



## Inflammatory response and the endothelium

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**Abstract** Antiphospholipid-mediated endothelium perturbation plays a role in antiphospholipid syndrome (APS)-associated vasculopathy. Antiphospholipid antibodies activate endothelium both in vitro and in vivo experimental models by inducing a pro-inflammatory/-coagulant phenotype; the antibodies recognize  $\beta_2$  glycoprotein I ( $\beta_2$ GPI) on human endothelial cells (EC) from different parts of the vasculature.

In spite of such large in vitro evidence, few studies have addressed the issue whether or not a comparable endothelial perturbation might be detectable in vivo.

We investigated several indirect ex vivo parameters of endothelial dysfunction: plasma levels of soluble adhesion molecules (sADM), soluble thrombomodulin (sTM), von Willebrand factor (vWF) and tissue plasminogen activator (t-PA) by solid-phase assays. The study included: patients with primary antiphospholipid syndrome ( $n=32$ ), with the syndrome secondary to non-active systemic lupus erythematosus (SLE,  $n=10$ ), six patients with persistent antiphospholipid positivity at medium/high titre without any clinical manifestation of the syndrome. Fifty-two age and sex matched healthy subjects have been enrolled as controls. In addition, circulating endothelial cells identified by flow cytometry and the brachial artery flow-mediated vasodilation (FMV) were evaluated in 26 patients (20 primary and 6 lupus syndromes) and 30 healthy controls.

**Abbreviations:** aPL, anti-phospholipid antibody; EC, endothelial cell; LA, lupus anticoagulant;  $PGI_2$ , prostacyclin;  $TXA_2$ , thromboxane; APS, antiphospholipid syndrome;  $\beta_2$ GP,  $\beta_2$  glycoprotein I; sADM, soluble adhesion molecules; LPS, lipopolysaccharide; TF, tissue factor; HUVEC, human umbilical cord vein endothelial cells; HS, heparan sulphate; t-PA, tissue plasminogen activator; IL, interleukin; TNF, tumor necrosis factor; TLR, Toll-like receptor; sTM, soluble thrombomodulin; vWF, von Willebrand; SLE, systemic lupus erythematosus; FMV, flow-mediated vasodilation; ANCA, anti-neutrophil cytoplasmic antibody.

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Plasma levels of soluble adhesion molecules did not differ from controls, while a significant increase in von Willebrand factor titres ( $P < 0.05$ ) was found. No significant difference was found regarding the number of circulating endothelial cells and flow-mediated vasodilation.

As a whole, these findings do suggest that antiphospholipid antibodies per se are not able to support a full-blown endothelial perturbation *in vivo*. As shown in antiphospholipid syndrome experimental animal models, a *two-hit hypothesis* is suggested.

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The ability of anti-phospholipid antibody (aPL) to interact with endothelial cells (EC) was initially suggested by Carreras and Vermeylen [1]. They demonstrated that lupus anticoagulant (LA) positive plasmas can suppress prostacyclin (PGI<sub>2</sub>) release by vascular endothelium and in turn affect the balance between endothelial PGI<sub>2</sub> and platelet thromboxane (TXA<sub>2</sub>) favouring an anti-phospholipid syndrome (APS) hypercoagulable state.

More recently, aPL IgG fractions were shown to up-regulate endothelial expression of adhesion molecules (ADM) and leukocyte adherence *in vitro* [2]. At the same time, our group demonstrated that such an activity was related to the anti- $\beta$ 2 glycoprotein I ( $\beta$ 2GPI) antibody specificity [3,4]. These findings confirmed and extended the observation that anti-cardiolipin IgG fractions activated EC in the presence of  $\beta$ 2GPI [2]. Following these studies, other groups have confirmed this finding by using both polyclonal and monoclonal aPL [5–8]. Interestingly, aPL-mediated activation with ADM up-regulation and pro-inflammatory cytokine secretion was also found with human brain and skin primary EC cultures, suggesting that such an effect might be generalized to EC from different anatomical sites [9].

It is important to point out that EC activation has been reproduced in an *in vivo* experimental animal APS model by Pierangeli et al. [7]. We recently described an additional experimental *in vivo* model, in which the infusion of IgG fractions with strong anti- $\beta$ 2GPI activity but not control IgG can induce leukoaggregation in the mesenteric rat microcirculation when small amounts of lipopolysaccharide (LPS) are also injected at the same time (Fischetti et al., 2004, personal communication).

## The pleiotropic aPL effect on endothelial cells

Several *in vitro* studies have shown that incubation of EC with aPL can generate different effects on endothelial function.

In fact, besides the interference with the eicosanoid metabolism and the induction of a pro-adhesive phenotype, *in vitro* EC incubation with aPL was reported to be able: (i) to up-regulate pro-inflammatory and chemokine synthesis and secretion [5–8], (ii) to modulate tissue factor (TF) expression on the cell membrane [10,11], (iii) to interfere with the protein C/S activation (review in Ref. [12]), (iv) to displace annexin V binding to the cell membrane [13], (v) to induce pre-pro-endothelin (ET)-I synthesis [14], (vi) to favour an apoptotic process [15,16] and (vii) to interact with late endosomes [17] (Table 1).

As a whole, these effects might cooperate in sustaining the endothelial perturbation that has been suggested to play a pivotal pathogenic role in APS-associated thrombophilic state.

## $\beta$ 2GPI as the main endothelial target antigen for aPL

The demonstration that APS sera might recognize antigenic determinants on EC membrane is the necessary requisite to explain any interaction between aPL and EC. By using an immunoprecipitation assay that specifically radiolabelled HUVEC apical cell surface proteins, two groups independently reported that APS sera immunoprecipitated several EC membrane proteins with a molecular weight ranging from 200 to 24 kDa [18,19]. Additional studies demonstrated that most of the endothelial binding of aPL positive IgG fractions was closely related to the anti- $\beta$ 2GPI activity (review in Ref. [12]). Moreover, affinity purified

**Table 1** Pleiotropic effect of aPL on endothelial cells

- Induction of a pro-inflammatory phenotype
- Tissue factor up-regulation
- Interaction with the protein C/S system
- Interaction with annexin V
- Interaction with the eicosanoid metabolism
- Induction of pre-proET-1 synthesis
- Induction of apoptosis
- Interaction with the late endosomes

polyclonal IgG or human monoclonal IgM with anti- $\beta$ 2GPI specificity were found to recognize the molecule on the EC surface membrane (review in Ref. [12]).

Plasma proteins can adhere to EC and the presence of  $\beta$ 2GPI on the endothelium was suggested to be, at least in part, due to such a general phenomenon [20]. Actually, extensive washing and culture in serum-free medium abolished the anti- $\beta$ 2GPI reactivity with human umbilical cord vein endothelial cell (HUVEC) monolayers that was in turn restored by the addition of purified  $\beta$ 2GPI (review in Ref. [12]). Interestingly, such a phenomenon was found with HUVEC monolayers but not with primary human EC cultures from skin or brain micro-circulation [9]. It has been suggested that EC might synthesize  $\beta$ 2GPI themselves, but this has not been substantiated (review in Ref. [12]).

Our own group demonstrated that  $\beta$ 2GPI binds to EC membranes through the putative PL-binding site—a highly positively charged amino acid sequence, located in the fifth domain of  $\beta$ 2GPI and previously shown to be involved in the binding to cardiolipin-coated plates (review in Ref. [12]). It has been suggested that heparan sulphate (HS)—the major proteoglycan of the vascular endothelium that constitutes the majority of the constitutive endothelial anionic sites (review in Ref. [12])—might be the corresponding negatively charged structure for the  $\beta$ 2GPI PL-binding site. In agreement with such a hypothesis, HUVEC treatment with heparitinase I, an enzyme able to cleave specifically the  $\alpha$ -*N*-acetyl-D-glycosaminidic linkage in HS, significantly down-regulates  $\beta$ 2GPI binding [9]. However, even the highest enzyme concentrations gave an inhibition only up to 65%. Although these findings indicate that HS is involved in  $\beta$ 2GPI binding, endothelial structures other than HS should be considered to bind  $\beta$ 2GPI.

Megalin—an endocytic receptor expressed on placenta, kidney and other tissues—was reported as a possible receptor for  $\beta$ 2GPI [21]. However, Cao et al. [22] showed that megalin-mRNA was not expressed in both human vein and arterial umbilical EC. Moreover,  $\beta$ 2GPI binding to megalin was reported to be  $\text{Ca}^{2+}$  ion-dependent; however, it is not affected at all when HUVEC are cultured in EDTA buffer (Meroni et al., personal communication). Altogether, these data are consistent with the fact that megalin is not likely involved in EC  $\beta$ 2GPI binding.

More recently,  $\beta$ 2GPI-binding proteins of ~70 and ~36 kDa from HUVEC and EAhy.926 cells were found to be identical in amino acid sequence to annexin II [23], an endothelial receptor for tissue plasminogen activator (t-PA) [24–26]. In addition, radio-

labelled  $\beta$ 2GPI was found to adhere at high affinity to Annexin II-transfected HEK 293 cells and the adhesion to HUVEC was inhibited by anti-annexin II antibodies [23]. The authors suggested that Annexin II might represent an EC membrane receptor for  $\beta$ 2GPI.

Whatever the endothelial receptor for  $\beta$ 2GPI is, all the reported findings do support the hypothesis that adhered  $\beta$ 2GPI offers suitable epitopes for anti- $\beta$ 2GPI antibodies either by making available high density immunogenic epitopes or by displaying new cryptic epitopes comparable to those detectable on ELISA plates either coated with anionic phospholipids or  $\gamma$ -irradiated.

### Endothelial activation by aPL

We and others have demonstrated that anti- $\beta$ 2GPI/aPL antibodies were able to induce a pro-inflammatory and a pro-adhesive endothelial phenotype through NF- $\kappa$ B activation in a similar way to that found with standard agonists such as IL-1 $\beta$ , TNF- $\alpha$  and LPS (review in Refs. [12,10]).

The NF- $\kappa$ B upstream EC activation pathways have been recently investigated and reported to be mediated by TRAF adapter family. TRAF-2 plays a critical role in the presence of TNF- $\alpha$ ; TRAF-6 is employed in IL-1 and LPS signalling pathways [27] and the MyD88 is identified as a further mediator molecule involved in the IL-1/LPS activation cascade. By using EC transfected with negative dominants of TRAF-2/-6 or MyD88 our group indicated that both human monoclonal IgM as well as polyclonal IgG with anti- $\beta$ 2GPI activity induced a signalling cascade comparable to that activated by LPS or IL-1 [28]. Additional studies on the phosphorylation time kinetic of the IRAK—the first kinase to be recruited by the IL-1/Toll-like receptor (TLR) superfamily—suggested that the autoantibodies activated EC through the TLR-4 involved in LPS pathway [28].

TLR are key components of the innate immune response able to recognize specific microbial products, including LPS, bacterial lipoproteins, peptidoglycan and bacterial DNA [29]. TLR expression is a widespread phenomenon in both lymphoid and non-lymphoid tissues; in particular, TLR-4 is found on EC, monocytes, neutrophils and dendritic cells [29].

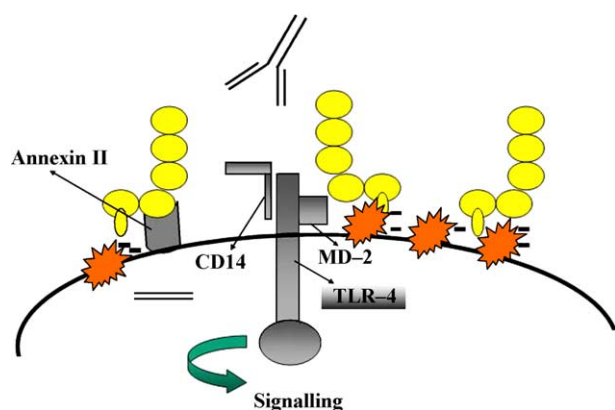
The relationship between TLRs and  $\beta$ 2GPI is apparently supported by the molecular mimicry shared by  $\beta$ 2GPI and different microbial pathogens—the natural ligands of TLRs [29,30,31,32,33]. We speculate that  $\beta$ 2GPI might adhere to EC through its PL-binding site and membrane anionic structures

and to interact with TLRs because of such a homology. In turn, anti- $\beta$ 2GPI antibodies might cross-link the complex eventually triggering the inflammatory response through the TLR cascade.

As stated before,  $\beta$ 2GPI can also bind annexin II. However, it has been reported that annexin II does not span the cell membrane, so the interaction between  $\beta$ 2GPI linked to annexin II and anti- $\beta$ 2GPI antibodies would not be sufficient to induce any intra-cytoplasmic signal and probably does require a yet unknown “adaptor” protein [23–26]. We speculate that  $\beta$ 2GPI behaves as a “bridge” between annexin II and TLR-4 on EC and at the same time it might offer suitable antigenic epitopes for the circulating autoantibodies. Antibody reactivity with such a complex could eventually cluster the right membrane molecules and trigger the cell signalling (Fig. 1).

### Do aPL induce an endothelial perturbation in vivo too?

There is evidence that  $\beta$ 2GPI can be expressed also on EC in vivo. Antisera specific for human  $\beta$ 2GPI give a clear staining on trophoblast vessels in term placentas [34,35]. However, in spite of the in vitro data with human primary skin EC cultures, we did not find a comparable endothelial staining in skin biopsies obtained from normal donors or patients



**Figure 1** Possible mechanisms for  $\beta$ 2GPI binding to endothelial cell membrane and cell signalling. Beta2GPI can bind to endothelial cell membrane via the electric charge interactions between the cationic phospholipid binding site and anionic structures or as a ligand for specific receptors (annexin II). The molecule can also interact with the adjacent TLR because of its homology with the microbial ligands for TLRs. Cell signalling might take place once circulating anti- $\beta$ 2GPI antibodies recognize the adhered molecules and cluster the complexes.

affected by inflammatory disorders (unpublished observations).

The question whether an endothelial perturbation comparable to that detectable in in vitro studies might be also detectable in vivo in APS patients has been addressed by few studies. Different indirect parameters of EC perturbation ex-vivo have been investigated with no definite conclusions. While some studies reported increased plasma levels of single soluble ADM or endothelial derived microparticles in APS patients, others did not confirm these findings [36–39]. Additional confounding variables—such as a concomitant associated immune-mediated systemic inflammatory disorder—did weaken the comparison. These findings are in contrast to those that demonstrate the aPL thrombogenic effect is closely mediated by ADM expression in a murine experimental model [40]. On the other hand, clear histological signs of endothelial activation have been reported only in biopsies taken from patients affected by the APS-related heart valvulopathy [41].

We investigated several indirect in vivo parameters of endothelial dysfunction in aPL positive patients: sADM: (sEselectin, sICAM-1, sVCAM-1), sTM, vWF and t-PA evaluated by solid-phase assays. Plasma levels of soluble ADM, as well as levels of sTM, vWF and t-PA have been reported to be increased in different disorders characterized by the presence of an endothelial perturbation [42,43]. These parameters have been investigated in: 32 patients with primary APS, 10 with APS secondary to systemic lupus erythematosus (SLE) and 4 SLE patients. All the included SLE patients displayed non-active disease (ECLAM score  $\leq 2$ ) in order to avoid any potential variables related to the immune-mediated inflammation sustained by the lupus disease itself. Additional two patients with persistent antiphospholipid positivity at medium/high titre but without any clinical manifestations and 52 age- and sex-matched controls were studied.

In addition a series of patients (20 primary and 6 SLE APS) was investigated for: (i) the presence of circulating EC identified by flow cytometry and (ii) the brachial artery flow-mediated vasodilation (FMV) at rest, during reactive hyperemia and after glyceryl nitrate. High levels of circulating EC have been reported in patients suffering from disorders characterized by an endothelial damage sustained by systemic immune-mediated inflammation such as in patients suffering from anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis or by ischaemic processes [44]. An impaired endothelial-dependent FMV has been also reported in comparable conditions [45].



Plasma levels of soluble ADAMs and sTM did not reach a statistical significance in APS patients in comparison with controls; t-PA levels showed an increase in patients that however did not reach the statistical significance. On the contrary, significantly increased vWF titres were found in APS patients. No significant difference between patients and controls was found regarding the number of circulating EC as well as the brachial artery FMV.

As a whole, these findings do support that aPL per se are not able to induce significant endothelial perturbation in vivo. This finding is in line with the results reported in the experimental animal models in which the infusion of aPL does not trigger clot formation or a strong endothelial activation in uninjured vessels unless a mechanical trauma or an inflammatory trigger is applied (Fischetti et al., 7, 2004, submitted). In this regard, a *two-hit hypothesis* has been suggested: aPL (*first hit*) increases the risk of thrombotic events by inducing a threshold endothelial perturbation; however, another thrombophilic condition (*second hit*) is required in order to trigger the clot formation. Moreover, the *two hit hypothesis* might also explain why patients persistently positive for aPL do display thrombotic events only occasionally.

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