REVIEW

Antiendothelial cell antibodies in vasculitis and connective tissue disease

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Antiendothelial cell antibodies (AECA) are a heterogeneous family of antibodies reacting with endothelial cell antigens. These antibodies are found in various diseases and recognise several antigen determinants. Different pathophysiological effects have been observed in *in vitro* experiments, which include direct or indirect cytotoxicity and endothelial cell apoptosis. Furthermore, some AECA activate endothelial cells, resulting in increased leucocyte adhesiveness, activation of coagulation and vascular thrombosis. In animal models, it has been shown that AECA could promote vascular damage. Neither the endothelial cell antigens nor their precise role in the pathogenicity of different diseases in which AECA are found is well characterised. Nowadays, it is not known whether AECA are an epiphenomenon accompanying vascular injury or whether they are pathogenic. It is controversial whether fluctuations in AECA titres are associated with disease activity during follow-up studies. This review summarises the present knowledge about AECA, AECA antigens and their potential role in the pathogenicity of vasculitis and connective tissue diseases.

in this field, and discusses the progress made in the debate about their potential pathogenic role.

AECA DETECTION

AECA are usually detected by ELISA using cultured human umbilical vein endothelial cells (HUVEC) as substrate.^{3–7} Generally, confluent endothelial cell monolayers are fixed before testing to avoid non-specific immunoglobulin (Ig)G binding and loss of cells. Fixation, however, induces permeabilisation of endothelial cell membranes and part of the AECA reactivity could be due to reaction with intracellular compounds. To avoid these artefacts, several groups use ELISAs with unfixed endothelial cells.³ Moreover, other techniques are used, such as immunofluorescence, radioimmunoassays, fluorescence-activated cell sorting, immunoblotting, immunoprecipitation, complement-dependent cytotoxicity (CDC) and antibody-dependent cytotoxicity (ADCC).³ Furthermore, endothelial cells other than HUVEC are sometimes used, such as cell membrane extracts, cells from renal or medullar microvessels, and cell lines.^{8–9} Each method and each substrate has a certain degree of specificity and sensitivity, and its own advantages and disadvantages. One perturbing element when comparing these tests is the variation between results, probably due to the modalities of antigenic preparations.¹⁰ Erroneous reporting of negative AECA may be owing to the lack of expression of certain target antigens on a specific substrate. Renaudineau *et al*² suggested the use of several endothelial cell substrates simultaneously to eliminate false-negative results.²

Heterophilic antibodies could sometimes be detected. Therefore, false-positive AECA could be reported owing to endogenous antibodies reacting with fetal calf serum (FCS) proteins from culture medium coated on ELISA plates. These results could be avoided by antibody absorption in FCS-containing dilution buffer or by washing cells free of FCS before plating.¹¹

Until now, to our knowledge, no standardised test or substrate exists for AECA detection, but concentrated efforts are currently being made.

PATHOGENIC EFFECTS

Direct cytotoxicity of AECA was reported only in few diseases. AECA could exert their pathogenic

The vascular endothelium has a pivotal position.¹ Antiendothelial antibodies (AECA) recognise a wide variety of antigens.² Their presence has been reported in connective tissue diseases, vasculitides and other inflammatory diseases (reviewed by Belizna *et al*³). The target antigens in these diseases are usually different and AECA possibly have several effects *in vivo*, explaining their complexity and heterogeneity.⁴ Although first described more than three decades ago,⁵ their pathophysiological role is still not completely understood, owing to the lack of precise characterisation of putative targets. Moreover, it is not established at what moment during vascular damage these antibodies are generated and whether they cause vascular dysfunction *in vivo*. Nevertheless, there is increasing evidence for the clinical importance and possible pathogenic role of AECA. They may interfere and control several endothelial cell functions, and therefore be a driving mechanism for vascular injury. This review discusses their role. Do AECA have a pathogenic role? Are they only “on the backstage” on the vasculitis theatre? Are they a marker of disease activity? This review summarises the present knowledge

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Abbreviations: ADCC, antibody-dependent cytotoxicity; AECA, antiendothelial cell antibodies; AHA, antihypertensive antibodies; APL, antiphospholipid; β 2-GPI, β 2-glycoprotein I; CDC, complement-dependent cytotoxicity; FCS, fetal calf serum; Hsp, heat-shock protein; HUVEC, human umbilical vein endothelial cells; SLE, systemic lupus erythematosus; SSC, systemic sclerosis

role either via CDC in patients with Kawasaki disease, or via ADCC mechanisms in those with Wegener granulomatosis or with microscopic polyangeitis.³

However, these data have not been confirmed.¹² In Takayasu's arteritis, some authors suggest that AECA are responsible for CDC.¹³

In 12 patients with Takayasu's arteritis, no sera showed ADCC at any of the effector:target ratios tested.^{13 14} Furthermore, this ratio was too high, suggesting a minor contribution of this mechanism during vascular injury in vivo. Rather than exerting a direct cytotoxicity, AECA could be pathogenic in vasculitis by activating endothelial cells, triggering the leucocyte adhesion to endothelial surfaces and cytokine production. However, recent experimental data suggest other AECA pathogenic mechanisms (fig 1).

Activation of endothelial cells

Incubation with AECA from patients with systemic lupus erythematosus (SLE) is followed by changes in expression of endothelial adhesion molecules such as E-selectin and intercellular adhesion molecule 1, and changes in the profile of cytokine secretion, with the production of pro-inflammatory cytokines (interleukin (IL)1 and tumour necrosis factor TNF α). This endothelial cell activation is dose dependent, as shown by in vitro incubation with serial AECA concentrations.¹⁵ Additional information comes from data showing the induction of a pro-inflammatory endothelial phenotype when endothelial cells are incubated with human monoclonal AECA from a patient with SLE.¹⁶ Endothelial cell activation is partially regulated by autocrine or paracrine actions of cytokines (IL 1).¹⁷ The signal transduction pathway in the expression of adhesion molecules induced by AECA seems to be via the mitogen-activated protein kinase cascade, but not exclusively.¹⁸ Therefore, endothelial cell activation seems to be mediated through nuclear factor κ B pathway and c-Jun N-terminal kinase-mitogen-activated protein kinases in response to TNF α .^{19 20} Interestingly, endothelial cell activation mediated by anti- β 2-glycoprotein I (β 2-GPI) antibody can be inhibited by statins. The capacity of these drugs to down regulate the expression of adhesion molecules suggests their possible use in various pathologies in which endothelial cell activation seems pathogenic.²¹

Induction of coagulation

Under physiological conditions, endothelial cells possess anticoagulant properties. Some AECA are responsible for the synthesis of tissue factor, the physiological initiator of coagulation. It has been found that after exposure to sera

from patients with SLE, HUVEC produced high amounts of tissue factor.²²

Moreover, when monoclonal AECA and anti- β 2-GPI antibodies derived from patients with Takayasu's arteritis and antiphospholipid syndrome were incubated with endothelial cells, they induced tissue factor production. Furthermore, tissue factor activity, tissue factor antigen and tissue factor mRNA were dose dependent on AECA titres.²³ Tissue factor activity was also time dependent; it was elicited by F(ab)2 fragments and completely disappeared when incubated with anti-tissue factor antibodies.

β 2-GPI can adhere to endothelial cells. The autoantibody binding activates a signalling pathway responsible for the translocation of nuclear factor κ B from the cytoplasm to the nucleus, and the activation of genes for up regulation of the adhesion molecule, pro-inflammatory cytokine and tissue factor.

Furthermore, the antiphospholipid antibodies interfere with the binding of annexin V, synthesis of endothelin I, induction of apoptosis and the protein containment/surveillance system.²⁴ Some studies have shown an association between antiphospholipid antibodies and low levels of free protein S,²⁵ and β 2-GPI interferes with the binding of protein S to its plasma inhibitor.²⁶

Altogether, these effects contribute to a pro-inflammatory and pro-coagulant endothelial phenotype. Since the description of antiheparin antibodies (AHA), much progress has been made to show how AECA induce a change in coagulant properties of endothelial cells. AHA have been reported in autoimmune diseases and their action has been reproduced in animal models. In endothelial cells, heparin sulphate represents the major glycosaminoglycan. AHA exert CDC on endothelial cells and form immune complexes with heparin. AECA binding to endothelial cells is followed by the cleavage and release of heparan sulphate on the endothelial cell surface, inducing pro-inflammatory and pro-coagulant consequences or apoptosis. This effect is specific for AECA, as elution studies failed to show inhibition with cardiolipin, anti-DNA, hyaluronate or chondroitine sulphate.² AHA are found in patients with SLE and correlate with renal and neurological disease. Furthermore, immunisation with glycosaminoglycan is followed by a systemic sclerosis (SSc)-like disease.²⁷ As AHA correlate with AECA activity, these antibodies could be considered to be part of the AECA repertoire.²⁸ Furthermore, it has been shown that autoantibodies to heparan sulphate may contribute to vascular injury via CDC mechanisms in MRL/lpr/lpr mice;²⁹ yet, the target antigens for AECA and AHA are incompletely characterised. Their molecular weight varies between 20

Figure 1 Pathogenic mechanisms for antiendothelial cell antibodies. ADCC, antibody-dependent cytotoxicity; β 2-GPI, β 2-glycoprotein I; CDC, complement-dependent cytotoxicity; EC, endothelial cell; PL, phospholipid.

and 200 kDa. Recently, the use of a monoclonal AECA to inhibit heparin binding to endothelial cells enabled the identification of a putative endothelial heparin receptor (a 45 000-M(r) heparin-binding polypeptide).³⁰

Apoptosis

Rather than exerting a direct cytotoxic effect, some AECA may induce endothelial cell apoptosis. Therefore, some authors reported apoptosis after incubation of human endothelial cells with AECA from patients with SSc. This phenomenon was inhibited by an anti-Fas ligand antibody, suggesting that apoptosis in SSc is mediated through CD95 (Fas).³¹ Adversely, some other authors report Fas-independent apoptosis.³²

If *in vitro* studies have suggested the AECA's role in mediating endothelial cell apoptosis, recent data led to suppose the same effects *in vivo*. Therefore, the injection of AECA-positive serum samples in normal chicken embryos from University of California at Davis line 200 chickens spontaneously developing a scleroderma-like disease was followed by an increased endothelial cell apoptosis.³³ AECA are often associated with antibodies to anionic phospholipids such as the phosphatidylserine. AECA and antiphospholipid (APL) antibodies seem to be different species of antibodies, although they may cross react.⁸ Anticardiolipin antibodies may behave as AECA, as absorption on to endothelial cells resulted not only in inhibition of AECA activity but also considerably reduced binding of anticardiolipin antibodies. In contrast, AECA binding could not be inhibited by incubation with cardiolipin.³⁴ However, AECA activity is partially related to APL. Hence, the incubation of endothelial cells with AECA is followed by the translocation of phosphatidylserine with the exposure of phosphatidylserine on the outer face of the endothelial cell membrane, followed by apoptosis.

APL antibodies recognise and activate endothelial cells.^{8, 35} They do not recognise anionic phospholipids, but plasma proteins bound to anionic surfaces such as β 2-GPI and prothrombin. Therefore, APL antibodies react with endothelial cells, mainly by reacting with β 2-GPI on the cell surface. β 2-GPI can adhere to endothelial cells via the annexin II receptor and negatively charged structures (heparin-like molecules) bound by the phospholipid-binding site of the molecule. Furthermore, a subset of APL antibodies recognises annexin V and induces endothelial cell apoptosis.³⁶ Adhesion of β 2-GPI to endothelial cells offers suitable epitopes for circulating APL antibodies that can induce endothelial cell activation.^{37–39} Moreover, a new antigen in patients with SLE—namely, heat-shock protein 60 (Hsp60)—is the target for anti-Hsp60 antibodies that bind to endothelial cells and induce phosphatidylserine exposure, followed by apoptosis, thus providing a target for anti-phosphatidylserine.⁴⁰

The incubation of human endothelial cells with APLs is followed by production of tissue factors and up regulation of adhesion molecules. Cell activation is associated with translocation of nuclear factor kappa B and with a signalling cascade similar to that triggered by the toll-like receptors.⁴¹

This activation seems to be mediated by β 2-GPI and incriminated in the adhesion of the leucocytes to the vessel wall.

However, *ex vivo* analysis of several parameters of endothelial cell dysfunction suggests that APLs alone could not be responsible for endothelial cell perturbation, and support a two-hit pathogenic hypothesis.⁴²

ANTIGEN CHARACTERISATION

AECA are generally not specific for endothelial cells.³ Endothelial cell-specific antigens for AECA are found only in a few cases, such as in patients with Kawasaki disease.¹⁴ Antigens recognised by AECA include constitutively expressed or cytokine-induced cryptic antigens, as well as

adherent molecules.³ As expected, various antigens are found when several types of substrates are used. Although not a major antigen determinant for AECA, human leucocyte antigen class I determinants are also endothelial cell antigens. A 25% reduction in AECA binding was reported after serum samples from patients with systemic vasculitides were incubated with a crude extract of extracellular matrix components.⁴³ Extracellular matrix components may also be target antigens for AECA, such as collagen types II, IV and VII, vimentin or laminin.^{3, 43} Antilaminin antibodies were reported in patients with SSc and in those with primary Raynaud's phenomenon, whereas antivimentin antibodies were found in patients with SLE.³ Human leucocyte antigen class II determinants, present only on activated endothelial cells, could also be target antigens for AECA. In patients with Kawasaki disease, however, they are not major antigenic determinants.^{3, 14}

Proteinase 3 could represent another potential cryptic target antigen. Mayet and Meyer zum Buschenfelde⁴⁴ have shown that ANCA recognise proteinase 3 translocated into the endothelial cell membrane; but this finding could not be reproduced by others.⁴⁵ Several molecules could bind to endothelial cells and become so-called planted target antigens for AECA via presumed charge-mediated mechanisms, a DNA–histone bridge or a specific receptor. Examples are myeloperoxidase, DNA or β 2-GPI,³ which might adhere to endothelial cells during incubation of endothelial cells with sera of patients. Subsequently, positive AECA are reported, but in reality anti-myeloperoxidase, anti-DNA or anti- β 2-GPI antibodies are detected.

Moreover, β 2-GPI could also be produced by the endothelium itself, or it could be a bovine β 2-GPI from the medium used when testing AECA.⁴⁶

DNA represents a planted or constitutively expressed antigen for AECA.⁴⁷ A strong association was reported between AECA and anti-DNA antibodies in patients with SLE.³ Chan *et al*⁴⁷ showed that this binding was partially caused by DNA–anti-DNA immune complex binding, and enhanced by histones. It was also shown that the binding of monoclonal anti-DNA antibodies to HUVEC was reduced by 20%, owing to treatment of HUVEC with deoxyribonuclease, suggesting presence of DNA on the endothelial cell surface.⁴⁷ Finally, some AECA recognise antigens exclusively present in microvascular but not in macrovascular endothelial cells.⁴⁸ Several phenotypic and functional differences are noticed between endothelial cell antigens from microvascular and macrovascular sites.⁴⁹ Differences in their nutritional requirements and responses to growth and migration stimuli are also reported.⁵⁰ In light of these findings, the use of endothelial cells from vessels of different sizes seems to be indicated during AECA assays.

Regarding scleroderma, two antigens were described—namely, a 95–100-kDa doublet and thrombomodulin.⁵¹ In patients with rheumatoid arthritis, a 44-kDa antigen was described, which was no more found with the disappearance of clinical vasculitis.⁵² In patients with Kawasaki disease, the antigens are not well characterised, but some cytokine-inducible epitopes may be important. AECA binding was detected by ELISA on both unstimulated and cytokine-treated endothelial cells.⁵³ In patients with Wegener granulomatosis, protein bands of 25, 68, 125, 155 and 180 kDa were detected.⁵⁴ It is not established whether proteinase 3 and myeloperoxidase are also endothelial cell epitopes for AECA. Also, in patients with SLE, different groups reported antigens with molecular weights ranging from 15 to 200 kDa.³

Recently, IgG AECA from patients with SLE have shown a shared reactivity to a 60-kDa endothelial cell surface polypeptide, the human Hsp60, responsible for endothelial

cell apoptosis.⁴¹ Much progress with respect to the identification of the target antigens for AECA has been brought about by new techniques (proteomics), one of which is the construction of expression libraries of complementary DNA to messenger RNA extracted from endothelial cells and transfected into prokaryotic or eukaryotic cells; the obtained sequences are compared with those of the genes from the data bank.⁵⁵

Molecular cloning strategy enabled the identification of a novel panel of candidate endothelial autoantigens in patients with SLE. After detecting AECA by ELISA, western blotting was carried out on samples from two patients and a HUVEC cDNA expression library was screened with their sera for identifying the autoantigens. Among them, the endothelial cell-specific plasminogen activator inhibitor, ribosomal P protein P0, ribosomal protein L6, elongation factor 1 α , adenylylase-associated protein, DNA replication licensing factor, profilin II, and human endothelial-associated lupus autoantigens 1 and 2⁵⁵ were autoantigens. In one of the two patients with SLE, antibodies to ribosomal P protein were predominant. Furthermore, levels of these antibodies directly correlated with AECA levels and clinical scores. Correlations between specific antibodies recognising defined antigens with clinical manifestations were described in patients with SLE.^{44 46 52 54 56} Another recent technique, two-dimensional electrophoresis, is also promising. When combined with western blot analysis using protein extracts from a hybridoma cell line, it enabled the identification of antigens such as calreticulin, tubulin, vimentin and Hsp70.⁵⁷

ANIMAL MODELS

Animal models further supported the pathogenic role of AECA. In 1988, Matsuda⁵⁸ immunised guinea pigs with cultured endothelial cell membrane products and found high AECA titres, resulting in proliferative changes in the mesangial matrix and proteinuria. Injection of antibodies to endothelial cell antigens, such as angiotensin-converting enzyme or factor von Willebrand, promoted experimental lung or renal injury.⁵⁹ An idiopathic experimental model was created by Damianovitch.⁶⁰ The active immunisation of BALB/c mice with AECA from a patient with Wegener granulomatosis triggered the production of mouse AECA and vasculitis, reflecting direct evidence for AECA pathogenicity.⁶⁰ Finally, it is presumed that xenoantibodies or isoantibodies, reactive with transplantation antigens, may result in severe endothelial cell damage, as has been suggested in experimental models.^{61 62}

Allografts are carried out between members of the same species, and xenografts are carried out between members of different species; rejection is therefore usually expected after a short delay following the grafting (hyperacute rejection) via humoral mechanisms. Several data suggest that a mechanism of the graft damage is related to antibodies against the xenograft endothelium. It seems that AECA activate the classical complement pathway, which promotes endothelial cell activation and hyperacute rejection. In a pig-to-rhesus model, techniques for lowering the circulating AECA levels (plasma exchange) permitted a delayed rejection.⁶² However, recent data suggest that several other mechanisms are associated with hyperacute rejection, and that monitoring of the peripheral antibody responses is required in pig-to-primate xenograft recipients.⁶³

AECA AND DISEASE ACTIVITY

A fluctuation of AECA titres with disease activity was reported in patients with systemic vasculitis.^{64 65} These data, however, could not be confirmed by others.⁶⁶ Although a temporal relationship between AECA titres and disease relapses has not been shown, AECA may represent a

predictive marker for relapse for AECA-positive patients, but ANCA-negative patients had an increased risk for the clinical relapse.^{67 68} In patients with SLE, correlation between AECA and disease activity suggests that AECA are markers of disease activity, as was also shown for anti-DNA antibodies.^{69 70} AECA may be markers of disease severity as well. Therefore, high AECA prevalence has been associated with vascular lesions, kidney involvement, anticardiolipin antibodies, thrombosis^{71 72} and pulmonary hypertension.⁷³ Furthermore, correlations between AECA recognising defined antigens with clinical manifestations were reported.⁷⁴

However, data from a study conducted on 48 patients are rather contradictory.⁷⁵ Therefore, when evaluating different biological parameters to distinguish the quiescent and active phases of lupus nephritis, no correlation between AECA titres and disease was found. In SSC, AECA are more often found in patients with complications such as digital ischaemia, pulmonary hypertension⁷⁶ and alveolocapillary dysfunction.⁷⁷ AECA are closely correlated with pulmonary fibrosis⁷⁸ and vasculitis,⁷⁹ and AECA testing can identify subsets of patients with SSC with different prognoses.⁸⁰ In patients with rheumatoid arthritis, AECA levels are higher in patients with renal or neurological complications.^{56 81} Vasculitis lesions are correlated with high AECA titres in patients with rheumatoid arthritis and in those with Kawasaki disease.⁷⁹

CONCLUSION

Several findings raise the possibility that AECA might be pathogenic in connective tissue disease and vasculitis. Moreover, some studies suggest that AECA may be useful markers for disease activity. However, further study is needed to clarify their clinicopathological significance.

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