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Anti-Endothelial Antibodies Interfere in Apoptotic Cell Clearance and Promote Thrombosis in Patients with Antiphospholipid Syndrome

Audrey Graham, Isobel Ford, Rona Morrison, Robert N. Barker, Mike Greaves, and Lars-Peter Erwig

Antiphospholipid syndrome is an important cause of recurrent thrombotic events. The pathogenesis of the thrombosis remains unclear, but it has been suggested that anti-phospholipid Abs, which are laboratory markers for the disease and include species capable of binding to vascular endothelial cells, play an important role. We hypothesized that these anti-endothelial Abs promote thrombosis through interference with clearance of dying cells. We show that healthy endothelial cell monolayers effectively remove apoptotic endothelial cells, but this clearance is markedly inhibited by serum or IgG from patients with antiphospholipid syndrome and anti-endothelial Abs. In addition, patient sera or IgG opsonize apoptotic endothelial cells and cause enhanced Fc-mediated uptake by professional phagocytes. Importantly, the delayed clearance of apoptotic cells by healthy endothelial cells and the enhanced Fc-mediated macrophage uptake each result in procoagulant consequences, as judged by increased thrombin generation. The effects on apoptotic cell clearance were reproduced by a mAb derived from a patient with antiphospholipid syndrome, which binds to endothelial cells and is thrombogenic in experimental models. Taken together, our data support a novel, dual mechanism by which anti-endothelial Abs are prothrombotic in antiphospholipid syndrome by inhibiting removal of procoagulant apoptotic cells and by diverting their clearance to provoke inflammatory and prothrombotic changes in professional phagocytes.

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4 Abbreviations used in this paper: APS, antiphospholipid syndrome; SLE, systemic lupus erythematosus; EC, endothelial cell; AEC, apoptotic EC; HEC, healthy EC; NEC, necrotic EC.

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apoptotic cells by their healthy neighbors (15). Therefore, we postulated that healthy EC have a key role in the removal of apoptotic EC and that autoantibody binding perturbs this clearance process and thereby augments a prothrombotic milieu. Delayed clearance of apoptotic cells by neighboring EC would diminish the anti-inflammatory response normally associated with effective and specific apoptotic cell clearance (16). Additionally, the delay may trigger recruitment and activation of professional phagocytes to remove the dying EC, resulting in further prothrombotic changes. The apoptotic cells in APS patients would be coated with anti-endothelial Abs and the uptake of such opsonized particles by macrophages is generally thought to provoke proinflammatory responses, including the release of platelet-activating prothrombotic, arachidonic acid metabolites (17). Specifically, in APS, it has been previously suggested that both EC and macrophages activated by phagocytosis are alternative potential sources of intravascular tissue factor (18–21). Therefore, our aim was to test the hypothesis that anti-endothelial Abs delay the clearance of apoptotic EC by their healthy neighbors and also opsonize these cells for macrophage uptake, and by this dual mechanism enhance a prothrombotic environment on the endothelial surface.

Materials and Methods

Patients and control donors

The protocol was approved by the institutional research ethics committee. Blood samples were collected into Vacutainers (BD Biosciences) with or without trisodium citrate anticoagulant. Serum was separated by centrifugation of clotted blood and stored at –80°C. Patients for this study were selected based on two criteria. First, subjects were strongly positive for lupus anticoagulant which was assessed using a panel of well-standardized assays (5, 20) and all selected plasmas were positive in at least two assays. The assay panel consisted of a dilute Russell’s viper venom time with side scatter characteristics, and populations were gated to exclude dead cells. Photographic images were taken using a Zeiss microscope. The cells were typically 70–90% apoptotic. HEC were rendered necrotic (NEC) by heating for 30 min at 60°C before microwaving for 1 min (23).

Preparation of macrophages

Macrophages were prepared by isolating PBMC from the citrate-anticoagulated blood of healthy donors by density gradient centrifugation as previously described (24, 25). Monocytes were separated from the PBMC by positive selection with CD14 beads and a MACS LS Separation Column (Miltenyi Biotec) according to the manufacturer’s instructions. Purified monocytes were plated onto 24-well tissue culture plates (Corning) at 1 × 10^6 cells/ml in Macrophage-SFM Medium (Life Technologies) containing 10% autologous serum (or recalcified plasma) and matured over 5–8 days. Fluorescent EC were counted using an inverted-phase fluorescence microscope with rhodamine filter. Three fields of 100 cells were examined in duplicate wells and the percentage of EC containing at least one ingested cell was determined. The results are shown as absolute changes in percentage uptake compared with control (cells preincubated with healthy control serum). Photographic images were taken using a Zeiss inverted fluorescence microscope and Axiovision software. To investigate the effect of serum or IgG from subjects with APS, PKH26GL-labeled EC were preincubated with 10% control or APS serum or IgG (0.1 mg/ml) in M199 for 2 h before presentation. To confirm the observations made by light microscopy, endothelial uptake by macrophages was also assessed by dual-color flow cytometry of CD11b-allophycocyanin (Sigma-Aldrich)-labeled macrophages that had been incubated with PKH26GL-labeled AEC. Data were acquired by a FACS Calibur (BD Biosciences), with CellQuest software (BD Biosciences) used for analysis. Negative isotype controls and single positive controls were performed to allow accurate breakthrough compensation. Gates and instrument settings were set according to forward and side scatter characteristics, and populations were gated to exclude dead cells.

### Table I. Patient Ab profile

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<tr>
<th>Patient</th>
<th>Lupus Anticoagulant</th>
<th>Anti-Cardiolipin Ab IgG</th>
<th>Anti-Cardiolipin Ab IgM</th>
<th>Anti-Endothelial Ab IgG</th>
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<td>2</td>
<td>4</td>
<td>109</td>
<td>68</td>
</tr>
</tbody>
</table>

Notes: Shows that the patient sera were all positive for lupus anticoagulant and importantly positive in the endothelial cell ELISA. As expected they were heterogeneous for anticardiolipin, anti-β2-glycoprotein, and antiprothrombin expression. NA, Not assessed.
or clumped cells. A total of 10,000 events was collected. Data shown are representative of at least five separate experiments.

APS mAb

Mouse-human hybridoma cell line IS4 was a generous gift from Dr. P. Chen (University of California, Los Angeles, CA). The human monoclonal IgG, IS4, was isolated from a patient with primary APS and is known to bind to cardiolipin and EC (26). IS4 cells were cultured in RPMI 1640 medium (Life Technologies) supplemented with 10% heat-inactivated FCS, 1% nonessential amino acids, 1 mM sodium pyruvate, 15 mM HEPES buffer (pH 7.3), 2 mM l-glutamine, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B (all from Sigma-Aldrich). The cell medium supernatant was sampled regularly for testing with human IgG anti-cardiolipin Abs (Varelisa Cardiolipin IgG Antibodies ELISA kit; Sweden Diagnostics). Aliquots of pooled supernatants from anti-cardiolipin-positive cultures were stored at –80°C. PKH26GL-labeled

FIGURE 1. Phagocytosis of AEC by HEC monolayer. A, Fluorescent light microscopy showing representative image of an EC (arrow) that has ingested a PKH26GL-labeled (red) AEC (original magnification, 1:40). B, Fluorescent light microscopy showing representative image of EC that have ingested PKH26GL-labeled AEC with subsequent degradation and perinuclear localization. C, Electron micrograph of AEC (as indicated by arrow) engulfed by a HEC (original magnification, 1:5000). D, Percentage of HEC containing HEC, NEC, or AEC after incubation. Mean ± SEM of eight separate experiments.

FIGURE 2. Effect of patient serum or IgG on phagocytosis of AEC by HEC. A, The effect of preincubation with serum from patients (p1–p7) compared with serum from a pool of healthy control subjects (c1) on phagocytosis of HEC, NEC, and AEC by HEC monolayer. Results are expressed as arithmetic difference in percentage uptake vs control for sera from seven individual patients; median and range. B, The effect of preincubation with IgG purified from patient sera compared with IgG from a pool of healthy control subjects (C1) on phagocytosis of HEC, NEC, and AEC by HEC monolayer. Results are expressed as arithmetic difference in percentage uptake vs control for IgG from seven individual patients; median and range.
HEC or AEC were incubated with pooled supernatant (IgG concentration 0.1 mg/ml) for 2 h before presentation to endothelial monolayer or to macrophages as above.

**RGD peptide to block integrin-mediated uptake of apoptotic cells**

Arg-Gly-Asp-Ser (RGDS) peptide or control Arg-Gly-Glu-Ser (RGES) peptide (both from Sigma-Aldrich) was incubated with the EC monolayers at 1 mM for 30 min at 37°C before the addition of PKH26GL-labeled HEC, AEC, or NEC as previously described (27).

**Blocking Fc receptors in macrophage phagocytosis**

Macrophages were incubated with 45 μg/ml mouse anti-human CD16 (FcγRIII; Sigma-Aldrich) and/or 30 μg/ml mouse anti-human CD32 (FcγRII; Santa Cruz Biotechnology) or irrelevant mouse monoclonal IgG (MOPC-31; Sigma-Aldrich) for 2 h before the presentation of serum-incubated AEC.

**Measurement of procoagulant activity and thrombin generation assay**

**Endothelial phagocytosis.** HEC or AEC in serum-free M199 were incubated with control or patient serum (cell concentration 10^6 cells/ml) at 37°C for 2 h, then centrifuged and the pellet resuspended in serum-free medium for thrombin generation assay.

**Macrophage phagocytosis.** AEC in MAC-SFM medium (10^6 cells) were incubated with patient serum, control serum, or medium, as before, then presented to plated macrophages (10^6/well) in 96-well plates. Noningested cells and medium were aspirated, followed by a single gentle wash, then macrophages were harvested by scraping into PBS to a concentration of 2.5 × 10^7/ml.

The cell suspension was diluted to a volume of 400 μl (EC, 1:2; macrophages, 1:1 with PBS) and 1 ml of normal pooled plasma was added. After incubation for 3 min at 37°C, 1 ml of 20 mM CaCl₂ was added. At 0, 1, 2, 3, and 4 min, then every 2 min until clotting occurred, 150-μl aliquots were removed into 150 μl of S2366 substrate (Quadratech) in a 96-well microtiter plate, incubated for 90 min, and read on a spectrophotometer at 405 nm. A standard curve was constructed from human thrombin (Sigma-Aldrich) for 2 h before the presentation of serum-incubated AEC.

**Results**

**Uptake of AEC by HEC**

The first part of our hypothesis was that autoantibodies binding to AEC would critically alter their clearance and thus perpetuate a procoagulant state. First, we showed that HEC cultured to a confluent monolayer consistently take up AEC that are presented to the monolayer in suspension. Fig. IA shows a representative fluorescent microscopic image of HEC illustrating the engulfment of PKH26GL-labeled AEC and (Fig. 1B) the subsequent degradation within the cells and perinuclear localization. Uptake was further confirmed by the preparation of sections for electron microscopy in which whole AEC were seen to be engulfed by HEC (Fig. 1C).

Uptake was quantified by fluorescent microscopy by our standard phagocytosis assay (25) using HEC and NEC as controls and presented as the percentage of HEC in the monolayer containing ingested cells (Fig. 1D). Apoptotic cell uptake is mediated through recognition of specific changes on the apoptotic cell surface by a number of bridge molecules and receptors, including integrins (15). The RGDS peptide has been extensively used previously to inhibit integrin-mediated recognition of apoptotic cells by various phagocytes (27). We found that preincubation of endothelial monolayers with Arg-Gly-Asp-Ser (RGDS) peptide (but not RGES control peptide) significantly inhibited the uptake of AEC by 40.7% ± 4.7% (mean of five experiments) but had no effect on uptake of NEC or HEC, demonstrating that phagocytosis of AEC by HEC is specific and at least partly integrin dependent.

**Effect of APS sera on endothelial phagocytosis of AEC**

Our hypothesis predicts that patient sera containing anti-phospholipid/anti-EC Abs would interfere with apoptotic cell recognition by EC layers. Therefore, we examined whether preincubation of labeled AEC with serum from 7 APS patients and 20 healthy volunteers affected their uptake by HEC. There was a significant reduction (p = 0.016) in uptake of AEC (Fig. 2A) after preincubation with patient serum. It should be noted that this effect was observed in all patients tested and was not dependent on whether the patients were positive for anti-cardiolipin or...
β₂-glycoprotein I or prothrombin Abs. In contrast to the effects on AEC, the uptake of HEC or NEC was not significantly altered by preincubation with patients’ sera (Fig. 2A).

The inhibitory component of APS serum resides in the IgG fraction
To test whether the effect of patients’ sera on apoptotic cell uptake is Ab mediated, we repeated the uptake experiments, comparing the effects of the purified IgG fraction from individual sera of patients with the pooled IgG fraction from five healthy control subjects. The results show a similar level of inhibition to that observed with the unfractionated individual sera for apoptotic cell uptake, but little effect on viable or necrotic cell uptake. This confirms that the inhibitory component of APS sera on apoptotic cell uptake resides in the IgG fraction (Fig. 2B).

Phagocytosis of apoptotic EC by human macrophages
In addition to impaired clearance of apoptotic cells by neighboring nonprofessional phagocytes, we proposed that the subsequent Ab-mediated recognition of opsonized cells by professional phagocytes may initiate immune activation (28) and prothrombotic consequences. Therefore, we went on to examine whether APS sera or IgG fractions from patients not only inhibit uptake of AEC as shown above, but also enhances their phagocytosis by macrophages. Preincubation of AEC with four of the five APS sera (Fig. 3A) and all five IgG fractions tested (Fig. 3B) significantly increased phagocytosis by macrophages compared with control serum. The median difference between phagocytosis associated with APS IgG compared with control IgG was +35.2% (33.0–39.6%).

These findings were confirmed by dual-color flow cytometry using PKH-labeled AEC preincubated with patient or control serum as appropriate and then presented to CD11b-allophycocyanin-labeled human monocyte-derived macrophages. Fig. 3C shows a representative experiment illustrating the enhanced uptake of AEC following incubation with patient serum.

The role of Fc receptors in phagocytosis of opsonized EC by macrophages
To determine whether the serum effects on macrophage uptake were indeed a consequence of opsonization of EC rather than direct macrophage activation, we conducted experiments in which macrophages were incubated with serum before AEC were presented. We found that macrophage pre-exposure to patient sera or IgG fractions had no significant effect on uptake (data not shown). This is consistent with the interpretation that incubation of AEC with patient sera or IgG opsonized the apoptotic cells and deviated the macrophage recognition mechanism toward Fc-mediated uptake. Therefore, we incubated macrophages with either blocking Abs to FcRII (CD32) or FcRIII (CD16) alone or in combination. Treatment with anti-CD32 alone or in combination with anti-CD16 negated the enhanced phagocytosis that was associated with patient sera (Fig. 4A). Anti-CD16 alone had no significant effect, nor did an irrelevant Ab (mouse IgG clone MOPC-31; Fig. 4A).

APS mAb mimics effects of patients’ sera and IgG
To confirm that the effects of patient sera and IgG fraction on apoptotic cell uptake by both HEC and macrophages are mediated by specific Abs, we also tested a monoclonal APS Ab (IS4). IS4 was chosen because it had previously been shown to bind to EC in addition to its activity against cardiolipin and to induce thrombosis in experimental models (26, 29). Incubation of AEC with the Ab caused a significant reduction in uptake by HEC (Fig. 4B) similar to that observed after incubation with patient serum. Furthermore, incubation of AEC with the IS4 Ab significantly enhanced phagocytosis by macrophages compared with AEC incubated in control serum (Fig. 4C). The increase was similar in magnitude to the effects of patient serum or isolated IgG.

Procoagulant consequences of failed or altered clearance
Finally, we went on to confirm whether failed or altered clearance of AEC indeed induced procoagulant consequences. Our experiments confirmed that the rate of thrombin generation is increased in AEC exposed to patient serum in comparison to AEC or HEC incubated with serum from healthy donors (Fig. 5A). Furthermore, we tested whether the rate of thrombin generation by macrophages was enhanced after ingestion of AEC incubated with patient sera when compared with AEC preincubated with healthy control serum or unchallenged macrophages. Patient sera and IgG fractions tested significantly increased thrombin generation (Fig. 5B).
These results suggest that anti-endothelial Ab binding may promote a procoagulant environment in APS through a dual mechanism: inhibition of apoptotic cell clearance by neighboring nonprofessional phagocytes along with opsonization and recognition by macrophages.

Discussion

Anti-phospholipid Abs have been associated with thrombosis but the mechanisms are not completely understood. In this study, we tested whether Ab binding to endothelium may contribute to thrombosis through both inhibition of apoptotic cell clearance by neighboring EC and increased uptake of opsonized AEC by macrophages. We show that HEC monolayers effectively clear AEC, but incubation of AEC with serum or IgG with anti-endothelial reactivity from patients with APS markedly inhibits engulfment of apoptotic cells. Furthermore, this impaired clearance is associated with enhanced recognition of opsonized AEC by professional phagocytes. Importantly, these effects on apoptotic cell clearance can be replicated by the IgG fraction of patient sera and by a monoclonal anti-endothelial APS Ab. Finally, we show that the shift in apoptotic cell clearance from uptake of apoptotic cells by its neighbors to opsonized cells by macrophages causes enhanced thrombin generation. Thus, our data establish a novel dual mechanism by which anti-endothelial Abs promote prothrombotic changes in patients with APS and potentially other autoimmune-mediated diseases.

Our results are consistent with a large body of literature showing that a growing list of cells, including fibroblasts, endothelial, epithelial, smooth muscle, and stromal cells, can take up apoptotic cells (30, 31). Apoptotic cells are normally cleared by specific recognition mechanisms independent of classical opsonization (15, 25). Uptake of AEC by HEC is no exception, since the process was inhibited by RGDS peptide, which has been extensively used previously to block integrin-mediated recognition of apoptotic cells (27). Effective clearance of dying cells is physiologically essential for tissue remodeling, the effective function of the immune system, and is associated with anti-inflammatory consequences (16, 28). Clearance failure has been implicated in autoimmunity, mainly because intracellular Ags are clustered on the surface of apoptotic cells (22) and immunization with apoptotic cells can trigger the immune response and result in the production of autoantibodies (16, 28).

In this study, we show that antiphospholipid sera which bind to EC critically interfere with clearance of AEC by their healthy neighbors and confirm that this blockade results in prothrombotic changes. The inhibitory effects were observed regardless of variable binding to $\beta_2$-glycoprotein and prothrombin. Endothelial Abs have previously been implicated in causing programmed cell death (7, 8), and their effects on subsequent clearance may be exacerbated by the exposure of further antigenic sites following apoptosis induction. In particular, phosphatidylserine exposure occurs early during apoptosis and would provide sites for binding $\beta_2$-glycoprotein I, prothrombin, and annexin V, the antigenic targets for anti-phospholipid Abs. This would be expected to result in clustering of Ag allowing recognition by the classic antiphospholipid Abs present in APS in the same way that human monoclonal antiphospholipid Abs selectively bind to membrane phospholipid and $\beta_2$-glycoprotein I on apoptotic monoblastoid cells (34).

In addition, we show here that antiphospholipid/antiendothelial sera not only interfere with uptake by HEC, but opsonize AEC, resulting in enhanced clearance by professional phagocytes via Fc-mediated recognition, rather than receptors specific for apoptotic cells. In this regard, observations that the pleiotropic effects of statins may include modulation of the prothrombotic effects of anti-phospholipid Abs on EC could be highly relevant (35). Uptake of Ab-coated particles by professional phagocytes has previously been shown to cause proinflammatory changes including tissue factor expression (18) and the release of platelet-activating prothrombotic arachidonic acid metabolites (17, 28). The prothrombotic effects of macrophages that engulfed opsonized AEC were confirmed in the current study. Coagulation activation may promote inflammation, also, through the recently described C5a convertase activity of thrombin (36). It may be noteworthy that recent evidence suggests that anti-phospholipid Ab-induced pregnancy failure in a murine model involves coagulation activation but is a complement-dependent inflammatory process rather than due to fibrin formation (37). Importantly, in the prototypical autoimmune disease SLE, Ab-mediated opsonization of apoptotic cells also promotes enhanced phagocytosis by macrophages and is proinflammatory through increased expression of TNF-α (38). It may be argued that the consequences of uptake are not solely dependent on the recognition mechanism, but are also determined by the nature of the ingested particle. However, recent data studying phagosome maturation of ingested cells show that opsonized apoptotic and opsonized viable cells exhibited similar rates of phagosome maturation that were distinctly different from those of uncoated apoptotic cells (25).

The rational design of specific, effective therapy for APS will depend on understanding fully the pathogenesis of the disease. Our data, in context with previous observations, provide evidence for an additional novel, dual mechanism contributing to thrombosis in APS; first, anti-endothelial Abs provoke persistence of procoagulant apoptotic EC and, second, divert their clearance to cause inflammatory...
changes in professional phagocytes. Interestingly, an APS mAb reactive with EC in addition to cardiolipin, which is known to be directly thrombogenic in murine models of microcirculation and thrombosis (26, 29), recapitulates similar effects on uptake of AEC and consequent opsonization and Fc-mediated clearance by macrophages. This not only strongly supports our hypothesis that thrombosis in APS is at least partially driven by aberrant Ab-mediated clearance of AEC, but also raises the intriguing possibility that the dual mechanism proposed here for the initiation of thrombosis in APS may apply to other autoimmune-mediated thrombotic diseases in which endothelium-reactive Abs are a frequent feature (39, 40). Our data provide a novel mechanism contributing to the prothrombotic state of patients with autoimmune disorders and further analysis may lead to the development of more specific therapies.

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Disclosures

The authors have no financial conflict of interest.

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