Pathophysiologic insights into the antiphospholipid syndrome

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Summary
The antiphospholipid syndrome (APS) is characterized by venous and/or arterial thrombosis and severe pregnancy morbidity in presence of antiphospholipid antibodies (aPL). While there is compelling evidence that aPL cause the clinical manifestations of APS, the underlying mechanisms are still a matter of scientific debate. This is mainly related to the broad heterogeneity of aPL. There are three major types of aPL: The first one binds to (anionic) phospholipids, e.g. cardiolipin, in absence of other factors (cofactor independent aPL). The second type binds to phospholipids only in presence of protein cofactors, e.g. β2-glycoprotein I (B2GPI) (cofactor dependent aPL). The third type binds to cofactor proteins directly without need for phospholipids.

It is widely believed that cofactor independent aPL (type 1) are associated with infections and, more importantly, non-pathogenic, while pathogenic aPL belong to the second and in particular to the third type. This view, in particular with regard to type 1 aPL, has not been undisputed and novel research data have shown that it is in fact untenable. We summarize the available data on the pathogenic role of aPL and the implications for diagnosis of APS and future research.

Schlüsselwörter
Antiphospholipidsyndrom, Antiphospholipid-Antikörper, β2-Glykoprotein I, Thrombose

Zusammenfassung

The antiphospholipid syndrome (APS) is characterized by recurrent venous and/or arterial thrombosis or severe pregnancy morbidity in presence of antiphospholipid antibodies (aPL) (1, 2). There is currently ample evidence that aPL are causally related to the clinical manifestations of the APS (3).

The wide heterogeneity of aPL has been a challenge for diagnostic tests as well as for the elucidation of the pathophysiologic mechanisms of the disease. Currently, most researchers believe that aPL which bind directly to phospholipids in absence of any cofactors (see below) are irrelevant for the pathogenesis of APS (4–7).

According to the current opinion, cofactor independent aPL occur mainly in the context of infections and are non-pathogenic.

However, a closer look at the available literature including very recent data shows that this concept should be revised.

In this review we summarize the available data on pathogenicity of aPL based on the available literature and our own data. Therefore, it is helpful to consider criteria that should be fulfilled in order that an aPL-specificity may be considered patho-
Antiphospholipid syndrome pathophysiology

Subtypes of aPL found in APS patients

There are 3 major subtypes of aPL as listed in ▶ Table 1:

- **Type 1 aPL** bind to (anionic) phospholipids in absence of any cofactors (cofactor independent aPL).
- **Type 2 aPL** bind to phospholipids only in presence of specific protein cofactors, e.g. β2-glycoprotein I (β2GPI) (cofactor dependent aPL).
- **Type 3 aPL** bind directly to cofactor proteins, mainly β2GPI. This type is considered to belong to aPL even though its antigen is not a phospholipid.

One point to keep in mind is the large number of potential protein cofactors. While β2GPI is the only cofactor protein that has been included into the classification criteria of APS (2), several other proteins may be involved. These include prothrombin, members of the annexin family and other proteins.

For the sake of clarity and focus we will only discuss β2GPI. Data on non-criteria cofactors in the literature are sketchy and their role has been reviewed in the past (9, 10).

Limitations of the classification

While the distinction of 3 aPL subgroups appears plausible and is of theoretical value, it has several limitations.

First of all, it is not easy to assign aPL to one type. The assays used in clinical diagnostics cannot differentiate between type 1 and type 2 aPL. This can only be achieved reliably if purified immunoglobulins rather than serum or plasma is used in the immunoassays for aPL.

Second, and more important, there is an apparent overlap between the subtypes. In particular, type 2 and type 3 show significant overlap. Furthermore, there are aPL which bind to phospholipids alone but also to β2GPI. We and other groups have isolated monoclonal human aPL with such overlapping antigen specificity (11–14).

Pathogenesis

Currently, most researchers believe that only type 2 and 3 aPL induce APS while type 1 aPL are irrelevant for the pathogenesis of APS. In fact, it is postulated that aPL directed against β2GPI (type 3) are the most pathogenetical – if not only – relevant type of aPL (4–7).

For better understanding and evaluation of this pathogenetic concept it is worthwhile to recapitulate the history of the detection of the different aPL subtypes. Cofactor dependence was first described in 1990 by two groups (15, 16). These authors showed that at least some aPL bind to cardiolipin only in presence of serum proteins which they called cofactors. β2GPI was identified as one major cofactor. Later on, antibodies directed against the cofactor proteins themselves were detected in APS patients. These observations have been confirmed repeatedly thereafter.

However, the prevalence of cofactor dependent (type 2) vs. cofactor independent (type 1) aPL in APS patients is still not known exactly. This is mainly related to technical difficulties to distinguish between type 1 and type 2 aPL using diagnostic assays. Currently available immunoassays are not suitable for this task, because human serum used in these assays contains sufficient quantities of cofactors (15, 16).

In fact, today there is no immunoassay available that can differentiate type 1 from type 2 reliably from whole serum. One assay format based on massive dilution of patient serum has been devised but is hampered by low sensitivity (17).

The need to isolate immunoglobulin fractions devoid of any cofactors before analysis has precluded larger patient studies to assess the frequency of the 2 types of aPL. The available literature on this issue suggests that a significant proportion – if not the majority – of APS patients harbor type 1 antibodies, which are considered non-pathogenic (18–20). Numbers range from 40–100%. In fact, in our own studies the prevalence of type 1 aPL is higher than that of type 2 aPL (20). While these studies were small (5–35 patients), they unequivocally prove that the presence of type 1 antibodies in APS patients is common. Thus, the first criterion, i.e. presence in APS patients, is fulfilled for all 3 types of aPL.

Pathogenic mechanisms in vitro

There is a vast literature on potentially pathogenic effects of aPL in vitro. While these include effects on plasmatic coagulation,
there is broad consensus today that the relevant targets of aPL are cells involved in vascular homeostasis and placental function. There is good evidence that aPL can in vitro activate
- platelets,
- leukocytes,
- endothelial cells and
- trophoblast cells.

But again, the underlying mechanisms appear to be quite diverse. In the following we will briefly review the currently known and proposed signaling pathways of aPL.

Signaling via endosomal NADPH-oxidase

We have recently shown that cofactor independent aPL (type 1) are internalized into the endosomal/lyosomal compartment by monocytes and plasmacytoid dendritic cells where they activate NADPH-oxidase 2 (NOX2) (21).

Activation of NOX2 induces several downstream responses. These include induction of proinflammatory and procoagulant genes, e.g. TNFα and tissue factor, and components of the inflammasome, but also non-transcriptional responses, e.g. rapid translocation of TLR7 or TLR8 (TLR: toll-like receptor) from the endoplasmic reticulum to the endosome (20–22). Lack of NOX2 by genetic deletion or pharmacologic blockade of endosomal NOX2 by niflu mic acid abolishes all downstream effects of type 1 aPL.

Since we used monoclonal aPL rather than a heterogeneous mixture we can assign this signaling pathway to specific binding properties of aPL. Interestingly, strictly cofactor dependent aPL (type 2 and 3) do not activate this pathway in our cell systems (Müller-Calleja et al. unpublished data).

Thus, at least some type 1 aPL induce cellular activation via endosomal NOX2.

Signaling via TLR4 and annexin A2

Several investigators have shown that monocytes, endothelial cells and platelets can only be activated by aPL when β2GPI is present. This suggests that the active molecule is a complex of β2GPI and anti-β2GPI (type 3 or type 2 aPL). Depending on the experimental setting it has been shown that cellular effects depend on the presence of TLR4 (23, 24) or annexin A2 (25–27).

Since in most studies either cells lacking one of these two membrane proteins were used or these proteins were targeted by specific antibodies or other inhibitors, it appeared that aPL signal via independent mechanisms involving one of these proteins.

While this may still be the case, McCrae et al. (27) presented data which suggest that these proteins form a complex on the cell surface which is required to mediate the effects of aPL.

Signaling via LRP8

De Groot et al. (28, 29) have shown that complexes of β2GPI and anti-β2GPI (type 3 and type 2 aPL) can activate platelets and perhaps other cells via LRP8. Since LRP8 belongs to the LDL-receptor family and binds apolipoprotein E, it is well conceivable that it can also bind other proteins with affinity to lipids and structural similarities to apolipoproteins as β2GPI. Again, their data show that only the antigen-antibody complex is able to induce cellular responses, very similar to the data for TLR4 and annexin A2.

Thus, all three subtypes of aPL have been shown to induce cellular responses that might lead to the clinical manifestations of APS.

Thrombotic effects in vivo

In order to show pathogenic effects of aPL in vivo several animal models have been developed which either analyze pregnancy loss or thrombus formation induced by aPL. In particular the in vivo thrombosis models are quite heterogeneous with regard to the type of the
- analyzed blood vessel,
- needed stimulus to induce thrombus formation,
- kinetics of thrombus formation, and
- size and composition of the thrombi induced in the animals (Tab. 2).

Due to this heterogeneity of models and the aPL preparations used it is in many cases only possible to state that aPL induce or rather accelerate thrombus formation in an animal model.

Tab. 2  In vivo thrombosis mouse models used in antiphospholipid syndrome research. aPL: antiphospholipid antibody; IgG: immunoglobulin G

<table>
<thead>
<tr>
<th>Blood vessel analyzed</th>
<th>Stimulus to induce stimulus</th>
<th>Thrombus formation kinetics</th>
<th>Major blood component involved</th>
<th>aPL analyzed</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>femoral artery</td>
<td>pinch injury</td>
<td>thrombus forms and resolves within minutes</td>
<td>platelets</td>
<td>patient IgG</td>
<td>30–33</td>
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<td></td>
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<td>monoclonal aPL</td>
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<tr>
<td>cremaster muscle arterioles</td>
<td>laser injury</td>
<td>thrombus forms and resolves within minutes</td>
<td>platelets</td>
<td>patient IgG</td>
<td>35</td>
</tr>
<tr>
<td>mesenteric microcirculation</td>
<td>FeCl3 injury</td>
<td>occlusive thrombus forms within minutes</td>
<td>platelets</td>
<td>patient IgG</td>
<td>37</td>
</tr>
<tr>
<td>Vena cava</td>
<td>flow reduction</td>
<td>occlusive thrombus forms within 24–48 h</td>
<td>platelets</td>
<td>mouse</td>
<td>34</td>
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<td></td>
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<td>red blood cells</td>
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<td>plasmatic coagulation factors</td>
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It remains an open question, if all aPL behave similar in these models. If patient IgG fractions were used, it is not known which aPL among these fractions were in fact responsible for the observed effects.

The most widely used thrombosis model in APS research has been introduced by Pierangeli in the 1990s (30). It is based on a pinch injury of the femoral artery followed by a rapid thrombus formation within minutes. Thrombi resolve again within 30 minutes, but usually much faster. Since these authors did not stain blood components, the composition of thrombi is not known for sure, but the kinetics suggest that they consist of platelets, mainly. Several different approaches to apply aPL in this model have been taken, among them

- patient IgG,
- human monoclonal aPL, and
- endogenous mouse aPL induced by immunization (30–33).

All investigations showed increased thrombus formation at the site of injury in aPL treated mice. In particular, the experiments with 7 human monoclonal IgG-aPL isolated from 2 patients shed some light on the relevant properties of pathogenic aPL (32, 33). The 2 most thrombogenic aPL – IS2 and IS4 – both bound to cardiolipin in a cofactor dependent manner (type 2). While the cofactor for IS4 was β2GPI, the cofactor for IS2 remained unidentified but was not β2GPI. IS2 does also not bind to immobile β2GPI. Neither of the two aPL had lupus anticoagulant activity.

Two other aPL were also thrombogenic. Both were β2GPI-dependent anticardiolipin aPL. One of them – CL15 – showed lupus anticoagulant activity but did not bind to β2GPI.

These data show that different aPL (types 1–3) can induce thrombus formation in this model, and that neither specificity against β2GPI, nor dependency of β2GPI as cofactor, nor lupus anticoagulant activity are absolute and indispensable requirements.

Working with human monoclonal aPL we have recently shown in another thrombosis model based on flow reduction in the inferior vena cava that cofactor independent aPL (type 1) can accelerate venous thrombus formation dramatically (34). Interestingly, the signal transduction pathway via endosomal NOX2 of these monoclonal aPL described in vitro was shown to be relevant in vivo, as mice deficient in gp91phox, the catalytic subunit of NOX2, were protected.

While these studies with human monoclonal aPL provide significant insights into the underlying mechanisms – because aPL of defined specificity were employed – studies with sera or IgG fractions from APS patients are also of great relevance because their effects are representative for APS patients. However, it has been inherently difficult to associate the observed effects with specific aPL subtypes. This can be exemplified by two studies which claim that the thrombogenic effects observed in vitro are mediated by anti-β2GPI.

- Arad et al. (35) used a laser-injury based approach in the microcirculation of the cremaster muscle (△Tab. 2).
- Pericleous et al. (36) used the pinch-injury based model described above.

Both groups tried to purify anti-β2GPI by affinity chromatography. Going over their data it is obvious that affinity purification was not specific for anti-β2GPI. Arad et al. state “The antibody populations from the 3 patient sera studied that bound to β2GPI also had all anticardiolipin antibody activity.” Fig. 1 of Pericleous et al. (36) nicely shows that the affinity purified anti-β2GPI has significant anticardiolipin activity. Thus, their claim that they provide the “first direct evidence that aPL (antibodies against domain I of β2GPI) IgG from APS patients is prothrombotic in vivo” is not sufficiently backed by their data.

The best evidence that anti-β2GPI are pathogenic in vivo comes from the work of Ramesh et al. (37) which is built on previous in vitro data (28, 29). They show in mouse mesenteric microcirculation that LRP8 deficient mice are protected from the prothrombotic effects of human APS-IgG isolated from APS patients. According to their data, LRP8 mediates inhibition of endothelial nitric oxide synthase (eNOS) by aPL. While they do not show directly in vivo evidence that the causative aPL are anti-β2GPI, the combined in vivo and in vitro data strongly supporting their argument.

Thus, activation of LRP8 and perhaps further coreceptors by β2GPI/anti-β2GPI complexes with ensuing inhibition of eNOS is one thrombogenic mechanism of aPL.

### Pregnancy effects in vivo

Pregnancy models are more homogeneous because almost all are analyzing pregnancy outcomes in mice. The only differences here are the application or induction of aPL. Overall, there is ample evidence that aPL can cause pregnancy failure in vivo (38–42).

Some issues deserve to be mentioned specifically. As with thrombosis models, different aPL preparations have been tested in pregnancy models, including APS IgG fractions and monoclonal aPL. Also induction of aPL in the animals has been used. Again, only the monoclonal aPL permit conclusions on the pathogenic aPL populations.

It has been shown that aPL mediated pregnancy morbidity depends on activation of complement. Interestingly, these effects could be induced by cofactor independent aPL (40, 41). On the other hand, Ulrich et al. (42) demonstrated that some aPL apparently induce pregnancy complications via LRP8.

These combined data suggests that – similar to the observations in thrombosis models – there are at least two different mechanisms of aPL induced pregnancy failure. In summary, there is evidence that all subtypes of aPL can induce APS manifestations in vivo.

### Association with clinical manifestations of APS

It is beyond the scope of this article to review the numerous clinical studies on aPL and clinical events of APS. However, it should be noted that most of them were case-control studies – with all limitations of this type of study. Prospective data are still limited.
The relevance of different aPL in human thrombotic disease has been reviewed recently (43). The bottom line of this systematic review is that lupus anticoagulants show the strongest and most consistent association of all aPL with arterial and venous thrombotic events. Anticardiolipin antibodies are significantly associated with arterial and venous thrombosis, while anti-β2GPI antibodies are only significantly associated with arterial thrombosis. It should be noted though that the odds ratios for anticardiolipin and anti-β2GPI were similar, but not statistically significant for anti-β2GPI, perhaps due to the smaller number of clinical studies.

Another important aspect to keep in mind when interpreting these data is the fact that the assays for cardiolipin antibodies only detect such aPL but cannot differentiate between cofactor-independent and cofactor-dependent aPL. Two older reviews of the available clinical studies from 2003 come to similar conclusions (44, 45).

The major message of these reviews is that clinical data does not support the postulated dominant role for anti-β2GPI compared to other aPL in the pathogenesis of thromboembolic events.

With regard to anti-β2GPI it has been proposed that anti-β2GPI antibodies directed against an epitope in domain I of the protein (anti-domain I) represent the most pathogenic subpopulation of these aPL. This has in fact been corroborated in a prospective study (46). De Laat et al. show in a cohort selected for the presence of anti-β2GPI that the association with thromboembolic events of domain I antibodies is moderately higher than for other anti-β2GPI. However, the study clearly demonstrates that more than 70% of the patients who did not have domain I antibodies developed clinical features of APS. The role of other aPL, e.g. anticardiolipin, remains untouched.

Data regarding pregnancy morbidity are similar. The prospective PROMISSE study has shown that lupus anticoagulant is the only aPL predictive for poor pregnancy outcome in patients with aPL (47, 48). In absence of lupus anticoagulant, anticardiolipin and anti-β2GPI are not predictive, mainly due to poor specificity. This would suggest that the lupus anticoagulant assay for some reason is better suited to identify pathogenic aPL than the widely used immunoassays.

Conclusions and outlook

Recent in vitro and in vivo data on the pathogenicity of aPL show that the heterogeneity of aPL is most likely also reflected in heterogeneous pathogenic effects of different aPL subspecies. At least 2 independent pathways have been shown to be relevant in vivo:

1. Activation of endosomal NOX by anticardiolipin antibodies with subsequent induction of proinflammatory and procoagulant pathways. These antibodies do not need to be cofactor dependent (type 1 and 2).

2. Activation of LRПβ by β2GPI/anti-β2GPI complexes leading to several cellular effects including inhibition of eNOS (type 2 and 3).

Future work should be directed at delineating the relative contribution of different aPL and different signaling pathways to the pathogenesis of thrombosis and pregnancy morbidity. This will be a prerequisite for targeted therapeutic interventions to prevent these clinical manifestations of the APS.

Conflict of interest

The authors declare that there is no conflict of interest.

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Hämostaseologie 3/2017