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## Pathogenic mechanisms of the anti-phospholipid antibodies<sup>☆</sup>

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## ABSTRACT

The antiphospholipid syndrome (APS) is an autoimmune disease characterized by recurrent fetal loss, thrombotic events (arterial or venous) and hemocytopenic disorders associated to high titers of circulating aPL. Two variants of the APS have been described. Primary APS is a clinical entity without evidence of any other autoimmune disease and secondary APS is a clinical disorder mainly associated with Systemic Lupus Erythematosus (SLE). aPL are a widely group of immunoglobulins directed against different components or proteins factors. In 1990 three groups of researchers identified that β2GP-I is the mainly antigenic target of aPL in APS patients. There are evidences that show that more than one pathogenic mechanism is involved in the development of the APS. The best documented clinical manifestations associated with the APS are recurrent fetal loss and thrombotic disorders. The latter is based on observations in vivo in animal models and in vitro on the effects caused by aβ2GP-I antibodies from patients with APS or from animals which cause experimental APS.

The objective of the present paper is to show the pathogenic mechanisms that participate in the development of the APS. We also presented evidence that shows that aβ2GP-I induces pro-inflammatory, pro-adhesive and pro-coagulant disorder.

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### Mecanismos patogénicos de los anticuerpos antifosfolípidos

## RESUMEN

El síndrome de antifosfolípidos (SaF) es una enfermedad autoinmune caracterizada por abortos recurrentes, eventos tromboticos (arteriales o venosos) y hemocitopenias asociadas con títulos altos de aFL séricos. Se han descrito dos presentaciones de SaF: el SaF primario, que se presenta como entidad única y el SaF secundario o asociado principalmente a LEG.

Los aFL son un grupo heterogéneo de inmunoglobulinas dirigidas contra diversos componentes o factores proteicos. En 1990, tres grupos de investigadores identificaron a la β2GP-I como el principal blanco antigénico de los aFL presentes en los pacientes con SaF. Diversos trabajos han mostrado que existe más de un mecanismo patogénico involucrado en el desarrollo del SaF. Las manifestaciones clínicas mejor documentadas son los abortos recurrentes y las alteraciones tromboticas. Lo anterior se fundamenta en las evidencias observadas in vivo en modelos animales e in vitro causadas por los anticuerpos anti-β2GP-I (aβ2GP-I) de pacientes con SaF o de origen animal.

La presente revisión tiene como objetivo mostrar los mecanismos patogénicos que participan en el desarrollo del SaF. Presentamos, además, las evidencias que muestran que los aβ2GP-I inducen un estado proinflamatorio, proadhesivo y procoagulante.

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## Palabras clave:

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### Introduction

The first description of antiphospholipid syndrome (APS) was made in 1963, by Bowie et al, in a group of patients with systemic lupus erythematosus (SLE) who developed thrombotic events despite having circulating lupus anticoagulant.<sup>1</sup> Two decades later, a study in patients with SLE showed that thrombotic events

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were associated with the presence of circulating anticardiolipin antibodies (aCL),<sup>2</sup> so Graham Hughes described it as aCL syndrome.<sup>3</sup> Subsequently, however, it was proved that sera from these patients reacted not only against CL, but also against other phospholipids; thus the name of the syndrome was modified and extended to APS. Subsequently, it was noted that in addition to thrombotic events (venous or arterial), the presence of aCL was associated with recurrent foetal losses, neurological disorders, thrombocytopenia, haemolytic anaemia and livedo reticularis; symptoms which form part of the classification criteria for patients with APS, which may be associated with other autoimmune diseases. The best studied association is with SLE, defined as secondary APS (SAPS),<sup>4</sup> or in patients with clinical manifestations of APS but without evidence of another autoimmune disease, which is known as primary APS (PAPS).<sup>5-7</sup> In 1990, 3 research groups almost simultaneously demonstrated that the main antigenic target of antiphospholipid antibodies (aPL) was  $\beta$ 2-glycoprotein-I ( $\beta$ 2GP-I).<sup>8-10</sup> This event changed the course of investigations related to APS and, consequently, the study of  $\beta$ 2GP-I aroused great interest within the pathogenesis of APS.

The present work reviews the most relevant aspects of the evidence related to possible pathogenic mechanisms of aPL or  $\beta$ 2GP-I in the pathogenesis of APS.

### Antiphospholipid syndrome

APS is an autoimmune disease of unknown aetiology, which is the result of the interaction of environmental (e.g. infection), hormonal (e.g. higher prevalence in women) and genetic factors (association with MHC molecules, e.g. HLA-DR4, -DR7, -DR53). Currently, APS is defined as a clinical entity associated with the presence of thrombotic events (arterial and/or venous), repeated abortions, livedo reticularis, thrombocytopenia, haemolytic anaemia and neurological disorders with high titers of circulating aPL.<sup>11</sup>

### Antiphospholipid antibodies

aPLs are a heterogeneous family of immunoglobulins which recognize different protein components or factors (e.g. annexin V, prothrombin, protein C, protein S, among others), of which the most relevant are those directed against  $\beta$ 2GP-I. Due to the important association of IgG/IgM isotype a $\beta$ 2GP-I, these were included as part of the classification criteria for APS in 2006.<sup>12</sup> It is noteworthy that the name given to  $\beta$ 2GP-I as a cofactor for aPL is incorrect, since by definition a cofactor is a small organic molecule required for enzyme activity. Therefore, the term should not be used to define the molecules recognized by aPL associated with autoimmune diseases. Additionally, the importance of a $\beta$ 2GP-I was also demonstrated in our laboratory in 1995, in patients with APS.<sup>13</sup> The present work reviews the pathogenic mechanisms of a $\beta$ 2GP-I in APS which have the strongest scientific support.

### $\beta$ 2-glycoprotein-I ( $\beta$ 2GP-I)

$\beta$ 2GP-I or apolipoprotein H is a plasma protein present in all individuals in a concentration of about 200  $\mu$ g/ml. Studies in our laboratory show a greater concentration in healthy individuals of the female gender.<sup>14</sup>  $\beta$ 2GP-I is a highly glycosylated polypeptide chain consisting of 326 amino acids. Its molecular weight is 50kDa and approximately 30% of its weight is made up of carbohydrates. The  $\beta$ 2GP-I gene is located on chromosome 17q23-qter.  $\beta$ 2GP-I has 5 homologous domains, of approximately 60 amino acids each. The binding site of  $\beta$ 2GP-I to negatively charged phospholipids (e.g. cardiolipin, phosphatidylserine and phosphatidylinositol) or other molecules such as heparan sulphate, is located on the fifth (v) domain (amino acid sequence 281CKNKEKCC288) since

the 4 lysine (K) residues give the sequence a positive charge. However,  $\beta$ 2GP-I was described in 1961 and its physiological role was not identified until it was associated with aPL.<sup>8-10</sup> Thus it was understood that it participates in the physiological processes of anticoagulation.<sup>15</sup>

### Pathogenic mechanisms of aPL

According to the work of Evans et al,<sup>16</sup> the possible pathogenic mechanisms of aPL can be generally grouped into: 1) effect on procoagulant and anticoagulant mechanisms which take place in the membranes of some cells, and 2) activation of target cells and induction of the expression and secretion of various molecules.

In the study of the pathogenic mechanisms of aPL, different research groups have focused on thrombotic events and on abortions associated with aPL. However, due to the heterogeneity of the clinical manifestations, it is likely that more than one pathophysiological mechanism is involved in the development of the disease.

It is clear that APS is a disease mediated by antibodies. This statement is based on a perspective oriented towards a deep and thorough study of aPL. However, there is currently much evidence which shows that the elements of the cellular immune response, specifically CD4+ T lymphocytes, complement or play an important role in the pathophysiological mechanisms occurring in APS.

### Alteration of coagulation/anticoagulation mechanisms

Experimental evidence shows that some patients with APS may have antibodies which recognize protein C, protein S and thrombomodulin,<sup>17,18</sup> altering the coagulation/anticoagulation systems in which they participate and thus generating a prothrombotic state. In addition, it has also been shown that  $\beta$ 2GP-I inhibits the binding of protein C to phospholipids, favouring the event.<sup>19</sup> In 1981, Cosgriff reported that anti-thrombin III activity (the main inhibitor of factors IXa, Xa and thrombin) may be altered in patients with APS.<sup>20</sup>

Another molecule involved is annexin V (a protein which plays a thrombomodulator role in placental circulation and has a high affinity for negatively charged phospholipids). In 2000, Lakos et al reported that some patients with APS presented circulating antibodies directed against annexin V.<sup>21</sup> The presence of anti-annexin V antibodies is controversial. In 2001, Pasquier et al and Nojima et al measured anti-annexin V antibodies in patients with APS and found no association.<sup>22,23</sup>

On the other hand, while there is evidence that  $\beta$ 2GP-I has anticoagulant properties, the binding of a $\beta$ 2GP-I to  $\beta$ 2GP-I increases the affinity of the latter for the anionic phospholipids of cell membranes; thus, there is a competition between free  $\beta$ 2GP-I and the a $\beta$ 2GP-I/ $\beta$ 2GP-I complex for negatively charged phospholipids, which alters haemostatic reactions.

### Monocytes, endothelial cells and tissue damage associated to the presence of aPL

Tissue factor (TF) is a key protein in the activation of the coagulation cascade. It forms complexes with factor VIIa and phospholipids, activating factors IX and X. When the vascular endothelium is intact there is no expression of TF on the surface of cells. However, when it becomes activated under certain stimuli or loses its integrity, then TF is expressed in endothelial cells and in circulating monocytes. Using *in vitro* tests, Reverter et al demonstrated that there was an increase of TF expression in monocytes in the presence of IgG isotype aCL from APS patients who had presented thrombotic episodes. In contrast, the effect did not occur in the presence of IgG isotype aCL antibodies purified from SLE patients who had not

presented thrombotic events.<sup>24,25</sup> Additionally, this increase in TF also occurred in the presence of a $\beta$ 2GP-I antibodies from patients with APS. In 1999, Dobado-Barrios et al showed that the TF mRNA levels in mononuclear cells from PAPS patients were increased compared with mononuclear cells from healthy subjects, and that the levels of expression were higher in those patients who had suffered thrombotic events.<sup>26</sup> With regard to endothelial cells (EC), which are directly involved in the regulation of haemostasis, the presence of IgG isotype a $\beta$ 2GP-I purified from APS patients induced the expression of TF and adhesion molecules (selectin E, ICAM-2 and VCAM-1); this favoured a procoagulant state.<sup>27,28</sup> Additionally, Meroni et al demonstrated that in cultures of EC, the increase in adhesion molecules was accompanied by increased expression of proinflammatory cytokines IL-1 $\beta$  and IL-6.<sup>29</sup> In a very elegant manner, Pierangeli et al used a murine model of APS to demonstrate that the presence of a $\beta$ 2GP-I purified from APS patients induced the expression of adhesion molecules and the adhesion of leukocytes to vascular endothelium.<sup>30</sup>

On the other hand, several groups have shown the involvement of the complement system, specifically the activation of C3, C4 and C5, in foetal resorption and in thrombotic events in murine models.<sup>31–34</sup>

Tissue damage has recently been demonstrated at the placental level in foetal loss associated with APS. Using an *in vitro* system, Di Simone et al showed the binding of a $\beta$ 2GP-I to cytotrophoblasts, which affected their invasive capacity.<sup>35</sup> In addition, the binding of a $\beta$ 2GP-I decreased the synthesis of human chorionic gonadotropin (HCG). During their maturation process, trophoblasts expose negatively charged phospholipids on the outer face of the cytoplasmic membrane, thus favouring the binding of the a $\beta$ 2GP-I/ $\beta$ 2GP-I complex. The formation of the immune complex activates the thrombotic process by activating platelets via the Fc $\gamma$ II receptors with high affinity for the Fc portion of the immune complexes<sup>36</sup> and, jointly, several mechanisms which promote thrombotic events.

### Cellular immunity and APS

The first evidence showing the importance of T lymphocytes in APS was reported by Blank et al in 1995. Dr. Blank et al documented that the transfer of bone marrow cells with T cells or depleted of these, from mice with experimental APS to irradiated syngeneic mice, induced the development of clinical manifestations of APS (thrombocytopenia, prolonged partial activated thromboplastin time and foetal resorption) only in those mice which received the bone marrow cells containing T cells.<sup>37</sup> In addition to this, patients with APS presented circulating IgG and IgA isotype a $\beta$ 2GP-I, suggesting, prior to the experiments reported by Blank, that there is a collaboration between T and B lymphocytes in the activation of the autoimmune response in patients with APS. This has been confirmed *in vitro*, in trials in which it has been shown that peripheral blood mononuclear cells (PBMC) from APS patients have a greater proliferative effect in the presence of  $\beta$ 2GP-I than the PBMC from healthy subjects.<sup>38</sup> Studies carried out in our laboratory confirmed this phenomenon,<sup>39</sup> since the specific proliferative effect induced by  $\beta$ 2GP-I significantly decreased in the absence of CD4+ T lymphocytes. In 2001, Arai et al showed that the proliferative effect of  $\beta$ 2GP-I was located in the V domain, at the phospholipid binding site.<sup>40</sup> Another segment of the protein which is important in cell activation is one that includes amino acid position 247,<sup>40,41</sup> in which there is a polymorphism of the amino acids leucine and valine. A work carried out by Ito et al showed a higher proliferation of PBMC against a peptide containing this polymorphism (amino acids 244–264).<sup>41</sup> In 2003, we documented the existence of an association between the valine polymorphism at position 247 of  $\beta$ 2GP-I with high titers of IgG isotype a $\beta$ 2GP-I and thrombotic events.<sup>42</sup>

The study by Arai et al<sup>40</sup> detected an elevated production of IL-6 and INF $\gamma$  and a $\beta$ 2GP-I antibodies generated *in vitro* in PBMC culture supernatants from patients with APS. Inhibition of IL-6 with specific monoclonal antibodies (mAb) inhibited the production of a $\beta$ 2GP-I, whereas the inhibition of INF $\gamma$  with specific mAb did not affect their synthesis. In our laboratory we obtained and characterized clones of B cells from a patient with APS who consistently presented high serum titers of IgG, IgA and IgM isotypes a $\beta$ 2GP-I.<sup>43</sup> Cultures of B cells transformed with Epstein-Barr virus showed a high production of IL-6, which was associated with *in vitro* production of a $\beta$ 2GP-I.<sup>43</sup>

In addition to T and B lymphocytes, there are other cells involved in the development of the clinical manifestations of APS. Recently, the group of Salmon et al documented the importance of neutrophils in the generation of foetal resorption.<sup>33</sup> An experimental mouse model of APS showed that the absence of neutrophils decreased foetal resorption induced by IgG isotype aPL from APS patients.<sup>33</sup>

### Cytokines

The role of cytokines in APS has also been evaluated. The number of studies in this regard is smaller compared with the studies on aPL. One of the characteristics which converge between cellular and humoral immune response is the regulation by cytokines, which regulate the immune response. It is currently known that various stimuli have an effect on the balance or the generation of pro- and anti-inflammatory cytokines and, based on this synthesis, they have been classified into Th1, Th2<sup>44</sup> and recently Th17.<sup>45</sup> The role of cytokines in APS was demonstrated by Krause et al in 1999<sup>46</sup> in an experimental APS model. Krause induced APS by injecting BALB/c mice with a mAb with aCL activity, called H3, which was obtained from a healthy subject. The activity of the H3 mAb was neutralized with an anti-idiotypic antibody (anti-H3). An irrelevant anti-idiotypic antibody was administered as a control. Treatment with anti-H3 significantly decreased the number of Th2 cells (producers of IL-4 and IL-6) and increased Th1 cells (producers of IL-2 and INF $\gamma$ ), thus demonstrating the importance of the Th1/Th2 balance. In the same year, Visvanthan and McNeil showed an increased *in vitro* production of INF $\gamma$  with respect to IL-4 in cultures of PBMC from patients with APS, thus suggesting a Th1 polarization.<sup>47</sup> The authors suggested that increased production of INF $\gamma$  could be related to foetal loss associated with APS, and that during pregnancy the production of cytokines is polarized toward a Th2 response. Karakantza et al showed an *in vitro* increase in the production of INF $\gamma$  by CD4+ T cells from patients with APS.<sup>48</sup> This could also be supported by a high TNF production, as demonstrated by Berman in 2005 in a model of TNF-deficient mice, among which there was a low percentage of foetal resorption when administered purified aPL from APS patients.<sup>49</sup>

By contrast, Ito et al stimulated PBMC from APS patients with human purified  $\beta$ 2GP-I and observed a high *in vitro* production of INF $\gamma$  and IL-4, a response known as Th0.<sup>41</sup> One year later, Arai et al reported a high production of INF $\gamma$  and IL6 (Th0 pattern) by clones of auto-reactive CD4+ T lymphocytes.<sup>40</sup>

Regulation by cytokines plays an important role in APS. However, the published results are very heterogeneous, probably due to the characteristics of the syndrome *per se*. However, several points should be noted: 1) the documented studies have been conducted mainly *in vitro*; 2) the heterogeneity of the experiments makes it difficult to analyze the results (e.g. cultures stimulated with native and/or distorted  $\beta$ 2GP-I, determined cytokines, among others), and 3) the patterns of cytokines in murine models are more consistent than in humans. Advances in the study of APS and the participation of immune response elements require further studies in order to better understand the phenomena.

## APS as a pro-inflammatory state

APS was considered as a non-inflammatory clinical entity. However, recent evidence suggests that it is a pro-inflammatory entity or state. Recently, Hamid et al studied the *in vitro* expression of 18,400 genes in endothelial cells from umbilical cord (HUVEC).<sup>50</sup> They incubated the cells with IgG isotype  $\alpha\beta$ 2GP-I purified from patients with PAPS, then isolated the total RNA and, using microarrays, analyzed the pattern of gene expression. Compared with the control antibodies, in which no significant expression of genes was observed, the presence of  $\alpha\beta$ 2GP-I induced the expression of 101 genes. Among the overexpressed genes, it was found that a significant number of them corresponded to chemokine genes (CCL20, CXCL3, CX3CL1, CXCL5, CXCL2 and CXCL1), which are involved in the recruitment, chemotaxis and proliferation of mononuclear cells and/or granulocytes. This supports the hypothesis that APS is a pro-inflammatory state. The findings support the *in vivo* and *in vitro* studies which have shown an increased cell adhesion to EC caused by  $\alpha\beta$ 2GP-I and, consequently, the recruitment of inflammatory cells, mainly macrophages, in the placenta<sup>51</sup> and neutrophils; which can cause the loss of products in pregnant patients with APS.

Finally, although the inflammatory process in APS is not well accepted because the main studies show the syndrome as a disease mediated by antibodies, evidence shows that  $\alpha\beta$ 2GP-I from APS patients induce the activation of vascular endothelium via expression of adhesion molecules, recruitment of inflammatory cells (e.g. neutrophils and macrophages), probably by activation of chemokines, and participation of the complement. As a result of this,  $\alpha\beta$ 2GP-I in APS patients are able to induce a pro-inflammatory, pro-adhesive and pro-coagulant environment. These mechanisms are involved in the pathogenesis of the syndrome.

## Conclusions

APS is an autoimmune disease of multifactorial origin. It can appear alongside other autoimmune diseases (mainly SLE), in which case it is known as secondary APS, and there is also an entity in which only the clinical features of the syndrome are manifested, which is known as primary APS. The main antigenic target of aPL present in patients with APS is  $\beta$ 2GP-I. However, there may be antibodies against other proteins (annexin V, prothrombin, etc.).

Studies of the pathogenic mechanisms of  $\alpha\beta$ 2GP-I have mainly focused on thrombotic events and repeated abortions. However, due to the heterogeneity of the clinical manifestations present in these patients there is a high possibility that more than one pathophysiological mechanism is involved. In general, the pathogenic mechanisms of APS can be classified into two: 1) those which alter the pro- and anti-coagulant mechanisms, and 2) those which activate cells and consequently increase the expression and secretion of various molecules.

Additionally, the involvement of auto-reactive CD4+ T cells specific for  $\beta$ 2GP-I has been shown as part of the components of the cellular immune response in APS. Lastly, evidence shows that  $\alpha\beta$ 2GP-I have the ability to induce a pro-inflammatory, pro-adhesive and pro-coagulant environment. These processes are involved in the pathogenic mechanisms of APS.

## Conflict of interest

The authors declare no conflict of interests.

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