Human β2-Glycoprotein I Binds to Endothelial Cells Through a Cluster of Lysine Residues That Are Critical for Anionic Phospholipid Binding and Offers Epitopes for Anti-β2-Glycoprotein I Antibodies[1,2]

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β2-Glycoprotein I (β2GPI) is a phospholipid-binding protein recognized by serum autoantibodies from the anti-phospholipid syndrome both in cardiolipin- and β2GPI-coated plates. We found that: 1) recombinant wild-type β2GPI bound to HUVEC and was recognized by both human monoclonal IgM and affinity-purified polyclonal IgG anti-β2GPI anti-phospholipid syndrome Abs; and 2) a single amino acid change from Lys^{286} to Glu significantly reduced endothelial adhesion. Double and triple mutants (from $Lys^{284,287}$ to $Glu^{284,287}$, from $Lys^{286,287}$ to $Glu^{286,287}$, and from $Lys^{284,286,287}$ to $Glu^{284,286,287}$) completely abolished endothelial binding. A synthetic peptide (P1) spanning the sequence Glu^{274}–Cys^{288} of the β2GPI fifth domain still displayed endothelial adhesion. Another peptide (P8), identical with P1 except that Cys^{281} and Cys^{288} were substituted with serine residues, did not bind to HUVEC. Anti-β2GPI Abs, once bound to P1 adhered to HUVEC, induced E-selectin expression and up-regulated IL-6 secretion. Control experiments conducted with irrelevant Abs as well as with the P8 peptide did not show any endothelial Ab binding nor E-selectin and IL-6 modulation. Our results suggest that: 1) β2GPI binds to endothelial cells through its fifth domain; 2) the major phospholipid-binding site that mediates the binding to anionic phospholipids is also involved in endothelial binding; 3) HUVEC provide a suitable surface for β2GPI binding comparable to that displayed by anionic phospholipids dried on microtiter wells; and 4) the formation of the complex between β2GPI and the specific Abs leads to endothelial activation in vitro. ***The Journal of Immunology,* 1998, 160: 5572–5578.**

Human β2-glycoprotein I (β2GPI)[4] is a phospholipid (PL)-binding protein that is required for the binding of autoantibodies in sera from patients with the anti-phospholipid syndrome (APS) to cardiolipin (CL)-coated plates (1–3). It is not yet known whether anti-β2GPI Abs recognize epitopes displayed by native β2GPI when available at increased density or new epitopes expressed after binding of the molecule to negatively charged structures (4–6). Anti-β2GPI Abs may be purified from both primary and secondary APS sera. Such serum anti-β2GPI Abs have been found to be associated with thrombotic events and/or fetal loss, suggesting a potential pathogenic role in APS (7–13).

β2GPI is composed of five highly conserved subunits called sushi domains or complement control protein repeats (14, 15). The major PL-binding site on β2GPI has been identified as a highly positively charged amino acid sequence, Lys^{282}-Asn-Lys-Glu-Lys-Lys^{287}, in the fifth domain of the molecule (16–20).

β2GPI binds not only to negatively charged substances such as PL (21), heparin (22), and lipoproteins (23), but also to cell membranes such as activated platelets (24–26). Recently, several groups demonstrated that β2GPI is able to bind to resting endothelial cells and to be recognized by monoclonal and polyclonal anti-β2GPI Abs (27–30). While platelet binding has been related to the large expression of anionic PL on platelet cell membranes after activation, a comparable phenomenon is unlikely on resting endothelial cells, which do not display such PL distribution changes (31).

The aim of the present study was to investigate whether β2GPI binds to endothelial cells through the same amino acid sequence that is involved in binding to anionic phospholipids. If β2GPI-endothelial cell binding mirrors that found in the solid phase CL assay, it is reasonable to assume that endothelial cells can represent one of the natural structures to which β2GPI binds, offering suitable epitopes to circulating anti-β2GPI Abs. In this regard, it is

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[4] Abbreviations used in this paper: β2GPI, β2-glycoprotein I; APS, anti-phospholipid syndrome; CL, cardiolipin; PL, phospholipid; Sf, *Spodoptera frugiperda*; NHS, normal human serum.

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useful to point out that Ab binding to β2GPI adhered on endothelial membranes has been shown to cause, at least in vitro, cell activation that induces the expression of proinflammatory and proadhesive molecules (27, 30, 32).

Materials and Methods

Native and wild-type β2GPI

Native β2GPI was purified from human serum and characterized as previously described (13, 33). Wild-type synthetic molecule was obtained by insertion of cDNA for human β2GPI into the baculovirus viral DNA BacPAK 6 for expression in insect cells (*Spodoptera frugiperda* (Sf)) (20). Wild-type β2GPI was purified from supernatants of transfected insect cells, its purity assessed by SDS-PAGE, and subjected to N-terminal amino acid sequencing as detailed previously (20). As well as native β2GPI, the wild-type molecule: 1) displayed PL-binding properties, since it bound to CL-coated plates and inhibited the binding of native iodinated β2GPI to CL in solid phase; 2) expressed epitopes recognized by affinity-purified anti-β2GPI Abs in an anti-β2GPI ELISA; and 3) displayed cofactor activity, since it was recognized by anti-β2GPI Abs in a CL-ELISA (20).

β2GPI mutants

Site-directed mutagenesis was performed to assess the role of individual amino acids in the Lys^{282}-Lys^{287} loop for the PL-binding and anti-β2GPI activity as detailed (20). Four mutants were obtained: 1k, with a single amino acid change from Lys^{286} to Glu; 2k and 2ka, with a double amino acid change (from $Lys^{286,287}$ to $Glu^{286,287}$ and from $Lys^{284,287}$ to $Glu^{284,287}$ respectively); and 3k, with a triple amino acid change (from Lys ^{284,286,287} to Glu ^{284,286,287}). In comparison to native and wild-type β2GPI, mutant 1k displayed 1) reduced PL-binding activity, 2) lower cofactor activity for anti-β2GPI Abs in a CL-ELISA, and 3) decreased inhibition of the binding of iodinated native β2GPI to CL-coated plates. Double or triple mutants 2k, 2ka, and 3k lost the PL-binding activity and did not display any cofactor activity for anti-β2GPI Abs in CL ELISA or the ability to inhibit iodinated native β2GPI binding to CL. While mutations in the Lys^{282}-Lys^{287} loop altered the properties of the PL-binding site, they did not affect the recognition of all the mutants by purified anti-β2GPI Abs in an anti-β2GPI ELISA (20).

β2GPI synthetic peptides

The peptides (P1, P8) spanning the residues of the PL-binding site of the fifth domain of β2GPI, were synthesized by the Queensland Institute of Medical Research (Brisbane, Australia) as previously described (17, 34). Residue numbers in the native protein and amino acid sequence were 274–288, GDKVSFFCKNKEKKC for P1, and 274–288, GDKVSFFSKNKE KKS for P8, respectively (34). The residue GDKVSFF has been shown to be recognized by anti-β2GPI GR1D5 mAb, while the residue CKNKEKKC displays PL-binding site activity (34). Accordingly, both P1 and P8 were able to inhibit, in a dose-dependent manner, the binding of anti-β2GPI GR1D5 mAb to β2GPI. However, P8 did not display any cofactor activity, since the substitution of C^{281} and C^{288} with serine residues affected the PL-binding activity (34).

Human monoclonal anti-β2GPI Abs

Three human mAbs of the IgM isotype derived from patients with APS were used. GR1D5 and TM1G2 have been characterized previously as reacting with human β2GPI. TM1B9, which did not display reactivity to β2GPI, was used as a negative control. The characterization of the mAbs had been previously reported in detail (35).

Detection and affinity purification of anti-β2GPI Abs

Anti-β2GPI Abs were detected by a solid phase ELISA as described (10, 13, 27, 30). Sera from two patients with a primary APS were collected, and the whole IgG fractions were purified on protein G-Sepharose (HiTrap Protein G, Pharmacia Biotech Europe Uppsala, Sweden) as previously reported (13). The anti-β2GPI Abs were then affinity purified using solid phase, linked β2GPI as previously described (13). The characteristics of the patients and of the affinity-purified preparations have been reported in detail previously (13, 30).

Endothelial cell culture

HUVEC were isolated from normal term umbilical cord vein by collagenase perfusion and cultured as previously reported (27). Endothelial cells were grown in standard medium, washed three times with HBSS (Flow Laboratories, Irvine, U.K.) (200 μl/well), and cultured for 5 h in serum-free medium (HyQ-CCMTM 1, HyClone Laboratories, Logan, UT) to remove adherent serum proteins including β2GPI supplied by bovine serum present in the culture medium.

Binding of β2GPI and synthetic peptides to endothelial cells

A cell-ELISA was performed to determine whether any of the anti-β2GPI Abs (monoclonal and polyclonal) bound to endothelial monolayers through the adherent β2GPI and synthetic peptides. Serum-free endothelial cell cultures grown to confluence in 96-well microtiter plates were incubated with: 1) human native β2GPI (100, 50, and 25 nM); 2) recombinant wild-type β2GPI (100, 50, and 25 nM); 3) recombinant β2GPI mutants (100, 50, and 25 nM); 4) synthetic peptides (up to 1000 μM); and 5) with serum-free medium for 1 h at 37°C. The unoccupied binding sites in the wells were then blocked with HBSS-0.5% gelatin for 1 h at 37°C, and the plates where washed twice with HBSS. Affinity-purified anti-β2GPI IgG and the IgM mAb preparations, ranging from 100 to 3.1 μg/ml, were added to the wells. After a 2-h incubation, followed by three washes, the plates were incubated with alkaline phosphatase-conjugated goat anti-human IgG or IgM (Sigma Chemical, St. Louis, MO) for 90 min. After four washes, chromogenic substrate (50 μl of *p*-nitrophenylphosphate (1 mg/ml)) in 10% diethanolamine buffer, pH 9.8, was added to each well and incubated for 30 min. OD was read at 405 nm (Titertek Multiskan ELISA reader; Lobsystein, Finland) as previously described (27).

Endothelial E-selectin expression

E-selectin expression was evaluated by a cell ELISA as previously described (27, 30). Briefly, confluent HUVEC monolayers in serum-free medium were incubated with human purified β2GPI (100 nM) or synthetic peptides (800 μM) for 1 h at 37°C. The cultures were then incubated with: 1) human IgM anti-β2GPI mAb GR1D5 (at final protein concentrations ranging from 3.1 to 100 μg/ml); 2) human irrelevant IgM mAb TM1B9 (100 μg/ml); 3) affinity-purified IgG anti-β2GPI (100 μg/ml); or 4) normal human serum (NHS) IgG (100 μg/ml) in a final volume of 200 μl for 4 h at 37°C. After incubation, the cells were washed twice with RPMI 1640 (Life Technologies, Grand Island, NY), 2.5% FCS (Flow Laboratories), and incubated for 60 min at room temperature with 100 μl/well of murine monoclonal IgG specific for E-selectin at a final dilution of 1/1000 (Serotec, Kidlington, Oxford, U.K., catalogue no. MCA883). After three more washes, the cells were fixed with 3% paraformaldehyde for 15 min at room temperature. Cells were then washed three times and incubated for another 60 min at room temperature with 100 μl of peroxidase-conjugated goat anti-mouse IgG (Cappel, Cochranville, PA). After four washes with RPMI 1640, 2.5% FCS, and one more with PBS alone, 100 μl of orthophenylendiamine (0.5 mg/ml in sodium citrate buffer (10^{-1} M), pH 5, plus 0.01% H_2O_2) were added. The OD values were read at 450 nm, after a 30-min incubation, by a semiautomatic reader (Titertek Multiskan). The following control cultures were also performed: 1) HUVEC incubated with human purified β2GPI (100 nM) or with synthetic peptides (up to 1600 μM) alone; and 2) HUVEC in serum-free medium alone. As a positive control, additional cultures were incubated in the presence of 50 U/ml of human IL-1β (British Bio-Technology Products, Oxford, U.K.) using the same experimental conditions.

IL-6 production by endothelial cultures

To investigate endothelial IL-6 secretion, HUVEC cultures in serum-free medium were incubated with: 1) medium alone, 2) human purified β2GPI (100 nM), or 3) synthetic peptides (P1 or P8; 800 μM) for 1 h at 37°C. After two washes with HBSS, the human IgM GR1D5 or the irrelevant control TM1B9 (100 μg/ml) were added to the cultures at a final volume of 200 μl/well. As a positive control, HUVEC in serum-free medium were incubated with 50 U/ml of human rIL-1β, and the experiment was performed as described above. After the incubation period (6 h at 37°C), the plates were centrifuged at 800 rpm at 4°C for 10 min, and the cell-free supernatant was removed for IL-6 determination.

IL-6 was measured as hybridoma growth factor (HGF) on the indicator 7TD1 cell line, as previously reported (30).

Results

Binding of native and wild-type β2GPI and mutants of β2GPI to HUVEC monolayer in vitro

HUVEC cultured in serum-free medium were incubated with: 1) purified native human β2GPI, 2) recombinant wild-type β2GPI, and 3) 1k, 2k, 2ka, and 3k mutants of β2GPI at several concentrations. Endothelium-bound β2GPI was then evaluated as anti-β2GPI (GR1D5) mAb binding to the monolayers. In contrast to the

Table I. *Binding of GR1D5 anti-β2GPI mAb to endothelial cells incubated in the presence of native β2GPI, recombinant wild-type β2GPI, or recombinant β2GPI mutants*[a]

	Protein Concentrations (nM)		
	100	50	25
Native β2GPI	849 ± 91[b]	594 ± 49	176 ± 65
Wild-type β2GPI	915 ± 54	933 ± 38	329 ± 86
Mutants of β2GPI			
1K	528 ± 84	437 ± 93	297 ± 69
2K	275 ± 67	167 ± 35	191 ± 25
2Ka	202 ± 54	179 ± 32	214 ± 45
3K	130 ± 25	157 ± 32	117 ± 51

[a] GR1D5 anti-β2GPI mAb was used at 100 μg/ml. GR1D5 anti-β2GPI mAb incubated with HUVEC in serum-free medium without exogenous β2GPI gave the following background binding values: 134 ± 21 (mean of triplicate experiment ± SD). Comparable experiments performed with the control human IgM mAb (TM1B9) did not show any significant endothelial binding.

[b] OD values ($\times 10^{-3}$) are expressed as mean ± SD of triplicate experiments.

irrelevant human IgM mAb (TM1B9), GR1D5 anti-β2GPI human IgM mAb recognizes both human native and recombinant wild-type β2GPI on HUVEC monolayers (Table I). When endothelial cells were incubated with the 1k mutant, GR1D5 binding values were 58% of those obtained in the presence of recombinant wild-type β2GPI. No mAb binding was observed when HUVEC were incubated in the presence of mutants lacking the PL-binding properties (2k, 2ka, and 3k) or with serum-free medium. Comparable results have been obtained with another human anti-β2GPI TM1G2 mAb (data not shown). All of the human mAbs were used at a final protein concentration of 100 μg/ml, which was shown to display optimal binding (see Fig. 1). GR1D5 mAb binding to HUVEC was dependent on the final amount of native β2GPI, of recombinant molecules, or of peptides, respectively, added to the

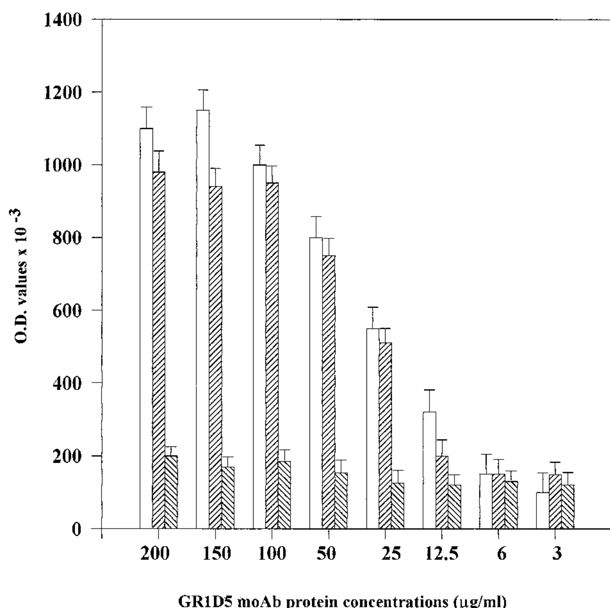

FIGURE 1. Endothelial GR1D5 binding at serial protein concentrations in the presence of native β2GPI (100 nM; □) or synthetic peptides P1 (800 μM; ▨) or P8 (800 μM; ▧). Values are expressed as OD ($\times 10^{-3}$) values; mean ± SD of triplicate experiments. Negligible binding was found by incubating HUVEC with the control mAb TM1B9 or with cells in serum-free medium (data not shown).

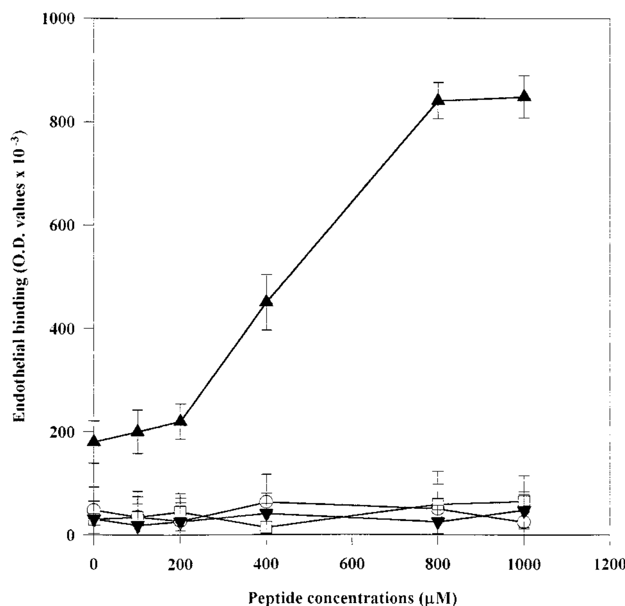

FIGURE 2. Recognition of adhered synthetic β2GPI peptides to HUVEC by the human anti-β2GPI mAb GR1D5 (100 μg/ml) and the control mAb TM1B9 (100 μg/ml). GR1D5 binding to serum-free HUVEC in the presence of synthetic peptides P1 (▲) or P8 (▼) (0 to 1000 μM) is shown. Parallel experiments were conducted with TM1B9 in the presence of the same peptides, P1 (○) or P8 (□). mAb binding is expressed as OD ($\times 10^{-3}$) mean values ± SD of triplicate experiments.

cultures (Table I). Wild-type β2GPI supported Ab binding to endothelial cells at concentrations of 50 and 25 nM, higher than for purified human native β2GPI.

Binding of synthetic β2GPI peptides to endothelial monolayers

HUVEC cultured in serum-free medium were incubated with a synthetic peptide (P1) possessing the amino acid sequence that binds to negatively charged PL. The synthetic peptide (P8) does not bind to PL and was used as a negative control. The human anti-β2GPI IgM (GR1D5) mAb that recognizes the common residue GDKVSFF was used to identify the peptides on the endothelial monolayer. Figure 2 shows that GR1D5 was able to bind HUVEC in the presence of P1. Endothelial GR1D5 binding was directly related to the amount of P1 added to the cultures, displaying maximum binding at a concentration of 800 μM. No binding was found with HUVEC incubated with the control peptide (P8) or with HUVEC cultured in serum-free medium alone. Comparable experiments performed with the irrelevant mAb TM1B9 displayed negligible endothelial binding. Control endothelial monolayers cultured in the presence of the optimal human native β2GPI concentration (100 nM) (30) displayed a clear GR1D5 binding (920 ± 32, mean OD values [$\times 10^{-3}$] ± SD of three determinations).

Two affinity-purified anti-β2GPI polyclonal IgG Abs (patients M.B. and B.A.) were able to recognize human β2GPI and synthetic peptide P1. Table II shows the binding of the affinity-purified anti-β2GPI IgG to endothelial monolayers in the absence or presence of human β2GPI or in the presence of increasing concentrations of P1 and P8 peptides. It can be seen that affinity-purified anti-β2GPI IgG preparations did not bind to endothelial monolayers cultured in serum-free medium and that the addition of human native β2GPI restored the binding. The same IgG fractions exhibited a dose-dependent binding to P1 but not to P8 on HUVEC monolayers. Control human IgG did not change their background

Table II. *Binding of NHS- and affinity-purified anti-β2GPI IgG fractions to HUVEC incubated in the presence of β2GPI synthetic peptides or of native β2GPI*

Peptide Concentrations	affinity-purified M.B.	affinity-purified B.A.	IgG NHS[a]
P1			
1000 μM	880 ± 58[b]	700 ± 51	182 ± 71
800 μM	850 ± 50	510 ± 28	115 ± 55
400 μM	430 ± 58	380 ± 35	194 ± 51
200 μM	220 ± 65	228 ± 25	189 ± 31
P8			
1000 μM	385 ± 60	250 ± 58	181 ± 54
800 μM	390 ± 55	230 ± 51	192 ± 62
400 μM	280 ± 65	200 ± 65	173 ± 32
200 μM	260 ± 32	170 ± 35	116 ± 39
Native β2GPI 100 nM	1100 ± 28	980 ± 39	189 ± 26
Without β2GPI	188 ± 32	179 ± 47	216 ± 38

[a] IgG preparations were used at the concentration of 100 μg/ml.
[b] Results are expressed as OD values ($\times 10^{-3}$); mean ± SD of triplicate experiments.

Table IV. *E-selectin expression induced by anti-β2GPI Abs on HUVEC incubated in the presence of either human purified β2GPI or the synthetic peptides P1 and P8*

	HUVEC in Serum-Free Medium Incubated with			
	Serum-free medium alone	Native β2GPI (100 nM)	P1 (800 μM)	P8 (800 μM)
GR1D5 (100 μg/ml)	113 ± 50[a]	1080 ± 130	1003 ± 98	95 ± 41
TM1B9 (100 μg/ml)	113 ± 41	94 ± 48	100 ± 21	100 ± 38
affinity-purified M.B. (100 μg/ml)	93 ± 35	1175 ± 109	531 ± 85	92 ± 65
affinity-purified B.A. (100 μg/ml)	90 ± 24	990 ± 59	700 ± 99	85 ± 42
NHS IgG (100 μg/ml)	93 ± 21	83 ± 36	38 ± 12	89 ± 35
IL-1β (50 U/ml)	1298 ± 75	1283 ± 98	1293 ± 93	1282 ± 105
Medium	76 ± 36	71 ± 31	65 ± 22	78 ± 35

[a] E-selectin expression was evaluated by a cell-ELISA as described in *Materials and Methods* and expressed as OD values ($\times 10^{-3}$); mean ± SD of triplicate experiments.

endothelial binding under any of the experimental conditions employed. Table III shows the dose-dependent binding of affinity-purified anti-β2GPI IgG to HUVEC monolayers incubated in the presence od optimal concentrations of native β2GPI or P1 or P8 peptides. Control NHS IgG did not display any endothelial binding even at highest protein concentration (100 μg/ml).

E-selectin expression induced by Ab binding to synthetic peptides on HUVEC

Since it has been demonstrated recently that both polyclonal and monoclonal anti-β2GPI Abs can up-regulate adhesion molecule expression after endothelial binding (27, 30), we investigated whether affinity-purified IgG and human anti-β2GPI mAbs directed against endothelium-bound P1 (and P8) were able to modulate E-selectin expression on HUVEC. To this end, HUVEC in serum-free medium were incubated with either native human β2GPI (100 nM) or peptide P1 (800 μM) or P8 (800 μM). E-selectin expression was then measured in the presence of mAbs, of affinity-purified anti-β2GPI IgG, or of the respective controls. Endothelial cultures treated with 50 U/ml of human rIL-1β were used as positive control for E-selectin expression. Table IV shows that, in contrast to IL-1β, both monoclonal and polyclonal anti-β2GPI Abs did not induce any E-selectin expression on HUVEC under serum-free medium conditions. The addition of human purified β2GPI as well as P1 (but not P8) induced E-selectin. As shown in Figure 3, GR1D5 induced E-selectin expression even at protein concentrations as low as 12.5 μg/ml. Parallel experiments with serial protein concentrations of affinity-purified IgG fractions showed that E-selectin was up-regulated at concentrations as high

Table III. *Binding of NHS- and affinity-purified anti-β2GPI IgG fractions, at serial protein concentrations, to HUVEC incubated in the presence of either β2GPI synthetic peptides or native β2GPI*

	P1 (800 μM)	P8 (800 μM)	Native β2GPI (100 nM)
affinity-purified M.B.			
100 μg/ml	880 ± 58[a]	375 ± 51	981 ± 41
50 μg/ml	650 ± 55	210 ± 55	700 ± 52
25 μg/ml	421 ± 50	180 ± 28	506 ± 39
12.5 μg/ml	215 ± 42	151 ± 35	302 ± 42
6 μg/ml	118 ± 24	129 ± 27	200 ± 34
3 μg/ml	105 ± 32	115 ± 38	162 ± 36
affinity-purified B.A.			
100 μg/ml	775 ± 36	139 ± 55	854 ± 32
50 μg/ml	400 ± 31	125 ± 18	421 ± 35
25 μg/ml	226 ± 26	142 ± 25	317 ± 22
12.5 μg/ml	119 ± 32	162 ± 32	129 ± 12
6 μg/ml	135 ± 29	145 ± 27	124 ± 32
3 μg/ml	145 ± 21	115 ± 25	115 ± 23
IgG NHS			
100 μg/ml	122 ± 14	116 ± 31	132 ± 22

[a] Results are expressed as OD values ($\times 10^{-3}$); mean ± SD of triplicate experiments.

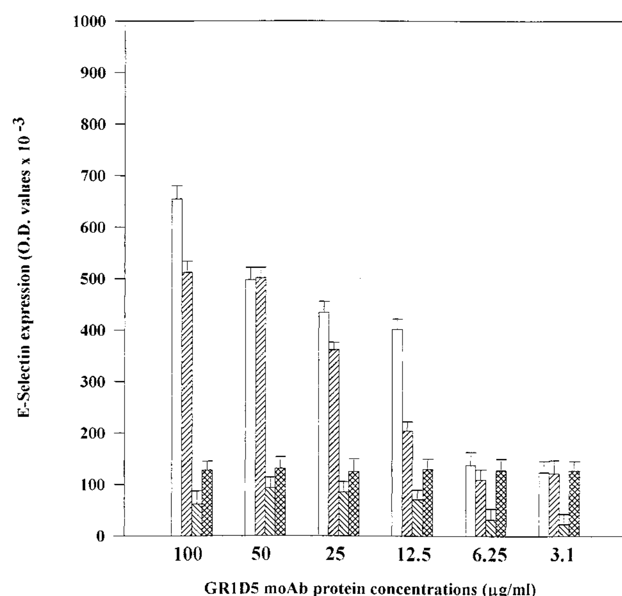

FIGURE 3. Anti-β2GPI mAb GR1D5 recognizes the synthetic peptide P1 bound to HUVEC and induces E-selectin expression in a dose-dependent manner. GR1D5 was used at serial protein concentrations (100–3.1 μg/ml). E-selectin expression was investigated in HUVEC cultured in the presence of human β2GPI (100 nM; □), or synthetic peptides P1 (800 μM; ▨) or P8 (800 μM; ▧), or in serum-free medium alone (▩). Values are expressed as OD ($\times 10^{-3}$) values; mean ± SD of triplicate experiments.

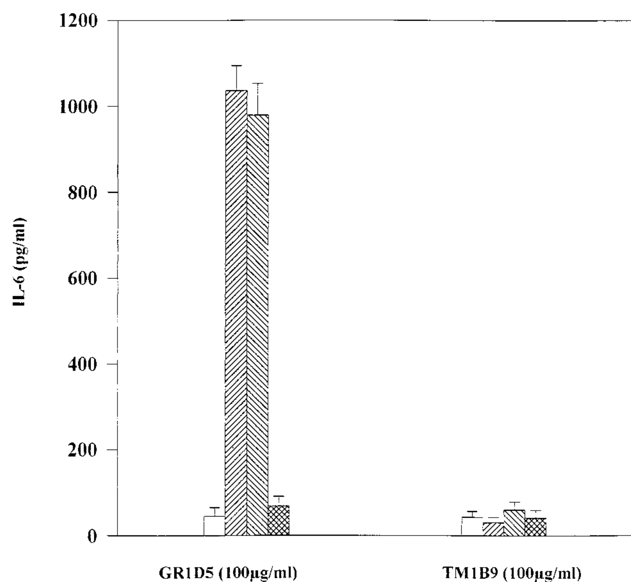

FIGURE 4. Endothelial IL-6 secretion by HUVEC incubated in the presence of β2GPI or synthetic peptides and anti-β2GPI mAbs (GR1D5 and TM1B9). IL-6 secretion was evaluated in supernatants of HUVEC cultured in serum-free medium (□) or in the presence of β2GPI (100 nM; ▨), P1 (800 μM; ▧), or P8 (800 μM; ▩). IL-6 levels are expressed as mean pg/ml ± SD of triplicate determinations.

as 25 μg/ml (data not shown). To confirm the specificity of this phenomenon, HUVEC monolayers were also incubated in the presence of high P1 and P8 concentrations alone: no E-selectin expression was demonstrated even at peptide concentrations higher (1600 μM) than those used in the experiments performed in the presence of the anti-β2GPI Abs (data not shown).

To exclude that endothelial activation was, in part or entirely, related to endotoxin contamination of the Ab preparations, parallel control experiments were also performed in the presence of Polymyxin B (5 μg/ml; Sigma Chemical). The Ab-induced E-selectin expression did not change. These findings suggest that endotoxin contamination does not play a major role in Ab-mediated endothelial activation (data not shown).

Ab binding to β2GPI synthetic peptides on endothelial cells up-regulates IL-6 production

To evaluate whether E-selectin expression was associated with other parameters of endothelial cell activation, we also investigated whether GR1D5 mAb was able to induce IL-6 production once bound to P1 adhered on HUVEC. Figure 4 shows that incubation of GR1D5 mAb with serum-free HUVEC pretreated with P1 up-regulated IL-6 secretion in an equivalent manner to monolayers incubated with purified human β2GPI. Endothelial cell monolayers incubated with comparable amounts of P8 and GR1D5 mAb displayed levels of IL-6 production similar to those found in control HUVEC cultures in serum free-medium alone. Control TM1B9 mAb did not modify IL-6 background production under any of the experimental conditions studied. Endothelial cell cultures performed in the presence of human rIL-1β (50 U/ml) secreted high levels of IL-6 in the supernatants (1050 ± 50 pg/ml, mean values ± SD of triplicate experiments).

Discussion

The binding of β2GPI to anionic structures seems to be a prerequisite for allowing Ab recognition of the molecule (3, 36). Anti-β2GPI Abs have been found to display low affinity binding and to require increased Ag density such as that offered by β2GPI bound to anionic structures (3, 5, 13). Alternatively, some authors have reported that conformational changes occurring in bound β2GPI might expose new epitopes eventually recognized by anti-β2GPI Abs (18).

It has been demonstrated that a highly positively charged amino acid sequence, Lys^{282}-Asn-Lys-Glu-Lys-Lys^{287}, located in the fifth domain of β2GPI, is the PL-binding site involved in the binding to CL-coated plates (16, 17, 20). A single amino acid substitution from Lys^{286} to Glu (mutant 1k) decreased the binding of β2GPI to CL in a significant manner. Further substitutions—from $Lys^{286,287}$ to $Glu^{286,287}$ (mutant 2k), from $Lys^{284,287}$ to $Glu^{284,287}$ (mutant 2ka), and from $Lys^{284,286,287}$ to $Glu^{284,286,287}$ (mutant 3k)—completely abolished the ability of β2GPI to bind to CL-coated plates (20).

Our data show that recombinant wild-type β2GPI binds HUVEC monolayers in a comparable manner to the native human molecule. Bound β2GPI was recognized both by human IgM mAbs and by affinity-purified polyclonal IgG anti-β2GPI Abs from two patients with APS. Using the recombinant β2GPI mutants, we demonstrated that the endothelial cell binding was comparable to that obtained in the CL-ELISA. Anti-β2GPI Ab binding was significantly reduced when HUVEC were incubated with mutant 1k, and it was completely abolished with 2k, 2ka, and 3k mutants of β2GPI. These data suggest that β2GPI binds to human endothelium by the major phospholipid-binding amino-acid sequence in the fifth domain. To confirm this observation, we used two different peptides: P1, which contains the sequence Cys^{281}-Lys-Asn-Lys-Glu-Lys-Lys-Cys^{288} previously found to display PL-binding activity, and P8, in which the substitution of Cys^{281} and Cys^{288} with serine residues abolished PL-binding (17, 34). Both peptides contain additional residues (GDKVSFF) that have been reported to be an epitope for human monoclonal IgM anti-β2GPI AbGR1D5 (34). The lack of reactivity of GR1D5 with HUVEC incubated in the presence of P8 and its dose-dependent binding to HUVEC incubated in the presence of serial concentrations of P1 further support the involvement of the PL-binding site in the adhesion of β2GPI to the endothelium. Interestingly, two affinity-purified polyclonal anti-β2GPI IgG fractions from patients with APS were shown to recognize P1 adherent to endothelial monolayers. The latter finding is consistent with the presence in APS sera of Ab specificities that react also with epitopes present in the fifth domain of the molecule (34).

We have previously reported that although anti-β2GPI GR1D5 mAb binds to P1 when it is complexed with CL, it is unable to react with microtiter plates coated with the same peptide in the absence of CL (34). It is reasonable to conclude that the binding of P1 to endothelial monolayers offers a suitable Ag density or the optimal peptide conformation, as is found in CL-coated plates.

To detect anti-β2GPI mAb binding, endothelial monolayers, as well as CL-coated plates (34), require a much higher peptide concentrations than that used with native β2GPI. These findings might be consistent with the higher affinity displayed by the PL-binding site in the native molecule than in the peptides and/or with the higher avidity of the native β2GPI for anionic PL (or equivalent endothelial structures) due to the presence of more than one PL-binding site. On the other hand, the affinity and/or avidity of the anti-β2GPI mAb itself could be higher for its epitope expressed on the native molecule than on the peptide.

The binding between anti-β2GPI Abs and β2GPI (or the P1 peptide) anchored to endothelial membranes activates the cells as demonstrated by the up-regulation of E-selectin expression and IL-6 secretion. These findings confirm previous data on endothelial

cell activation obtained with whole IgG fractions from APS sera (32) or with both human monoclonal and polyclonal affinity-purified IgG anti-β2GPI Abs (27, 30). In addition, our results indicate that a linear amino acid sequence of the fifth domain of β2GPI, which includes the major phospholipid-binding site, is one of the epitopes for anti-β2GPI Abs that bound to human endothelial cells. It has been suggested that the membrane binding of IgG aPL Abs might affect cell activation through the involvement of FcγR (37). The demonstration that anti-β2GPI IgM mAbs induce endothelial cell activation, in the presence of peptide P1, rules out this possibility.

β2GPI is a normal plasma glycoprotein whose endothelial cell binding might represent a physiologic event related, for example, to the cell uptake of lipid-carrying proteins (23). Accordingly, β2GPI adhesion to endothelium has been described even in normal placental vessels in in vivo studies (33, 38). Recently, Takeya et al. (39) demonstrated that anti-β2GPI mAbs exert lupus anticoagulant activity by enhancing the binding of β2GPI to phospholipids. Such an effect may account for the stabilization of the binding of β2GPI also to endothelial cell membranes and for the changing of a physiologic binding into a pathologic one. Moreover, the clustering effect of anti-β2GPI Abs on endothelial-adhered β2GPI molecules may also be the molecular means by which APS sera perturb the endothelial function in vitro.

In conclusion, our results are consistent with a model of β2GPI acting as an endothelial cell target Ag for circulating anti-β2GPI Abs in APS. β2GPI binds endothelial cell membranes through the predicted PL-binding site; its binding is apparently able to trap and increase Ag density and/or to induce conformational changes suitable for Ab binding. The final event is represented by functional changes in the endothelium that might be instrumental in promoting a procoagulant tendency. However, despite high levels of circulating autoantibodies and such in vitro findings, there are no evidences for an in vivo Ig deposition in the patients' vascular lesions (40). In this regard, it is useful to point out that anti-β2GPI Abs have been characterized as low affinity Abs (13, 41). Therefore, in vitro experimental conditions might offer the best Ag density and the optimal Ab binding that could be affected in vivo by detachment (or engulfment) of the Ab or the β2GPI/anti-β2GPI complexes from endothelial membranes. In such a way, endothelial binding could induce the activation state without a huge Ig deposition detectable by standard immunohistochemical analysis. Thrombosis would require a second triggering factor, thus explaining why patients with persistent serum autoantibodies display clotting events only occasionally and in the absence of detectable Ig deposits.

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