies constitute the main autoantibody specificity in the sera of patients with antiphospholipid syndrome (APS). There is evidence that anti-beta2GPI antibodies induce the precoagulant activity of the endothelium by cross-linking the beta2 glycoprotein I (beta2GPI) on the cell surface. Since beta2GPI lacks intracellular domains, homology with other molecules such as CD40 that could initiate signaling, was extensively searched. A 86% homology between the amino acid position 239-245 of the CD40 and 7-13 of the beta2glycoprotein was found. The CD40 peptide corresponding to amino acids 239-245 of the CD40 molecule was synthesized and coupled to a multiple antigenic peptide carrier. Antibodies to CD40 peptide were found in 61.5% APS patients (n = 39), in 72.7% of systemic lupus erythematosus (SLE) positive for anti-beta2GPI antibodies (n = 11) and 31.6% of SLE negative for anti-beta2GPI antibodies (n = 19), but not in rheumatoid arthritis patients (n = 28) or controls (n = 36). Antibodies to CD40 peptide were associated with arterial thrombosis and/or brain microinfarcts. Affinity purified anti-CD40 peptide antibodies as well as affinity purified anti-beta2GPI antibodies recognized both, the beta2GPI and the CD40 peptide. The specificity of this recognition was confirmed with homologous and heterologous inhibition experiments. Confocal microscopy experiments demonstrated this cross-recognition of CD40 and beta2GPI molecules, by the purified anti-CD40 peptide antibodies, at the protein level. Thus, antibodies reacting with the beta2GPI can react and potentially activate different cells which express CD40 molecules at their surface.

Blood Coagulation, Fibrinolysis and Cellular Haemostasis

Anti-CD40 antibodies in antiphospholipid syndrome and systemic lupus erythematosus

Panayiotis G. Vlachoyiannopoulos, Clio P. Mavragani*, Efi Bourazopoulou*, Anthi V. Balitsari, John G. Routsias

Department of Pathophysiology, Medical School, National University of Athens, Athens, Greece

Summary

Anti-β2glycoprotein I (anti-β2GPI) antibodies constitute the main autoantibody specificity in the sera of patients with antiphospholipid syndrome (APS). There is evidence that anti-β2GPI antibodies induce the precoagulant activity of the endothelium by cross-linking the β2 glycoprotein I (β2GPI) on the cell surface. Since β2GPI lacks intracellular domains, homology with other molecules such as CD40 that could initiate signaling, was extensively searched. A 86% homology between the amino acid position 239-245 of the CD40 and 7-13 of the β2glycoprotein was found. The CD40 peptide corresponding to amino acids 239-245 of the CD40 molecule was synthesized and coupled to a multiple antigenic peptide carrier. Antibodies to CD40 peptide were found in 61.5% APS patients (n=39), in 72.7% of systemic lupus erythematosus (SLE) positive for anti-β2GPI antibodies (n=11) and 31.6% of SLE negative for anti-β2GPI antibodies (n=19), but not in rheumatoid arthritis patients (n=28) or controls (n=36). Antibodies to CD40 peptide were associated with arterial thrombosis and/or brain microinfarcts. Affinity purified anti-CD40 peptide antibodies as well as affinity purified anti-β2GPI antibodies recognized both, the β2GPI and the CD40 peptide. The specificity of this recognition was confirmed with homologous and heterologous inhibition experiments. Confocal microscopy experiments demonstrated this cross-recognition of CD40 and β2GPI molecules, by the purified anti-CD40 peptide antibodies, at the protein level. Thus, antibodies reacting with the β2GPI can react and potentially activate different cells which express CD40 molecules at their surface.

Introduction

Antiphospholipid syndrome (APS) is an acquired, immune-mediated thrombophilia, defined as a combination of thrombosis or pregnancy morbidity (recurrent abortions, fetal deaths, preeclampsia or eclampsia) with antibodies to phospholipids (aPLs) and more specifically, anticardiolipin antibodies (aCL) or lupus anticoagulant (LA) (1). These antibodies constitute a highly heterogeneous group in terms of binding specificity; they recognize, either, various phospholipids, phospholipid-binding proteins, like prothrombin, annexin V, or β2 glycoprotein I (β2GPI), or phospholipid-protein complexes (2-6). It has been suggested that β2GPI alone, or in complex with phospholipids is the main target of aPL/anti-β2GPI antibodies on the cell surface, and that antibodies recognizing β2GPI are considered rather powerful predictive factors for thrombosis (6).

Evidence suggests, that anti-β2GPI antibodies activate endothelial cells by inducing intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), E-selectin and tissue factor (TF) expression on the cell surface (7-9) and by secretion – in an autocrine manner – of proinflammatory cytokines, such as IL1-β (10). Endothelial cell activa-

tion by anti-β2GPI is mainly mediated by nuclear factor kB (NFkB) (11). Platelet activation by anti-β2GPI antibodies may also contribute to thrombotic complications in patients with APS (12).

Crystallographic studies suggest, that β2GPI has a five-domain fish-hook like structure and that binding to negatively charged phospholipids on the cell surface is actually achieved by its fifth domain (13). Interaction of the fifth domain with phospholipids, as well as heavy glycosylation of the third and fourth domains, makes it unlikely that these domains expose antibody binding sites. Thus, the first and second domains are most likely recognized by anti-β2GPI antibodies. Indeed, two recent studies, the first using recombinant domain-deleted β2GPI, and the second, using surface plasmon resonance, identified the first domain as the major target of anti-β2GPI antibodies (14, 15).

It is difficult to understand the mechanism of cellular activation after cross-linking of the surface β2GPI with anti-β2GPI antibodies, since β2GPI lacks any intracellular domain. We hypothesized that the signalling mediated by anti-β2GPI antibodies involves cross-reactivity of anti-β2GPI with a yet unknown cell surface protein, carrying an intracellular domain. A molecule which can be recognized by anti-β2GPI antibodies is the CD40, a member of the tumor necrosis factor receptor (TNFR) family of proteins, for the following reasons: (a) CD40 shares sequence homology with the 7-13 region on the antigenic first domain of β2GPI, (b) CD40 is constitutively expressed on vascular endothelium and human platelets as well as on the autoantibody producing B-cells, (c) engagement of CD40 (by anti-CD40 antibodies or CD40L) resembles many of the unexplained anti-β2GPI binding consequences as (i) NFkB expression, (ii) induction of adhesion molecules (E-selectin, VCAM) and tissue factor on the surface of endothelial cells, (iii) secretion of proinflammatory cytokines and (iv) platelet activation (16-20).

We provide evidence, which supports the hypothesis that cross-recognition of CD40 by anti-β2GPI antibodies takes place, as detected by ELISA and confocal microscopy experiments. In addition, we demonstrate that anti-CD40 reactivity is preferentially found in patients with APS and SLE positive for anti-β2GPI antibodies.

Materials and methods

Patients, clinical features and diagnoses

Consecutive patients, followed in our outpatient clinic, were diagnosed as APS (n=39) according to Sapporo criteria (1). Patients with SLE were diagnosed according to the American Rheumatism association criteria (21), and divided into those who were positive for anti-β2GPI antibodies, but without APS related features (SLE anti-β2GPI positive) (n=11), and those negative for anti-β2GPI (SLE/ anti-β2GPI negative) (n=19).

Furthermore sera from 28 rheumatoid arthritis (RA) patients, according to American College of Rheumatology Criteria (22), were tested. Sera from young, healthy individuals (n=36) (hospital personnel and blood donors) were tested in each ELISA experiment, as controls. Age at disease onset was considered the age by which the first finding, considered to be a criterion for the diagnosis of APS, SLE or RA, appeared.

Detection of thrombosis and pregnancy morbidity was based on the Sapporo criteria for the diagnosis of APS (1). Central and peripheral nervous system involvement was detected on the basis of the following: psychosis, focal infarcts, extrapyramidal disorders, cerebellar dysfunction, subarachnoid hemorrhage, aseptic meningitis, transverse myelitis, optic neuritis, cranial nerve palsies and peripheral sensorimotor neuropathy. In all these cases magnetic resonance imaging of the brain, brainstem and medulla, as well as cerebrospinal fluid examination, including protein levels and mini monoclonal bands determination was performed. The cases of cerebral infarcts, white matter microthrombi and demyelinating plaques were separately recorded. Renal involvement was defined according to the criteria proposed for the diagnosis of SLE (21). The diagnosis of pulmonary hypertension was based on clinical examination in combination with cardiac catheterization and heart ultrasonographic findings.

Sequence homology search

The sequences of β2GPI and CD40 were derived from the SWISS PROT data base with the names and access numbers: APOHβHUMAN, P02749 for the former and TNR5βHUMAN, P25942 for the latter. Sequence homologies between β2GPI and CD40 were performed using the scoring matrix blosum 62 with a window size equal to 7. Comparisons between the above proteins showed only one match reaching 86% homology between the amino acid position 239-245 of the CD40 and the amino acid position 7-13 of the β2GPI (Fig. 1).

Multiple antigenic peptide (MAP) synthesis

The peptide FPDDLPGSN corresponding to the amino acid positions 239-245 of the human CD40 extended by 4 amino acids at its aminoterminal and by one amino acid at its carboxy-terminal, was synthesized and coupled to multiple antigenic carriers (NH2-(Ac)QEINFPDDLPGSNT-MAP). Synthesis and high performance liquid chromatography purification was performed by Biosynthesis Inc. (Lewisville, TX, USA). This material, called "CD40 peptide" (CD40pep), was used as the antigen in an enzyme linked immunosorbent assay (ELISA) to detect anti-CD40pep reactivity in the sera of APS patients and controls.

Anti-CD40pep ELISA

Ninety-six well ELISA plates (NUNC, Covalink NH, FB, Batch 047395, Denmark) were coated with the CD40 pep

(150ng/well), under covalent binding conditions as follows: First, the following solutions were made: a) N-hydroxysulfosuccinimide (sulfoNHS, No 24510/CAS 106627-547, Pierce, Rockford, Illinois, USA), dialyzed in phosphate buffer saline (PBS) at a concentration 0.6mg/ml; b) 1-ethyl-3-(3-Dimethylaminopropyl) Carbodiimide (EDC, Sigma) dialyzed in PBS at a concentration 0.63mg/ml; c) the CD40pep dialyzed in PBS at a concentration 4.5 μg/ml. The above solutions were then mixed at a volume 1:1:1 and 100 μl of the final mixture, containing 150 ng of the antigen, was incubated per well for 1.5h at 37 °C. After washing 3 times with Covabuffer 0.5M the wells were blocked with 200βl of highly pure albumin 2% in PBS (Blocking buffer) and incubated for 1h at 37°C. To make Covabuffer 0.5M, 11.7g NaCl and 2g MgSO4 were dialyzed in 200 ml of water for injection and 100μl of Tween 20 was finally added to this solution. Sera, diluted 1:300 in blocking buffer (100μl/well), were incubated overnight at 4°C. After washing 3 times with covabuffer 0.5M, alkaline phosphatase conjugated anti-human IgG,γ-chain specific antiserum (Sera-Lab), diluted in 1:200 in blocking buffer was added to each well (100μl) and incubated for 1h at 37 °C. After washing 3 times with covabuffer 0.5M, substrate solution [p-nitrophenyl phosphate disodium (Sigma), 1 mg/ml in diethanolamine buffer, pH 9.8], (100 μl/well) was added to each well. Absorbance was read at 410 nm. A positive control gave optical density of 1.500 and a normal human serum an optical density of 0.250. According to our preliminary experiments, the anti-CD40pep positive sera were derived from the sera of APS patients positive for anti-β2GPI antibodies. Based on the fact that the CD40pep possessed homology to a β2GPI region, the cut-off point of our anti-CD40pep ELISA was established as the absorbance level of 0.450, defined as 2 SD above the mean of 36 normal sera.

In order to test the specificity of anti-CD40pep ELISA, all samples from patients with APS and SLE were tested in a new ELISA, performed under the same conditions undertaken in our original anti-CD40pep ELISA, using, as coating antigen, an irrelevant peptide, IASRYDQL (corresponding to the sequence 250-257 aa of Leishmania glycoprotein gp63) attached in 4 copies to a sequential oligopeptide carrier (SOC), [(IASRYDQL)$_4$SOC$_4$] (23).

Affinity purification of anti-CD40pep antibodies

The CD40pep (7mg) was coupled to EAH Sepharose 4B (Pharmacia® Biotech) (3.5ml), via carboxylgroups using the carboimide coupling method, according to manufacturers instructions. The pH of coupling was carefully selected at the level of 5.3 in order to achieve coupling preferentially through the γ-COOH group of glutamic acid. Serum diluted 1:2 in PBS was passed through the column and specific anti-CD40pep antibodies were eluted using glycine-HCl, pH=2.7. From a total of 154 mg, 2.875 mg specific IgG anti-CD40pep antibody was isolated.

Affinity purification of anti-β2GPI antibodies

A combination of affinity and cation exchange chromatography was used as previously described (6, 24-26). This method is based on the peculiarity of anti-β2GPI antibodies to tightly bind autologous β2GPI, only under certain conditions: more specifically, binding of serum β2GPI to negatively charged phospholipids results in exposition of the first domain of β2GPI to the anti-β2GPI antibodies and this happens either in the microtiter ELISA plates or in the bed of the affinity column. Therefore, first a cardiolipin/ cholesterol/ polyacrylamide affinity column was prepared as previously described (6, 24). Sera from two patients with APS expressing high titre of anti-β2GPI antibodies were passed through this column. The eluate from the above column was concentrated and dialyzed against buffer A, containing 75 ml stock A (1.5 ml acetic acid/ 1 L water), 175 ml stock B (27.2 g sodium acetate. 3H$_2$O/ 1 L water), 750 ml water and 2.9 gr NaCl. After two changes of buffer A overnight, dialy-
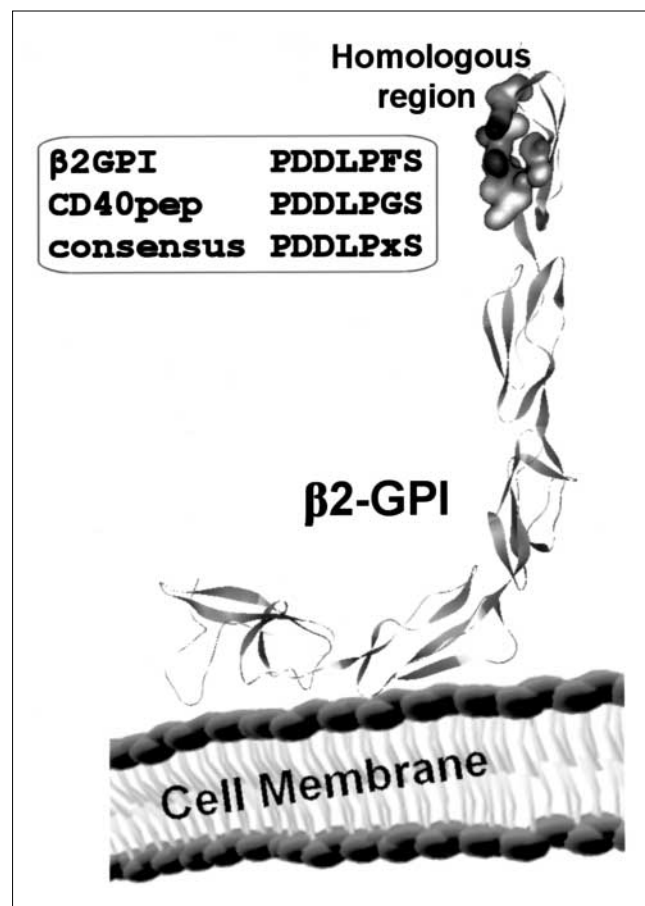
Figure 1: Localization of the CD40 homologous region, on the three-dimensional structure of β2GPI. The region of the domain I which possess homology with the CD40 is displayed using the Corey, Pauling, Koltun (CPK) representation. The sequence homology between the two molecules is depicted on the left panel.

sate was applied to a Mono-S cation (HR5/5) exchange column (Pharmacia) concentrated to 200 μL prior to application. Gradient was applied from 0 to 100% of buffer B (0.05 M acetate, 0.65 M NaCl) at 0.5 ml/min over 45 min, and fractions appearing on a chart recorder attached to a UV detector, which was connected to the column, were collected. All the protein peaks were evaluated in both aCL and anti-β2GPI ELISA, as well as in a modified aCL ELISA, which is similar to conventional aCL ELISA with the exception that the blocking factor was gelatine and not bovine serum, therefore it lacked β2GPI. Three peaks from each serum were obtained. With the exception of the last peak which contained the autologous β2GPI (6,24), the remaining peaks contained antibodies to β2GPI as detected by the anti-β2GPI ELISA performed in a cardiolipin independent fashion, using polystyrene irradiated microtitre plates (Lindro/Titertek; ICN Biomedicals, Horsham, PA 19044, Australian Nuclear Science and Technology Organization, Sydney, Australia), coated with 10 μg/ml β2GPI diluted in carbonate buffer, pH=8.5, 50 μl/well, as previously described (6). These peaks, although expressing also aCL activity in a conventional aCL ELISA (β2GPI dependent, since bovine serum containing β2GPI, was used as a blocking factor (6), did not express aCL reactivity in a modified aCL (β2GPI independent) ELISA (6) Therefore the serum fractions obtained by the combination of the above techniques, represented purified anti-β2GPI antibodies (6, 24, 25).

Inhibition experiments

The specificity of the binding of purified anti-CD40 pep antibodies was evaluated by homologous inhibition, using varying amounts of CD40pep or control peptide as inhibitor. Inhibitors at increasing concentrations ranging from 0 to 15 μg/ml were incubated at room temperature for two hours with purified anti-CD40pep antibody diluted at 20 μg/ml in PBS/albumin 2%, before their testing in the anti-CD40pep ELISA. Purified β2GPI was also used as inhibitor of the purified anti-CD40pep antibodies tested against CD40pep and also against-β2GPI in two parallel ELISA experiments.

FITC-labelling studies

Human purified anti-CD40pep. and normal IgG antibodies (1mg/mL) were mixed and incubated with fluorescein isothiocyanate (FITC) labelling solution (100 μg of FITC/1 mg IgG) in the dark for 1 h at room temperature, after dialysis against 0.1M carbonate-bicarbonate buffer, pH 9.20 for 2 h at room temperature. Unreacted FITC was removed by dialysing into phosphate buffer saline (PBS) at 4°C overnight.

Cell culture

Epstein-Barr virus (EBV)-transformed human B cells were generated in our laboratory as follows. B lymphocytes, from a patient with SLE, were separated from peripheral blood and EBV was obtained from the supernatant of confluent cultures of marmoset infected cells (B95-8) by filtration through 0·45-μm porous filters. One millilitre of these virus-enriched samples was added to 5-10×10⁶ cells and the cells were incubated overnight at 37°C. EBV-transformed B cells were then selected on the basis of their continuous proliferation in tissue culture flasks. EBV-transformed human B lymphocyte cell line, was cultured in RPMI 1640 medium (GIBCO BRL), supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 IU/ml streptomycin and 100 μg/ml penicillin at 37°C under 5% CO_2 in a humidified incubator.

Confocal microscopy

EBV-transformed B cells were isolated by centrifugation, washed with phosphate-buffered saline (PBS) and incubated with 5 μg/ml β2GPI (Fitzgerald, USA) for 45 min at 4°C. Prior to cell attachment onto slides by cytocentrifugation, cells were washed with PBS, and then fixed in methanol:acetone (1:1) for 10 min at -20°C. For single labelling immunofluorescence microscopy, cells were blocked with 1% bovine serum in PBS (blocking buffer) for 45 min, washed two times in PBS and incubated with 1mg/mL normal IgG in blocking buffer in order to occupy free Fc receptors on the cell surface. Subsequently, the cells were washed three times in PBS and then stained with anti-humanCD40-biotin (Serotec, UK) (1:100 in blocking buffer) or FITC-conjugated purified anti-CD40pep antibody, for 1h. Finally, after three washes in PBS, streptavidin-TRITC (1:200 in blocking buffer) was added, in anti-humanCD40 treated cells. For double labelling immunofluorescence microscopy, cells were blocked for 45 min, washed two times in PBS, incubated with 1mg/mL normal IgG, washed three times in PBS and then stained with primary FITC-conjugated human purified anti-CD40pep antibody and mouse anti-human β2GPI monoclonal antibody (Serotec, UK) (diluted at 7μg/ml and 1:100 in blocking buffer, respectively) for 1 hour. After, three washes in PBS, cells were incubated with secondary TRITC-conjugated anti-mouse IgG antibody (Jackson, USA) (1:200 in 1% bovine serum), washed three times in PBS and mounted with 50% glycerol solution in PBS. Nonspecific fluorescence was assessed by omitting the primary antibody from the immunolabeling reaction, replacing the human purified antibody with a non-relevant antibody (FITC-conjugated human purified normal IgG) or using cells without being pre-incubated with β2GPI. Confocal microscopy was performed with a Nikon laser scanning confocal microscope (PCM2000) equipped with a green/red Hene laser operated by EZ2000 software.

Statistical analysis

Contingency tables were used where indicated.
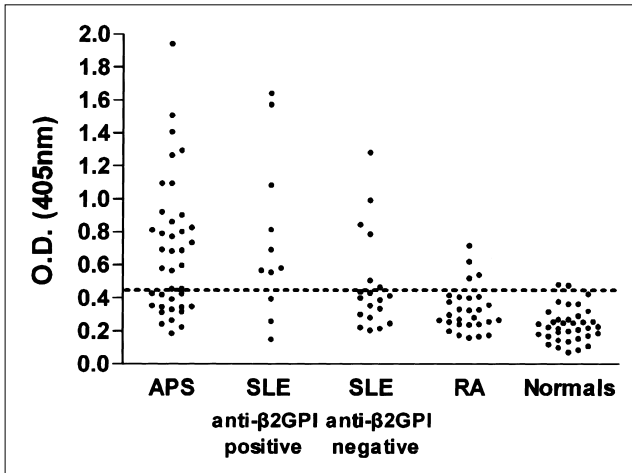
Figure 2: The reactivity of sera against the CD40pep, expressed as absorbance levels in the anti-CD40pep ELISA, in patients and controls. Dotted line represents the cut-off point of the assay.
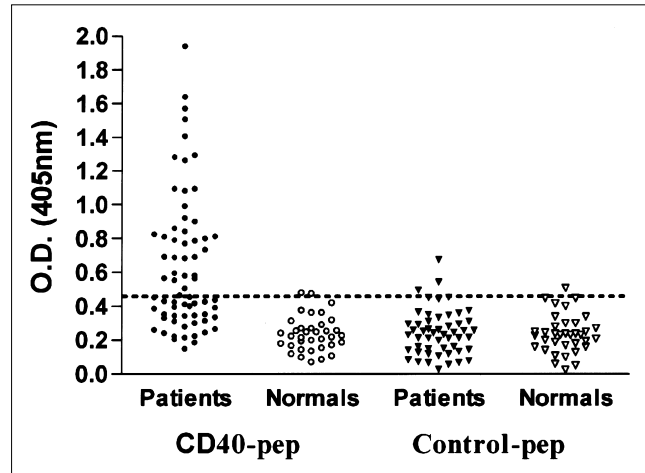
Figure 3: Comparison of the reactivity of the sera of patients and normal individuals against the CD40 peptide and an irrelevant peptide (control pep), both anchored to multiple antigenic peptide carriers. The reactivity of the sera against both peptides was tested in ELISA performed under covalent binding conditions. The irrelevant peptide was the peptide IASRYDQL corresponding to the sequence 250-257 aa of Leishmania glycoprotein gp63.

Results

Patients and their diagnoses

Thirty-nine patients with APS, 30 patients with SLE and 28 patients with RA were evaluated. The mean age ±SD (standard deviation) in the four groups of patients was respectively 37.81±12.40, 36.26±11.48, 37.18±12.63 and 49.81±12.48 years. In addition, the mean age of onset ± SD for the four groups was, respectively, 27.14±10.27, 28.37±12.25, 28.00±9.36, 38.04±15.34 years. The patients with APS and SLE did not differ in terms of age at study entry or age at disease onset. The RA patients were older at study entry and age of onset (p<0.05).
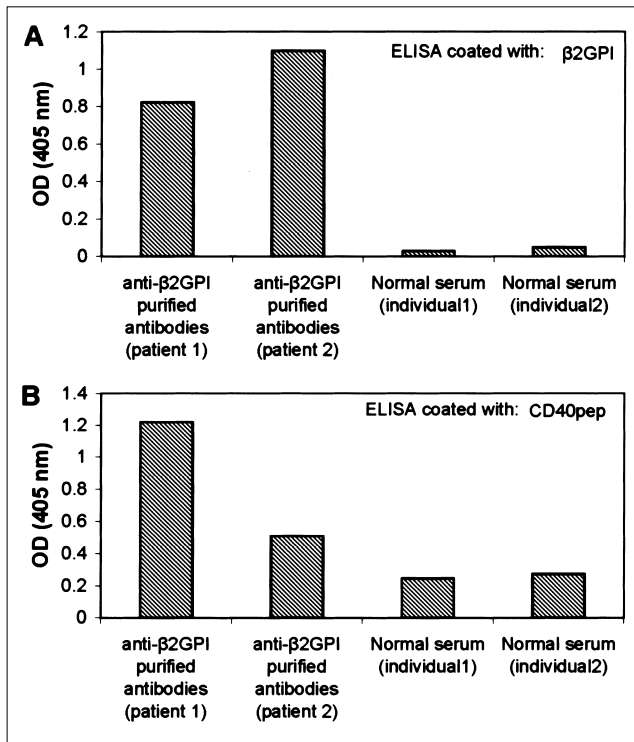
Anti-CD40 pep antibodies in patients and controls

The prevalence of anti-CD40pep positive sera was 61.5% for patients with APS, while it was 72.7% for SLE/anti-β2GPI positive, and 26.3% for SLE/anti-β2GPI negative patients. RA patients possessed CD40pep antibodies in their serum less frequently (14.3%), while only 5.1% of normal individuals gave absorbance values slightly higher than the cut-off point (Fig. 2).

The SLE and APS patients were divided into those positive and negative for anti-CD40pep antibodies, and the two groups were compared in terms of clinical and serological findings. Patients positive for anti-CD40pep antibodies were characterized by higher prevalence of arterial thrombotic events including arterial thrombosis and brain microinfarcts. This difference was of marginal statistical significance (15/37 vs 5/32, p<0.05).
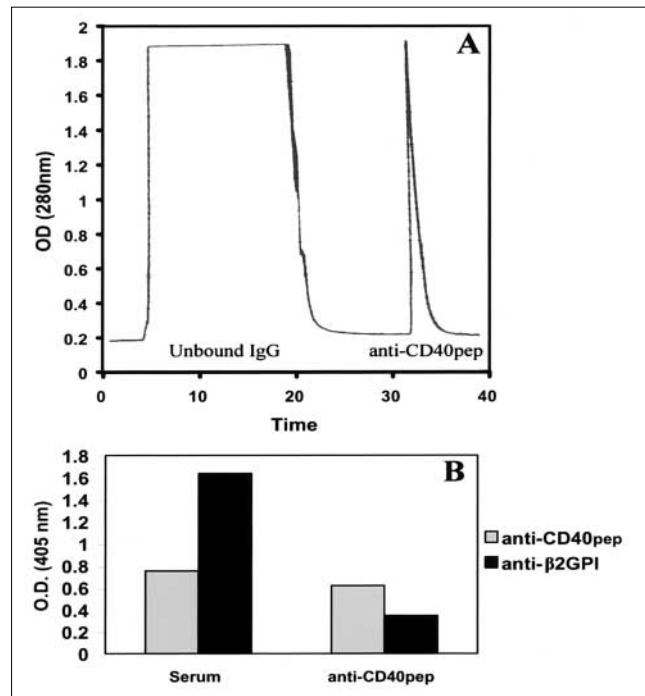
Figure 4: Chromatographic diagram of the affinity purification of anti-CD40pep antibodies, indicating the dissociation of anti-CD40pep from anti-β2GPI reactivity. The two peaks in panel A represent the unbound and eluted IgG, from a CD40pep affinity column. The anti-β2GPI and anti-CD40 reactivities of the whole serum IgG (prior its application in CD40pep column) and the purified anti-CD40pep antibodies (concentrated to the initial serum volume), from the anti-CD40pep affinity column are shown with black and dashed bars, respectively.

Figure 5: Affinity purified anti-β2GPI antibodies from the sera of 2 patients with APS recognize both the β2GPI antigen (panel A) as well as the CD40pep (panel B).
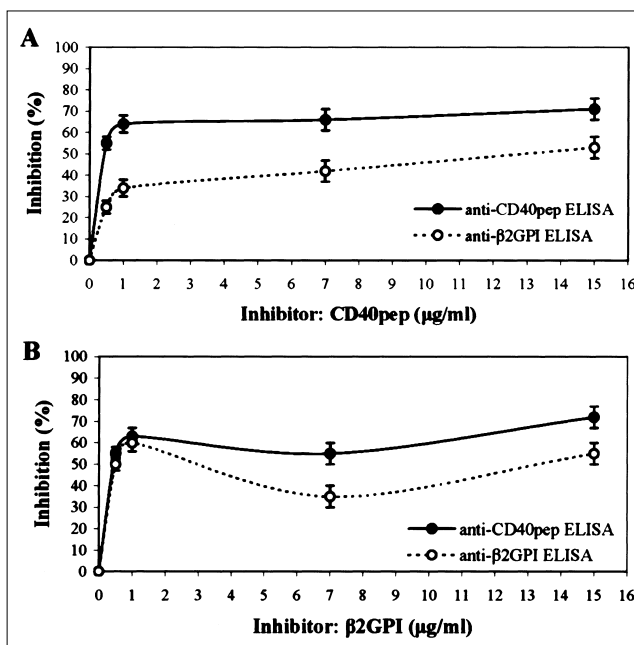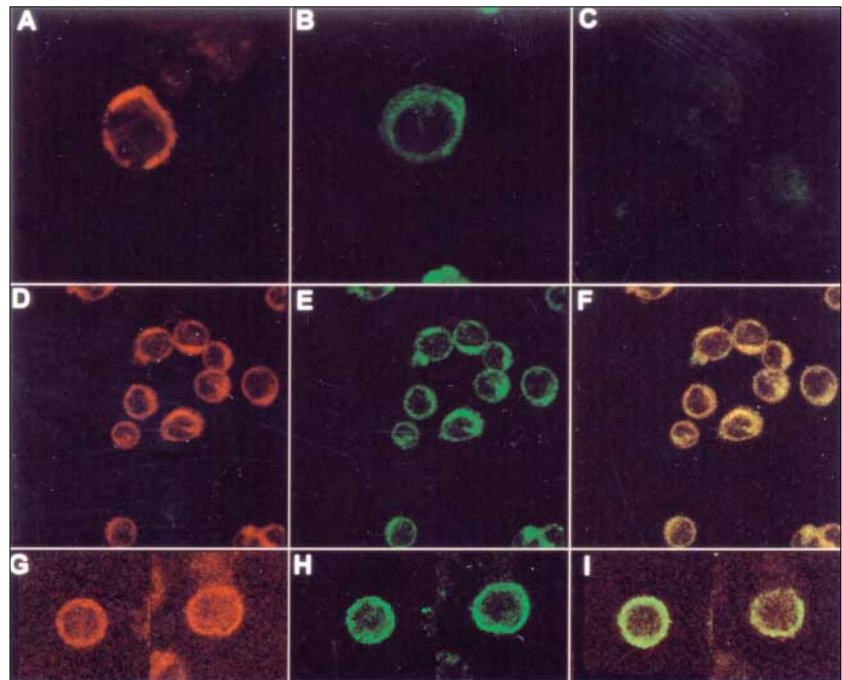

Figure 6: Purified anti-CD40pep antibodies tested in anti-CD40pep and anti-β2GPI ELISA. Inhibition experiments: Panel A: Pre-incubation of purified anti-CD40pep antibodies by CD40pep inhibited their binding to both, CD40pep and β2GPI, as detected by ELISA. Panel B: Pre-incubation of purified anti-CD40pep antibodies by β2GPI inhibited their binding to both, CD40pep and β2GPI, as detected by ELISA.

Specificity of anti-CD40pep ELISA

The specificity of anti-CD40pep ELISA was studied in two ways: First, an irrelevant peptide attached in four copies to a carrier [(IASRYDQL)$_4$SOC$_4$] was used as a coating antigen under the same conditions with the original anti-CD40pep ELISA. All the patients and normal sera were tested for reactivity against this irrelevant peptide. As shown in Figure 3 no reactivity against (IASRYDQL)$_4$SOC$_4$ was detected, either in patients or in normals. Second, we performed affinity purification of anti-CD40pep antibodies for a serum of an APS patient with high reactivity in the anti-CD40pep ELISA. As shown in Figure 4, purified anti-CD40pep antibodies recognized both the CD40pep and the recombinant β2GPI protein. Compared with the whole serum IgG, the purified anti-CD40pep antibodies retained almost the entire serum reactivity against CD40pep but they appeared less reactive against the β2GPI protein. On the other hand, the flow-through fraction of IgG possessed a large part of anti-β2GPI activity but almost no anti-CD40pep activity (data not shown). These observations suggested that anti-β2GPI antibodies represented a cluster of antibody molecules with different fine specificity , many of which did not recognize the homologous region with CD40 pep but other regions of the β2GPI molecule.

Cross-reactivity between anti-CD40pep and anti- β2GPI antibodies

Using a combination of affinity and cation exchange chromatography, we were able to isolate anti-β2GPI antibodies from two APS patients, which in parallel with the purified anti-CD40pep antibodies, were tested in both anti-β2GPI ELISA and anti-CD40pep ELISA. As shown in Figure 5, the anti-β2GPI purified antibodies recognize not only β2GPI (Fig. 5A) but also CD40pep (Fig. 5B). To confirm further the specificity of anti-CD40pep ELISA, as well as the specific recognition of β2GPI by purified anti-CD40pep antibodies, homologous and heterologous inhibition experiments were carried out as follows: Purified anti-CD40pep antibodies were tested in both, anti-CD40pep and anti-β2GPI ELISA. Inhibition curves were obtained either, by using as inhibitor CD40pep (Fig. 6A) or purified β2GPI (Fig. 6B). Pre-incubation of purified anti-CD40pep antibodies with the CD40pep inhibited their binding to solid phase CD40pep by 70% and their binding to solid phase β2GPI by 50% as detected in parallel anti-CD40pep and anti-β2GPI ELISA experiments (Fig. 6A). Pre-incubation of purified anti-CD40pep antibodies with β2GPI inhibited their binding to solid phase CD40pep by nearly 70% and their binding to solid phase β2GPI by almost 55% as detected in parallel anti-CD40pep and anti-β2GPI ELISA experiments (Fig. 6B).

Figure 7: Confocal microscopy experiments, using immortalized B lymphocytes of SLE patients as substrate.
I: Absence of exogenous β2GPI:
A: monoclonal anti-CD40 antibody, B: purified anti-CD40pep antibody, C: monoclonal anti-β2GPI antibody
II: Presence of exogenous recombinant β2GPI:
D and G: monoclonal anti- β2GPI antibody,
E and H: purified anti-CD40pep antibody,
F and I: merged images.

Cross-recognition of CD40 and β2GPI by anti-CD40pep antibodies at the cell membrane level

The ability of anti-CD40pep antibodies to recognize the CD40 and β2-GPI antigens at cell level, was explored by confocal microscopy. Immortalized B-cells from a patient with SLE were used as substrate. It was found that anti-CD40pep antibodies readily produced a clear membrane staining identical to that observed with an anti-CD40 monoclonal antibody (Serotec, UK) (Fig. 7A, B). On the other hand an anti-β2GPI monoclonal antibody (Fitzgerald, USA) gave no staining to the cells (Figure 7C). After exposure of the cells to exogenous recombinant β2GPI however, the anti- β2GPI monoclonal antibody was bound to the cell membrane (Fig. 7D, G). Confocal microscopy using purified anti-CD40pep antibodies as well as the anti-β2GPI monoclonal antibody against B-cells exposed to recombinant β2GPI, showed a clear co-localization pattern, supporting further the concept of cross-recognition of these two antigens (Fig. 7D-I).

Discussion

It is known that anti-β2GPI antibodies are able to activate both vascular endothelium and blood platelets, leading to increased adhesion molecule expression and proinflammatory cytokine secretion (7-10). These functions are associated with the thrombophilic diathesis which is a characteristic of APS. However, the signalling pathway following the binding of anti- β2GPI antibodies to β2GPI, remains a mystery, since β2GPI lacks any intracellular domain. It seems that many of these functions are mediated via an unexplained NFkB activation by anti-β2GPI antibodies (11).

In this study the cross-recognition of CD40 by anti-β2GPI antibodies was explored, in order to explain the intracellular signaling and cell activation following the exposure of endothelial cells to anti- β2GPI antibodies. This cross-recognition involves the 220-226 region of CD40 that shares sequence homology with a highly exposed part (7-13aa) of the antigenic first domain of β2GPI. A peptide analogue of the aforementioned region of CD40 was found to preferentially react with sera of patients with APS and SLE but not with RA or normal sera. Affinity purification of anti-CD40 antibodies retained their anti-CD40 reactivity but reduced their anti-β2GPI reactivity, as compared with the whole serum IgG. This observation may reflect the existence of additional antibody specificities targeting β2GPI molecule outside the region which exposed homology with the CD40. Nevertheless, the purified anti-CD40pep antibodies were found to recognize both, CD40pep and recombinant β2GPI molecules. The specificity of this cross-recognition was further demonstrated by specific homologous and heterologous inhibition experiments. Furthermore, affinity purified anti-β2GPI antibodies from two patients with APS recognized the CD40pep.

Although the homologous toCD40 region in ß2GPI (7-13aa) resides in a highly exposed region within its first – previously defined as antigenic –domain, the corresponding CD40 part (220-226aa) lies very close to its transmembrane region and probably is intracellular. Our experimental data, however indicated that the CD40 220-226 aa region can be actually recognized by the purified anti-CD40pep antibodies. In this regard, purified anti-CD40pep antibodies produced a membrane stain-

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ing of the cultured B-cells in the absence of exogenously added recombinant β2GPI. Under these conditions, no detectable β2GPI exists Therefore the observed anti-CD40pep staining can be logically attributed to an interaction with membrane bound CD40 molecules, hypothesis, which is further supported by the identical staining, produced by monoclonal anti-CD40 antibodies to the same cells. It is possible that under certain conditions (e.g. endothelial cells, triggered by cytokines) large parts of CD40 molecule could be externalized; it is well known that CD40 signaling in human dendritic cells is initiated within cholesterol-rich plasma membrane microdomains termed 'membrane rafts' (27).

The binding of autoantibodies to CD40 on the cell membrane can eventually mimic the interaction with its trimeric ligand that leads to multimerization of its cytoplasmic domain with subsequent signal transduction, propagated by interactions of tumor necrosis factor receptor-associated factor (TRAF) proteins, and NFkB activation (28-32). As CD40 is constitutively expressed on vascular endothelial cells, human platelets and B-cells, the initiation of its signaling pathway can lead to various activation effects on these cells, which are directly involved to thrombosis process and autoantibody formation, events that characterize APS and SLE.

Monoclonal anti-CD40 acts also as a B-cell mitogen on murine B-cells, which respond by proliferation (33). Although, thrombosis has not been observed in these mice, the B-cell effect of these antibodies can explain the self-perpetuation of immune reactivity against particular antigens in APS. In addition, CD40 associates with the Ku complex in the cytoplasm of B cells (34). The Ku complex is an autoantigen in SLE and is required from lymphocyte development and immunoglobulin class switch recombination (35). These findings suggest that antibodies recognizing CD40 on the cell surfaces exacerbate B-cell responses, recalling the hyperactivity of B-cells in SLE.

Engagement of CD40 resembles many of the unexplained, until now, anti-β2GPI binding consequences on endothelial cells such as: induction of adhesion molecules (E-selectin, VCAM) and tissue factor on their surface, as well as, secretion of proinflamatory cytokines (16-20). These events can perturb normal vascular endothelium, converting it into a procoagulant surface with a subsequent shift of the haemostatic balance towards clot formation. In this series of events key roles play the increased adhesion of leucocytes to activated endothelium under an environment of proinflammatory cytokines and the increased tissue factor expression that is known to result in the fibrin formation and platelet activation (36).

Ligation of platelet CD40 causes a-granule and dense granule resease accompanied by the classical morphological changes of the platelet activation (18). In addition b3-integrin activation and enhanced platelet-leukocyte adhesion is also observed (18). The latter effects of platelet CD40 ligation are important for the recruitment of leukocytes to the sites of thrombosis (e.g. sites of activated endothelium).

In conclusion, reactivity of the sera of patients with APS and SLE, against a peptide of the CD40 molecule which shares sequence homology with a highly exposed region of the first domain of β2GPI, was described. Our findings suggest that anti-β2GPI antibodies cross-reacting with CD40 molecules can potentially activate several cell types, initiating a coagulation process.

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