Anti-endothelial cell antibodies from lupus patients bind to apoptotic endothelial cells promoting macrophage phagocytosis but do not induce apoptosis

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Objective. Anti-endothelial cell antibodies (AECA) have been reported to induce apoptosis. We investigated the induction of apoptosis by these autoantibodies and their involvement in the removal of apoptotic cells.

Methods. AECA isolated from patients with active systemic lupus erythematosus (SLE) were incubated with human umbilical vein endothelial cells (HUVECs). AECA-positive sera were identified using a cell-based ELISA. Apoptosis was measured by morphology and phosphatidylserine externalization using flow cytometry with fluorescein isothiocyanate (FITC)-conjugated annexin V. Flow cytometry was used to investigate AECA binding to apoptotic cells using FITC-conjugated anti-human immunoglobulin G (IgG). Apoptotic endothelial cells were stained with a red dye (PKH26) and co-cultured with macrophages, and phagocytosis was visualized under phase contrast microscopy.

Results. AECA from patients with SLE did not induce apoptosis compared with normal IgG (nIgG) at any time point, as assessed by morphology (at 24 h, \( P = 0.167 \)) or phosphatidylserine externalization (at 24 h, \( P = 0.098 \)). However, there was increased binding of AECA to apoptotic endothelial cells (48.8 ± 11.9 compared with 25.8 ± 6.7% AECA binding to freshly isolated cells, \( P < 0.001 \)). These opsonized endothelial cells showed greater phagocytosis by macrophages (mean phagocytic index 24.9 ± 4.5%) when cells opsonized with nIgG were compared with AECA (34.8 ± 3.4% \( n = 5, P = 0.01 \)).

Conclusion. In conclusion, AECA bind to apoptotic endothelial cells but do not induce endothelial cell apoptosis. Macrophage phagocytosis is increased by opsonization of apoptotic endothelial cells by AECA, a proinflammatory mechanism of cell removal.

Key words: AECA, Apoptosis, Endothelial cells, Lupus, Macrophages.

Anti-endothelial cell antibodies (AECA) are a heterogeneous group of autoantibodies directed against antigens located in the membrane of endothelial cells. Since the original descriptions in the 1970s these autoantibodies have been demonstrated in a number of patients with connective tissue diseases, including systemic lupus erythematosus (SLE). AECA have been shown to correlate with disease activity, and are more often detected in SLE patients with active nephritis and low complement levels [1]. AECA have the potential to induce lesions since their targets, expressed on vascular endothelial cells, are readily accessible to these circulating antibodies. Obviously, the presence of AECA in vasculitides does not necessarily imply causation since their production may follow, rather than precede, endothelial cell damage [2]. However, a variety of \textit{in vitro} effects have been attributed to AECA and support their involvement in pathogenicity, including activation of endothelial cells with increased expression of adhesion molecules [3], and increased secretion of proinflammatory cytokines [3, 4], thereby facilitating the recruitment and trafficking of leucocytes into the inflamed vessels.

Anti-endothelial cell antibodies may exert some of their pathological effects by inducing endothelial cell apoptosis directly [5]. Not all studies have replicated this finding [6, 7] and have suggested that endothelial cell apoptosis is induced by antibody-dependent cell-mediated cytotoxicity rather than by the antibody directly inducing proapoptotic signal transduction pathways [7]. Some anti-endothelial cell antibodies might also target apoptotic cells specifically. For example, following induction of apoptosis, it is known that negatively charged phospholipids, including phosphatidylserine, are expressed on the external surface membrane of endothelial cells and allow binding of antiphospholipid antibodies in a \( \beta_2 \)-glycoprotein I (\( \beta_2 \)-GPI)-dependent manner [8]. Binding of \( \beta_2 \)-GPI auto-antibodies to apoptotic cells promotes enhanced phagocytosis by macrophages in a proinflammatory manner, with release of TNF-\( \alpha \) [9]. Many patients with SLE who have AECA present do not have antiphospholipid antibodies and it is currently unclear what role AECA play in the induction of apoptosis of endothelial cells and the removal of these apoptotic cells in lupus.
The aim of our study was to assess the ability of AECA isolated from patients with SLE to induce endothelial cell apoptosis and their role in the clearance of apoptotic cells.

Methods

Patients

Serum samples from 100 consecutive patients with SLE were a gift from the departments of rheumatology at the University of Birmingham and University College London. All patients had active lupus with renal involvement at the time of serum sampling and were positive for double-stranded DNA (dsDNA) antibody. The diagnosis of SLE was based on the criteria of the American College of Rheumatology [10]. Ethical permission was obtained from the local ethics committee and written informed consent according to the Declaration of Helsinki was obtained. The sera were screened for the presence of AECA using a cell-based enzyme-linked immunosorbent assay (ELISA) that has previously been described in detail [3]. Sera were also screened by ELISA for the presence of antiphospholipid antibodies (anti-β2-GPI and anticardiolipin activity) (Binding Site, Birmingham, UK) and samples that were positive were excluded from further analysis, resulting in 30 patients who were positive for AECA, and these samples were used in further studies. These patients included 27 female and three male patients, their ages ranging from 16 to 61 yr (mean 30 yr). Data on clinical disease activity corresponding to the patients’ samples were available from the British Isles Lupus Assessment Group (BILAG) index records [11]. The BILAG score ranged from 5 to 15 with a median of 10. Control samples were isolated from eight healthy donors (three men and five women), none of whom was positive for AECA as assessed by a cell-based ELISA.

ELISA for AECA

The sera were screened for the presence of AECA using an ELISA which has previously been described in detail [3, 12]. Briefly, serum samples were diluted 1:400 in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA; fraction V), and incubated for 60 min at room temperature with human umbilical vein endothelial cells (HUVECs) which had previously been briefly fixed with 0.1% glutaraldehyde. The bound IgG was detected by a further 60-min incubation with peroxidase-conjugated rabbit anti-human IgG (1:500; Dako, High Wycombe, UK), with subsequent quantification of peroxidase using o-phenylenediamine dihydrochloride and H2O2 in citrate–phosphate buffer (pH 5). Each plate always had a blank value obtained from the optical density given by the diluting medium, a standard highly positive control sample from a patient with scleroderma, and a negative control consisting of pooled serum from 24 healthy laboratory personnel. Results were expressed as an ELISA ratio (ER), calculated as ER = 100 × [(S – A)/(C – A)], where S is the absorbance of the sample and A and C are the absorbances of the negative and positive controls. Mean values were calculated from three replicate determinations.

Cell culture

HUVECs were isolated as previously described and cultured under standard conditions [3]. The cells were used at passage 2 and plated onto gelatin-coated 96-well microtitre plates. Cells were maintained in culture for 72-96 h before use.

K562 cells (derived from a patient with chronic myelogenous leukaemia) were cultured in RPMI medium with 10% fetal bovine serum (FBS), 100 μg/ml penicillin, 100 μg/ml streptomycin and 1 mM l-glutamine. To induce apoptosis, cells were deprived of FBS for a total of 120 h [13].

Human monocytes were isolated using discontinuous Percoll gradients as previously described [14]. Monocytes were prepared by retrieving cells at the 81 and 70% Percoll interface. These were then washed with PBS and resuspended to 5 × 106 cells/ml. One millilitre was added to each well of a 24-well plate and incubated for 1 h in serum-free Iscove’s Dulbecco’s Modified Eagle Medium (DMEM; Sigma-Aldrich, Poole, UK). Cells were washed with ice-cold saline to remove non-adherent cells and cultured for 8 days in Iscove’s DMEM with added 1% penicillin and streptomycin (Sigma) and 10% autologous serum to mature them into macrophages (>90% pure). This yielded 1.5–2 million macrophages per well at the time of the assay.

Isolation of IgG

IgG was prepared from patient and normal human donor plasma-derived sera using a Hi-trap protein G affinity column (Pharmacia Biotech, Uppsala, Sweden). IgG was eluted from the column with glycine hydrochloride, pH 2.8. The eluate was extensively dialysed against PBS (Sigma, Poole, Dorset, UK) and then concentrated using Vivaspin 15 centrifugal concentrators (Vivascience, Lincoln, UK). The total protein content was estimated by spectrophotometry [15]. All samples were endotoxin-negative as assessed using the Limulus amoebocyte lysate kit (Sigma).

Assessment of apoptosis

The ability of AECA to induce apoptosis was evaluated by morphology using Jenner–Giemsa staining. At least 100 cells per slide were counted. Translocation of phosphatidylserine to the outer cell membrane was assessed by binding of fluorescein isothiocyanate (FITC)-conjugated annexin V and analysed by flow cytometry according to manufacturer’s instructions (Sigma). Trypan blue staining was used to assess the proportion of necrotic cells. The Apoppercentage kit (Bicolor, Newtownabbey, UK) was used according to the manufacturer’s instructions. HUVECs were incubated with IgG at different concentrations for variable time periods. A combination of TNF (10 ng/ml) and cycloheximide (3 μg/ml) was used as an apoptosis-inducing agent to generate a positive control.

Flow cytometric analysis for binding of AECA to apoptotic endothelial cells

To assess AECA binding to apoptotic endothelial cells, endothelial cells were cultured in serum-free conditions, trypsinized, and then incubated in suspension for 1 h at 4°C with AECA or normal IgG (200 μg/ml), in solution containing PBS with 10% normal human serum. Endothelial cells were then washed three times with PBS containing 1% BSA and centrifuged at 1200 r.p.m. for 5 min at 4°C. Endothelial cells were incubated with saturating concentrations of FITC-conjugated F(ab)2 fragments of goat anti-human IgG (dilution 1:20; Binding Site) for 45 min and then washed and fixed with 1% paraformaldehyde. Prior to fluorescence-activated cell sorting (FACS) analysis, trypan blue staining was performed to ensure cell membranes were intact and positive staining was always less than 5%.

Phagocytosis of apoptotic endothelial cells

Endothelial cells were dyed with a red membrane dye, PKH26, according to the manufacturer’s instructions (Sigma).
Endothelial cells were then re-plated in serum-free media for 24 h until apoptotic, giving 60% apoptotic endothelial cells as assessed by morphology (data not shown). Apoptotic endothelial cells in suspension were incubated with AECA or normal IgG (200 μg/ml) for 1 h on ice, added at day 8 to human monocyte-derived macrophages at 1 × 10⁶ cells/well in a 24-well plate in RPMI medium, and incubated for 1 h at 37°C with 5% CO₂. The monolayer was then washed vigorously with ice-cold PBS to remove bound but uningested endothelial cells. Macrophages were then viewed under phase-contrast microscopy (Fig. 1). One hundred macrophages in five random fields were counted; the phagocytic index was expressed as the number of phagocytosed endothelial cells divided by the total number of macrophages and expressed as a percentage.

Statistics
Data are presented as the mean and SEM. Statistical significance was determined using unpaired t-test.

Results

Effects of AECA

We have previously shown that AECA isolated from patients with SLE are able to induce endothelial activation with increased expression of adhesion molecules, including intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) [3]. The ability of AECA in the absence of NK cells to induce apoptosis is contentious. We took the 10 AECA samples with highest binding on HUVEC ELISA and examined phosphatidylserine externalization using annexin V binding, and could show no difference in apoptosis induction compared with normal IgG (n = 8) (Fig. 2A), using a variety of concentrations of IgG (0–500 μg/ml) over a variable time course (0, 6, 12, 24 or 48 h) (Fig. 2B). Anti-annexin V antibodies have been detected in patients with SLE [16] and anticardiolipin antibodies have been shown to inhibit binding of annexin V [17]. We therefore examined apoptosis by morphology. Others have shown that maximal apoptosis induced by AECA occurred after 15 h of incubation [18]. Using all 30 samples, we incubated HUVECs with AECA (concentration 200 μg/ml) with endothelial cells over 24 h but could show no difference in the ability of AECA to induce endothelial cell apoptosis compared with normal IgG (P = 0.167) (Fig. 2C). Using a commercial kit for detection of apoptosis (Apopercentage), three AECA samples again showed no difference in apoptosis of HUVECs compared with three IgG samples from normal individuals at 13 h (P = 0.1853) and 24 h (P = 0.150).

Treatment of HUVECs with TNF-α has previously been shown to increase the binding of AECA [19]. Ten of 15 AECA IgG preparations were shown to have increased binding to HUVECs by ELISA following treatment with TNF-α (10 ng/ml for 24 h). There was a significant increase in binding of AECA samples following TNF-α treatment (204.9 ± 104 units before TNF-α treatment compared with 237.6 ± 122.5 units following TNF-α treatment (P < 0.001). There was no change in binding of normal IgG (Table 1). Despite increased binding of AECA in the HUVEC ELISA, there was no difference in the percentage of apoptosis induced compared with normal controls as assessed by morphology (28 ± 5% for AECA and TNF-α compared with 21 ± 6% for normal IgG and TNF-α; P = 0.08, n = 5). Cycloheximide is a potent inhibitor of protein synthesis and has been shown to induce apoptosis of endothelial cells that are stimulated with TNF-α. HUVECs were cultured for 24 h with both AECA (200 μg/ml) and cycloheximide (3 μg/ml); there was no difference in apoptosis compared with normal IgG (33 ± 5% for AECA + cycloheximide compared with 28 ± 2.5% for normal IgG + cycloheximide; P = 0.2, n = 3).

AECA can bind to apoptotic endothelial cells

The antigens binding AECA have not been confirmed and appear heterogeneous [20]. Previously AECA were shown to up-regulate phosphatidylserine expression on endothelial cells, allowing increased binding by anticardiolipin antibodies [8]. We were unable to show increased phosphatidylserine expression but wished to investigate whether these antibodies were also capable of binding apoptotic endothelial cells. HUVECs were cultured in serum-free media overnight and underwent spontaneous apoptosis. Apoptotic free-floating cells were observed 4 h after growth factor withdrawal and 40–45% of endothelial cells were apoptotic by 12 h (data not shown). There was increased binding of AECA IgG to apoptotic endothelial cells at 12 h compared with freshly isolated endothelial cells (48.77 ± 11.9% AECA binding to apoptotic endothelial cells compared with 25.8 ± 6.7% AECA binding to freshly isolated cells (P < 0.001, n = 10) (Fig. 3). To test if this increase in binding to apoptotic cells was a general mechanism or restricted to endothelial cells, K562 cells were induced to undergo apoptosis by culture in serum-free medium for 3 days (apoptosis confirmed by morphology) and then assessed for AECA binding as above. No difference was observed between binding of IgG and AECA (geometric means 15.29 ± 0.85 and 19.0 ± 1.75, respectively), illustrating that the augmentation in binding is specific to apoptotic endothelial cells.

AECA opsonized apoptotic endothelial cells are ingested in higher amounts than control cells

We and others have previously shown that opsonization of apoptotic cells by antibodies that recognize cell surface components frustrates the normal anti-inflammatory mechanisms of apoptotic cell removal, whereby macrophages engulf opsonized apoptotic cells with the release of proinflammatory cytokines [9, 14]. We took five AECA samples which had the highest binding to apoptotic endothelial cells (55 ± 15.5%) and investigated the ability of macrophages to phagocytose opsonized apoptotic endothelial cells. Macrophage phagocytosis was greater when apoptotic endothelial cells were opsonized with AECA compared with apoptotic endothelial cells that were incubated with normal IgG. The mean phagocytic index was 24.9 ± 4.5% when normal IgG was added, compared with 34.8 ± 3.4% (n = 5, P = 0.01) for AECA.
Discussion

This study provides further evidence for a role of AECA in promoting inflammation. AECA recognize and bind apoptotic endothelial cells, thereby promoting increased phagocytosis by macrophages, although we were unable to show that AECA directly induce endothelial apoptosis. This has important implications for the progression of inflammation as we and others have previously shown that opsonization of apoptotic cells by antibodies that recognize cell surface components frustrates the normal anti-inflammatory mechanisms of apoptotic cell removal, whereby macrophages engulf opsonized apoptotic cells with release of proinflammatory cytokines [9, 14, 21]. Recognition of apoptotic endothelial cells is a specific effect rather than the binding of autoantibodies to antigens contained in nuclear blebs. We have shown that the increased binding of AECA to apoptotic endothelial cells is specific, as there was no increased binding when using an irrelevant cell line that had undergone apoptosis. We cannot, however, formally exclude other autoantibodies, such as antihistone or antinucleosome antibodies, present in SLE sera binding to apoptotic cells.

According to this study, AECA promote the removal of apoptotic endothelial cells by macrophages in a similar manner to other autoantibodies present in patients with SLE, such as anti-β2GPI, and will thereby promote release of proinflammatory cytokines from macrophages, such as TNF-α and IL-12.
thromboxanes [9, 14]. Macrophages from patients with SLE have been shown to be defective in their abilities to remove apoptotic cells, and, interestingly, increased circulating endothelial cells have been found in vivo in patients with active SLE [22].

Persistence of circulating apoptotic endothelial cells may allow increased binding of AECA in vivo, leading to immune complex formation and subsequently induction of a vasculitic syndrome. Removal of these immune complexes by macrophages may allow autoantigen presentation to T cells, further promoting inflammation, as well as removal of apoptotic cells in a proinflammatory manner.

We were unable to demonstrate that AECA isolated from 30 patients with active SLE induced apoptosis, despite using several AECA concentrations and time points. This work is in agreement with Moscato et al. [6], who were also unable to induce apoptosis using AECA, with or without anti-dsDNA activity. In the paper by Bordron et al., only five of eight lupus AECA samples were able to induce apoptosis, suggesting that only a very selected proportion of AECA isolated from patients with SLE induce apoptosis [18]. However, despite a large number of AECA samples we were unable to induce apoptosis of HUVECs. The results in our study were obtained using a single type of endothelial cell, HUVECs, and it is well known that endothelia are different in functional and antigenic properties [23]. It has been demonstrated that endothelial cells from different organs express individual sets of antigens [24]. Thus lupus immunoglobulins might exert pro-apoptotic or activating effects on endothelial cells localized in one particular vascular bed and not in others.

In conclusion, our studies indicate that AECA do not induce apoptosis of HUVECs but they do show increased binding to apoptotic endothelial cells, promoting their removal by macrophages in a proinflammatory manner.

**Key messages**

- Anti-endothelial cell antibodies are common in systemic lupus and may be involved in pathogenesis by increased binding to apoptotic endothelial cells, promoting uptake by macrophages.
- Anti-endothelial cell antibodies do not cause damage by inducing apoptosis.

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**References**


