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PATHOGENIC MECHANISMS OF ANTI-ENDOTHELIAL CELL ANTIBODIES (AECA): THEIR PREVALENCE AND CLINICAL RELEVANCE

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1. Abstract

Anti-endothelial cell antibodies (AECA) represent a heterogeneous family of autoantibodies directed against structural endothelial proteins, as well as antigens adhering to endothelial cells. Although AECA immunoassays still show a high-interlaboratory variability, several findings suggest a pathogenic role of these autoantibodies in diseases characterized by endothelial damage. In this chapter, we analyze the knowledge about AECA prevalence, clinical relevance, and their pathogenic role in autoimmune diseases focusing in particular on systemic lupus erythematosus, antiphospholipid syndrome, systemic sclerosis (SSc), and systemic vasculitis.

2. Introduction

An increasing body of evidences in the last two decades have been reported on the presence of autoantibodies directed to a heterogeneous family of structural endothelial proteins, as well as antigens adhering to endothelial cells (ECs) in patients with diseases characterized by endothelial damage (Fig. 1). Vascular ECs play an important role in many processes such as blood pressure regulation, coagulation, fibrinolysis, angiogenesis, and blood cell activation during physiological and pathological processes [1]. So far,

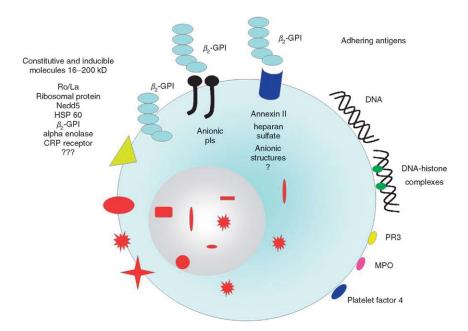


FIG. 1. Target antigens of AECA. AECA represent a heterogeneous group of antibodies direct to structural endothelial proteins (see left side of the figure), as well as to antigens adhering to endothelial cells directly or through endothelial receptors (see right side of the figure). Nedd5, intracytoplasmatic protein of the septin family; β_2 -GPI, β_2 -glycoprotein-I; HSP 60, heat shock protein 60; CRP, C-reactive protein; PR3, proteinase 3; MPO, myeloperoxidase.

anti-endothelial cell antibodies (AECA) have been correlated with both disease activity and vascular involvement in several diseases. Much effort has been made to determine whether AECA mediated-mechanisms underlie the development of diseases or merely represent markers of vascular damage. Intriguingly, endothelial antigens are readily accessible to circulating antibodies and a pathogenic role of AECA has been suggested by several authors. In particular, recent findings suggest that AECA may activate ECs by an upregulation of the cell adhesion molecule (CAM), or by inducing the secretion of proinflammatory cytokines. It has been suggested that AECA might also activate ECs through an interaction with toll-like receptor 4 (TLR4) because of the molecular mimicry between beta2-glycoprotein I (β_2 -GPI) and microbial structures that represent the natural ligand of TLR4. Finally, controversial results have been reported regarding the induction of ECs apoptosis by AECA, although it is well known that these autoantibodies bind to apoptotic ECs, thus increasing phagocytosis by macrophages and clearance of apoptotic cells [2-4].

In this chapter, we analyze the knowledge about AECA in autoimmune diseases focusing in particular on their pathogenic role in systemic lupus erythematosus (SLE), antiphospholipid syndrome (APS), systemic sclerosis (SSc), and vasculitis.

3. Methods of AECA Detection

Since the discovery of AECA, several studies have focused on their role as diagnostic tools for diseases characterized by an inflammation of blood vessels. Despite the growing interest in this field, AECA immunoassays still show a high-interlaboratory variability, most probably due to the lack of international standardization. Many methods have been developed to detect AECA, including immunofluorescence, immunoassays (ELISA and RIA) using fixed cells, Western blot (WB), and fluorescence-activated cell sorter analysis (FACS). Nevertheless, only a small number of comparative studies have been done and data concerning sensitivity and specificity of methods to detect AECA are frequently variable and inconclusive (reviewed in Ref. [5]). The major part of our knowledge on AECA reactivity comes from in vitro experiments with human umbilical vein ECs (HUVECs). Nevertheless, the isolation of primary HUVECs is laborious and the AECA reactivity obtained with different donors cannot easily be compared to each other because of their different origin. Moreover, HUVECs present a short life span and can be kept in culture only for short periods. Another point of debate is the fixation procedure of HUVECs, which may influence the AECA reactivity

against intracytoplasmic or membrane antigens. Interference from heterophilic antibodies, which recognize fetal calf serum (FCS) proteins, may be avoided by adding FCS to the dilution buffer [6]. Finally, different ECs from macro- and microvasculature have been used for AECA detection with different results. ECs from diverse tissues are also heterogeneous not only in respect to small vs large vessels but also in respect to their surface phenotype and protein expression. For instance, Renaudineau et al. [7] showed the highest prevalence of AECA reactivity in a large series of patients with SSc using microvascular bone marrow ECs in comparison to HUVECs. The need for reproducible results has increased the demand for immortalized EC lines, such as EA.hy926, generated by fusion of HUVECs with the human lung carcinoma cell line A549 [8], or the immortalized human microvascular EC line (HMEC-1) [9]. ECs derived from human umbilical and iliac veins and arteries transfected with a plasmid containing the Simian Virus 40 large T-antigen, may also be useful for the detection of AECA [10]. Immortalized human glomerular endothelial cell (HGEC) lines were also generated [11]. These cell lines maintain the morphologic and functional characteristics of HGECs even after several weeks in culture, thus providing a standardized substrate for AECA detection. Nevertheless, AECA detection on immortalized ECs may be subject to great variability and scientists interested in autoimmunity against ECs would prefer primary EC lines as substrate because these cells probably present more primary endothelial traits. Although the original cyto-ELISA remains the most widely used method for the detection of AECA, it is advisable to confirm a positive result using other methods, such as flow cytometry, immunoprecipitation, or WB, which appear to be difficult to use on a routine basis [12]. It is important to point out that heating sera should be avoided because this treatment increases AECA binding by a nonspecific mechanism in sera from both patients and healthy individuals [13]. ANA or RF in serum samples have been shown not to interfere with AECA detection by ELISA with nucleus-depleted lysates prepared from EA.hy926 [14].

Finally, because it has been reported that different AECA isotypes may be prevalent in different autoimmune disorders, as discussed elsewhere in this chapter, it should be pointed out that the AECA test should include determination of both IgG and IgM in most disorders with the addition of IgA in patients with Henoch-Schonlein purpura (HSP). In fact, while IgG AECA are predominant in most autoimmune conditions, IgM AECA have been shown to be particularly increased in Kawasaki disease (KD) probably reflecting the acute nature of this disorder. In addition, IgG and IgM AECA may exert diverse pathogenic role due to their differential ability to fix and activate the complement cascade as well as to induce ECs damage via antibody-dependent cytotoxicity.

4. AECA in Autoimmune Diseases

4.1. AECA IN SYSTEMIC LUPUS ERYTHEMATOSUS AND ANTIPHOSPHOLIPID SYNDROME

SLE is a protean autoimmune disease characterized by the largest number of detectable autoantibodies reactive with antigens localized in the nucleus, cytoplasm, or on the cell membrane (reviewed in Ref. [15]). Anti-dsDNA antibodies, and complement consumption are established markers of disease activity. A large number of studies have focused on the association of autoantibodies with specific symptoms of SLE: for example, it is well known that anti-dsDNA antibodies, as well as antibodies to the collagen-like region of the complement component C1q (anti-C1q), are associated with renal involvement in SLE [16]. Moreover, anti-Ro antibodies have been found to be a marker of neonatal lupus and subacute cutaneous lupus, and anti- β 2-glycoprotein I (anti- β_2 -GPI) and anti-cardiolipin (aCL) antibodies were significantly correlated with clinical thrombosis, abortion, and fetal loss. Despite the fact that most studies have shown a higher prevalence of AECA in SLE, the clinical relevance of these autoantibodies is still a matter of debate.

The prevalence of AECA in SLE largely range from 15% to 80%, depending on the method and patient selection. Nonetheless, the presence of vasculitis in SLE is frequently associated with AECA although these autoantibodies have been correlated with several other clinical manifestations of the disease such as lupus nephritis, pleurisy, thrombocytopenia, and neuropsychiatric symptoms [17-19]. Inflammatory or thrombotic vascular injury represents one of the most frequent lesions in SLE and endothelial perturbation induced by AECA may be involved in the earliest events in the pathophysiology of vascular tissue damage. Vessels of any size may be affected and therefore the clinical scenario of vasculitis in SLE is extremely wide. In 1987, Hashemi and coworkers described an association between AECA and vasculitis [20]. Thereafter, Del Papa et al. [21] showed that sera from SLE patients with vasculitis immunoprecipitated EC surface proteins ranging from 200 to 25 kDa. Song and coworkers described a higher positivity rate of AECA in patients with digital vasculitis as previously reported [19, 22]. In this study IgG-AECA showed a positive correlation with disease activity in SLE patients. Moreover, a significant correlation between the IgG-AECA titer and SLE disease activity index (SLEDAI) score modification was also observed in SLE patients after treatment [19]. Thus, AECA may be useful in the follow-up of patients; other soluble markers of EC dysfunction, such as von Willebrand factor, thrombomodulin, and soluble E-selectin, seem to be surrogate markers (an epiphenomenon) related to EC

damage [23]. It is possible that AECA may represent a marker of cell injury or a sign of polyclonal activation of humoral immune responses in other diseases such as in liver transplantation where AECA correlate with cytomegalovirus (CMV) infection [24, 25] and are associated with higher frequency of humoral allograft rejection [25]. AECA have been found in patients with SLE and urticarial vasculitis (UV) as well as in hypocomplementemic urticarial vasculitis syndrome (HUVS) [26]. D'Cruz and colleagues described a high prevalence of AECA, associated with reduced serum levels of complement, in SLE patients with UV, but not in primary UV or in SLE patients without UV [26]. The binding of AECA to ECs may be accompanied by the formation of immune complexes and complement fixation resulting in tissue damage. The anti-C1q antibodies seem to be a specific marker for HUVS [26, 27], but they may also be detected occasionally in SLE patients (with or without UV). It has been suggested that anti-C1q antibodies could contribute to EC damage by cross-linking C1q bound to the immune complexes on the surface of ECs [28].

The presence of AECA has also been associated with thrombotic vascular damage in patients with SLE as well as in primary or secondary APS [29]. In this regard, Dieude *et al.* showed that AECA from SLE patients recognized heat-shock protein 60 (HSP60) on the surface of HUVECs and induced apoptosis. A relationship between a high titer of anti-HSP60, the presence of lupus anticoagulant (LAC), and thrombosis in SLE patients was also observed [3]. Thus, the authors suggest that, in the pathophysiologic pathway of thrombosis, the induction of apoptosis by anti-HSP60 may represent the initial event that leads to aPL binding to the anionic phospholipid on the EC surface which may initiate the thrombotic cascade [3].

Anti-endothelial protein C receptor (EPCR) autoantibodies can be detected in APS patients and represent a novel risk factor for fetal death [30]. A higher percentage of AECA positivity was also observed in Sneddon's syndrome, a disease characterized by livedo reticularis and ischemic cerebral vascular disease [31].

It has been suggested that AECA of various isotypes may be associated with lupus nephritis in SLE [17, 32, 33]; in particular, the highest AECA levels have been detected in a large series of SLE patients with diffuse proliferative glomerulonephritis at the time of renal biopsy or with proteinuria and nephrotic syndrome [17]. AECA showed the highest titer in active lupus, whereas the mean antibody titer fell significantly as patients entered remission [34]. These findings have been further validated by a prospective study that suggested a role for AECA, in association with other laboratory parameters, as a marker of disease activity in SLE [35]. Autoantibodies reacting in immunoblot analysis with both 27- and 29-kDa endothelial cell antigens were correlated to the presence of glomerular capillary thrombi and heavy proteinuria [36]. In another study, patients with lupus nephritis, vasculitis, and hypocomplementemia showed IgG-AECA against a 66-kDa membrane antigen [18]. Antibodies against ribosomal P protein P0 have been identified by a molecular cloning strategy in SLE patients with active disease and nephritis [37]. In one patient, these autoantibodies correlated with total AECA levels as well as with anti-DNA antibody, disease activity, and renal involvement in a longitudinal follow-up. Nevertheless, to date, positive findings of both anti-dsDNA and anti-C1q antibodies are of higher specificity for active nephritis in SLE patients [16].

aPL, anti- β_2 -GPI, and AECA have been associated with various neurological manifestations in patients with SLE. These in vivo observations were further validated by a mouse model of APS, which developed neurological dysfunction and hyperactive behavior associated with aPL, anti- β_2 -GPI, and AECA after passive immunization with human aCL mAb [38]. We demonstrated an association between the presence of AECA and psychiatric manifestations, such as psychosis and depression in SLE, suggesting a possible organic mechanism underlying the psychiatric symptoms [39]. In our study no significant correlation was found between aCL, anti- β_2 -GPI, anti-Ro, antiglial fibrillary acidic protein, antiribosomal P protein, anti-dsDNA, and anti-nucleosome antibodies and psychiatric involvement in SLE patients. Moreover, no association was found between AECA reactivity and aCL and anti- β_2 -GPI antibodies. Remarkably, by screening a cDNA library from human umbilical artery ECs (HUAECs) with serum from an SLE patient with acute and active psychosis and elevated AECA serum level, we further identified one strongly reactive clone encoding the C-terminal region (C-ter) of Nedd5, an intracytoplasmic protein of the septin family [40]. IgGs specific to Nedd5 C-ter were present in 14 of 51 SLE patients (27.4%) by ELISA and the mean IgG reactivity to this protein was significantly higher in SLE patients with psychosis or mood disorders. On the contrary, no correlation was observed between the presence of anti-Nedd5 C-ter antibodies and the other clinical features. The results of these studies suggest a relationship between AECA and anti-Nedd5 antibodies with neuropsychiatric involvement in SLE, supporting the hypothesis of a biological origin of these disturbances.

4.2. AECA IN SYSTEMIC SCLEROSIS

Systemic sclerosis is a multisystemic disorder of the connective tissue characterized by immune abnormalities, microvascular injury, and fibrotic changes involving skin and visceral organs, such as the lungs, heart, gastro-intestinal tract, and kidneys. It is well recognized that vascular injury appears frequently in the clinic course of the disease—that is, Raynaud's phenomenon (RP)—and that vascular dysfunction represents one of the

primary events in the pathogenesis of the disease. Thus, it is not surprising that a significant amount of research by both clinical and basic scientists has focused on the identification of the clinical parameters and the pathogenic mechanisms of endothelial dysfunction, with AECA displaying great relevance in both fields of investigation.

AECA reactivity in scleroderma is quite common, having been detected with an overall prevalence varying from 19% to 85%, depending on the method used (ELISA, WB, IF), the Ig isotypes investigated, the substrate (tissue sections, source of human ECs) used, as well as the variability in the subtypes of SSc patients included (limited vs diffuse SSc) in the different studies (reviewed in Ref. [41]).

Considerable effort has focused on the identification of associations between AECA positivity and the clinical manifestations of SSc. An early study involving 31 patients with diffuse SSc (dSSc), 36 with limited SSc (lSSc) and 13 with primary RP (PRP) reported an increased prevalence of AECA with increased severity of clinical manifestations related to SSc [42]. If the analysis was limited to AECA IgG, these authors observed an AECA prevalence of 15% in patients with isolated RP, 39% in ISSc, and 77% in dSSc. An increased prevalence of AECA in dSSc compared to ISSc was also reported by Negi *et al.* [43] in 76 patients with SSc with a prevalence of 40% vs 13.5%. Although these results suggested an important role for AECA in identifying different clinical subsets of SSc, they have not been confirmed in later studies, in which no difference in AECA reactivity was observed between patients with ISSc and dSSc [44, 45].

Although the utility of AECA in differentiating the main clinical subsets of SSc is debatable, significant associations between AECA reactivity and some of the clinical manifestations of SSc have been consistently reported [42–45].

AECA were found to closely associate with capillaroscopy abnormalities as well as with severe digital ischemia and digital ulcers [42–44]. Together with vascular damage, the most striking clinical association of AECA observed in SSc relates to lung involvement. This is particularly important since lung involvement, manifested by either interstitial fibrosis and/or pulmonary hypertension, represents the most frequent cause of death in SSc [46].

Although evaluating lung involvement according to different clinical parameters and diagnostic procedures, at least four reports have demonstrated a strict association between AECA and one or more parameters of lung disease in SSc.

Salojin *et al.* [42] demonstrated lung involvement in 93% of AECA positive patients with SSc but only in 30% of those displaying no serum AECA reactivity. Unfortunately, no further characterization of lung disease (i.e., isolated reduction in diffusing capacity for carbon monoxide (DLco), pulmonary fibrosis, or pulmonary hypertension) and association with AECA

was available in this study. Negi *et al.* [43] found a significant correlation between AECA and pulmonary arterial hypertension; Pignone *et al.* [44] reported a strong association with both reduced DLco and with pulmonary artery hypertension but not with pulmonary fibrosis. This observation is of particular interest considering that isolated DLco reduction has been indicated as a predictor of subsequent development of pulmonary hypertension [47]. Finally, Ihn *et al.* [45] observed a significant association between AECA and reduced percent vital capacity (%VC), decreased DLco as well as pulmonary fibrosis, with the strongest association with severe interstitial lung disease.

Although prospective studies have not yet been carried out, all the available data suggest that AECA may have a profound clinical relevance in identifying SSc patients at major risk of developing more advanced vascular damage and severe pulmonary disease manifested either as interstitial fibrosis and/or pulmonary hypertension. Longitudinal studies on a large number of patients are awaited to confirm the possible clinical utility of AECA as disease markers in SSc.

Putative antigens responsible for AECA reactivity in sera of SSc patients have been identified by several groups as protein bands of various molecular weights obtained by WB analysis of sera reacting against cytoplasmic or membrane proteins extracted from HUVECs or ECs from other sources. However, in the absence of a molecular biology approach to identify the nature of the antigens observed, the exact endothelial antigenic targets recognized by AECA in SSc remain elusive. A first study reported the identification of several membrane endothelial cell specific antigens (more than 20 different bands at WB analysis) as the target of AECA in SSc patients [48]. In particular, reactivity against a membrane antigen of ~19 kDa was observed in more than 50% of the patients with SSc and in 100% of SSc patients with CREST syndrome and was shown to display anticentromere activity in IFI analysis. Although interesting, this observation has not been confirmed by subsequent reports. Ihn et al. [45] using HUVEC protein extracts observed reactivity of AECA positive sera, as previously assessed by cyto-ELISA on HUVECs, toward four major antigens recognizable as WB bands of 60, 90, 110, and 140 kDa. The most common response was observed against the 90-kDa antigen and purified anti-90 kDa antibodies displayed a cytoplasmic pattern at IFI identical to that observed in AECA positive sera. No such reactivity was observed using the same sera toward protein extracted from dermal fibroblasts, suggesting specificity of the immune response against endothelial antigens. Wusirika et al. [49] reported reactivity of SSc sera with pulmonary involvement against multiple bands between 38 and 110 kDa obtained by WB of protein extracts from HUVECs and pulmonary microvascular ECs. Although no further characterization of AECA antigenic

target in SSc is provided in these reports, it is clear that, as observed in other autoimmune conditions, AECA reactivity in SSc is rather heterogeneous and only the identification of the exact antigens responsible for AECA activity of SSc sera may provide important clues in understanding the pathogenic mechanisms underlying endothelial dysfunction and aggression in this disorder.

4.3. AECA IN SYSTEMIC VASCULITIS

Detection of AECA in sera of patients affected by systemic vasculitis is a relatively common finding, with prevalence varying greatly among the different forms of vasculitis.

Early reports demonstrated the ability of sera from children with acute KD to lyse HUVECs when pretreated in vitro with either IFNy or monokines [50, 51]. This effect was observed only in patients with acute febrile KD but not in the convalescent phase of the disease and was dependent on the presence of circulating IgM with AECA activity. However, later studies provided conflicting evidence on the effective role of AECA in KD. Guzman et al. [52] observed AECA in just 17% of 22 patients with KS and AECA were not able to differentiate KD from other febrile disorders of childhood resembling KD. Similar findings were reported by Nash et al. [53] in 58 children with acute KD, which showed no increased AECA reactivity compared to 35 children with febrile infections. Conversely, Kaneko et al. [54] detected IgM AECA in 73% of 22 KD children, half of which displayed complement dependent cytotoxicity in vitro against EC. Finally, Falcini et al. [55] reported AECA reactivity in 26% of KD patients, with increased incidence in the acute compared to the convalescent phase while Fujieda et al. [56] observed IgM AECA in 42% and IgG AECA in 26% of KD children, confirming the increased presence of IgM with AECA reactivity in the acute phase of KD. Although the effective role of AECA in KD is still matter of debate, there is sufficient evidence to support the concept that IgM AECA rise in a subset of patients in the acute febrile phase of the disease and may participate to endothelial inflammation leading to arteritis in KD.

More consistent evidence has been reported on AECA in anti-neutrophil cytoplasmic antibody (ANCA)-associated systemic vasculitis. Ferraro *et al.* observed AECA, mostly of IgG isotype, in 60% of sera of patients with microscopic polyangiitis (MPA) and 40% of those with Wegener's granulomatosis (WG). Importantly, absorption of ANCA did not affect endothelial binding of AECA suggesting that AECA and ANCA represent two different populations of autoantibodies [57]. Frampton *et al.* described increased AECA levels in ANCA-associated vasculitis with a significant correlation between the two antibodies. In addition, both AECA and ANCA correlated with disease activity [58]. These earlier results were later confirmed in an extensive evaluation of AECA reactivity in sera from 168 patients with ANCA-positive MPA or WG. AECA were present in about 60% of patients with ANCA-associated vasculitis (59% IgG and 68% IgM). Interestingly, and differently from KD, AECA from these patients were not able to induce complement-dependent cytotoxicity, even after pretreatment of ECs with cytokines, but a minority of sera induced an antibody-dependent cytotoxicity [59], as described elsewhere in this chapter. Although Del Papa *et al.* [21] observed in the majority of AECA-positive WG patients a relatively conserved precipitation pattern by WB analysis of EC surface extracts with five major bands of 25, 68, 125, 155, and 180 kDa, this finding awaits confirmation in larger studies; thus, as in other autoimmune conditions, the nature of the antigen(s) recognized by AECA in ANCA-associated vasculitis is currently unknown.

Two studies prospectively evaluated AECA (and ANCA) levels in patients with ANCA-associated vasculitis and assessed their relationship with disease activity [60, 61]. Both reports demonstrated that AECA were present in the majority of patients during the observation period and fluctuation of AECA was correlated with disease activity, with increased titer in disease relapses and reduced levels during remission phases. Although encouraging, these results do not provide definitive evidence on the possible role of AECA, alone or in association with ANCAs, as predictive factors of clinical relapses in ANCA-associated vasculitis.

Less information is available regarding the role of AECA in other forms of systemic vasculitis. In Takayasu arteritis (TA), Eichhorn *et al.* [62] reported a striking incidence of AECA of 95%, but this finding was not confirmed in a later study reporting an incidence of 33% [63]. Limited evidence is also available for Churg–Strauss syndrome (CSS), with Schmitt *et al.* [64] reporting AECA in 50% of 16 CSS patients but, in contrast with ANCAs, without any association with disease activity.

Finally, interesting findings have been reported regarding the presence of IgA AECA in patients with HSP. Fujieda *et al.* [65] observed IgA AECA in nearly 50% of HSP patients with nephritis but, interestingly, in no HSP patients without nephritis. Similar results were obtained by Yang *et al.* [66] who detected AECA IgA in 45% and 35% using HUVECs and human dermal microvascular endothelial cell (HDMECs) as substrates, respectively, with HUVEC AECA IgA titers correlating significantly with disease activity.

In summary, detection of AECAs in sera of patients affected by systemic vasculitis is a relatively common finding, with prevalence varying greatly among the different forms of vasculitis. Some of the differences observed may be related to the prevalent use of HUVEC as a substrate to detect AECA, despite the evidence that different form of systemic vasculitis target

preferentially vessels of different size. In the future, evaluation of AECA using as substrates ECs from macro- as well as microvasculature may be important to assess more precisely AECA reactivity in the various forms of vasculitis. In addition, this approach may also provide information on the preferential localization of antigenic targets in different forms of vasculitis and allow the identification of new antigenic specificities of AECA, which may reveal of pathogenic importance. In this regard, although inconclusive, preliminary attempts to detect AECA by using ECs from different sources in various autoimmune conditions suggest that this may represent a promising experimental approach [7, 49, 66, 67].

4.4. AECA IN OTHER DISEASES

AECA have been described in a number of systemic autoimmuneinflammatory diseases other than those already mentioned. For instance, increased AECA production was observed in patients with mixed connective tissue disease (MCTD) [67]. AECA from MCTD patients may activate ECs by the upregulation of E-selectin expression and may be implicated in the pathogenesis of MCTD. D'Cruz et al. [68] described an interesting association between AECA levels and interstitial lung disease in all forms of idiopathic inflammatory myopathies. In Behcet's disease (BD), the reported prevalence of AECA varied widely [69]. A significant association between AECA and thrombosis and vasculitis as well as disease activity in BD has been reported [70, 71]. Dinc et al. [72] described a low prevalence of AECA in a large series of Turkish Behcet's patients without significant differences from healthy controls. A new technique was described by Lee et al. [73] demonstrating for the first time that AECA IgM from sera of patients with active BD reacted strongly with human alpha-enolase, a protein of the HDMEC.

AECA were frequently detected in inflammatory bowel disease, and although they were associated with both active and extensive colitis, they seem to be less disease specific than other autoantibodies [74]. Nevertheless, at least in Crohn's disease, intestinal vascular injury mediated by AECA may be an important event [75].

Increasing evidence is accumulating on the possibility that AECA may play an important role in the development and progression of atherosclerotic lesions. Farsi *et al.* [76] described a higher rate of AECA in patients with unstable angina than in those with effort angina. Only 3 out of 12 AECA positive patients were also positive for anti- β_2 -GPI supporting the idea that β_2 -GPI is not the major AECA target antigen. Thus, AECA may contribute to the instability of angina and are associated with the high-restenosis rate after percutaneous transluminal coronary angioplasty. In contrast, patients with or without coronary atherosclerosis, confirmed by angiography, showed similar AECA levels [77]. The target antigen of AECA in acute myocardial infarction may be the endothelial protein C receptor, as reported by Montes *et al.* [78].

The AECA seem to be involved in the complex pathogenesis of vascular complications in diabetes [79], although others have suggested that they are only associated with coexisting autoimmune disorders [80]. A putative cytotoxic role of AECA vs ECs of the inner ear in immune-mediated sudden sensorineural deafness has been suggested [81, 82].

Understanding the relationship between autoimmune disease and infection has been a topic of interest for several decades. It is noteworthy that autoimmune diseases may be caused or triggered by infections. AECA are a common finding in several infections, such as HCV infection where these autoantibodies are often associated with mixed cryoglobulins [83]. Moreover, AECA associated with aCL, but not anti- β_2 -GPI, were found in HIV infection [84]. AECA and anti-epithelial human cell antibodies were described in severe acute respiratory syndrome (SARS) caused by infection with the SARS-associated coronavirus and in patients with leprosy [85, 86].

Natural AECA were described in healthy subjects and Ronda *et al.* [87] showed that these antibodies interact with living ECs, affecting their function after a ligand-receptor-like mechanism of internalization. Natural AECA showed a more restricted pattern of reactivity compared to AECA from SLE patients [88]. A significantly higher level of IgM-AECA was found during normal pregnancy compared with that in healthy nonpregnant controls and pregnant patients with SLE [89]. On the contrary, IgG-AECA levels were significantly higher in the serum of normal pregnant controls. Taken together, these findings support a physiological role of AECA in fetal tolerance of normal pregnant women, as well as a pathogenic role in impaired reproductive function frequently found in pregnant SLE.

5. Pathogenic Mechanisms of AECA

5.1. CYTOTOXICITY AND APOPTOTIC EFFECTS OF AECA

The pathogenic ability of AECAs to induce an endothelial perturbation is suggested by the demonstration of complement-dependent cytotoxicity on HUVECs from sera of patients with systemic vasculitis [50, 51, 54, 57, 90] and SLE [91]. In particular, early reports in the 1980s demonstrated that

purified IgM and, to a much lesser extent, IgG from patients with acute but not convalescent KD were able to lyse HUVECs, assessed by ¹¹¹In release, in the presence of fresh (but not heat inactivated) rabbit serum. Conversely, the simple incubation of KD IgM or IgG failed to induce any direct cytotoxicity; in addition, the lytic effect was observed only when HUVECs were prestimulated with either IFN γ [50] or monokines [51] suggesting that exposure of neo-antigen on endothelial cells was necessary for the binding of cytotoxic antibodies. The different preincubation time required for IFNy or monokines inducing binding of AECA and conferring cytotoxicity in vitro and absorption experiments with IFN_y- or monokine-treated HUVECs suggested that different epitopes were recognized by AECA from KD following different HUVECs stimulation. Similar results were reported in SLE by Moscato et al. [91] who showed that SLE sera and purified IgGs were able to fix complement in vitro leading to deposition of the complement component C3 and disruption of the endothelial monolayer. Noteworthy, differently from early reports on KD sera, this effect was observed in unstimulated HUVECs under basal conditions and suggests that cytotoxic SLE AECA may recognise different epitopes on ECs, which do not require endothelial activation by inflammatory cytokines.

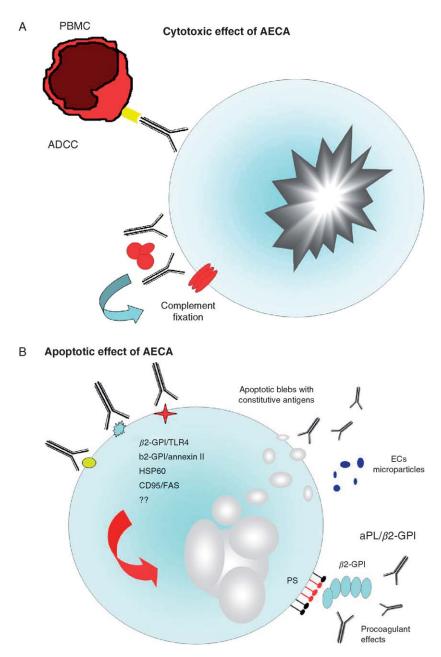
While complement-dependent cytotoxicity may be an important mechanism by which AECA induce endothelial perturbation and cell damage in SLE and systemic vasculitis, the ability of AECA to fix complement and induce complement-dependent cytotoxicity have not been always confirmed in other autoimmune diseases. In particular, reports in the mid- and late 1980s failed to demonstrate the ability of sera from patients with SSc to induce complement-dependent cytotoxicity *in vitro*. However, it has been shown that other mechanisms, namely antibody-dependent cellular cytotoxicity (ADCC) and induction of ECs apoptosis, may account for the pathogenic potential of AECA from SSc and other autoimmune diseases, including SLE and vasculitis.

The ability of AECA to induce ECs damage via an ADCC-like mechanism was originally demonstrated in the 1980s by *in vitro* evaluation of the cytotoxic effect of SSc sera on HUVEC monolayers [92–96] (Fig. 2A). The cytotoxic activity on ECs of either venous or arterial origin was assessed by morphological changes, decreased DNA [³H]thymidine incorporation, reduced fibronectin production, or increased ⁵¹Cr release and was present only in a minority of sera from SSc patients, ranging from 19% to 41%. In some cases, the same sera were also able to induce a cytotoxic effect on dermal fibroblasts [92], an observation which may have relevance in understanding the mechanisms leading to skin fibroblast dysfunction and which can be partially explained by possible cross-reactivity between endothelial

and fibroblast antigens as suggested by the evidence that absorption of purified IgG from SSc sera on dermal fibroblasts greatly reduced reactivity toward ECs [97]. Although some differences in the various reports exist, it is generally convincing that whole sera or purified IgG from SSc patients were not able to induce a cytotoxic effect on ECs per se or in the simple presence of complement, but that the cytotoxic mechanism was dependent on the presence of effector cells in the culture medium [96, 97]. *In vitro* coculture experiments using ECs, SSc sera, and peripheral blood mononuclear cells (PBMCs) demonstrated that the cytotoxicity was dependent on IgG with AECA reactivity and the presence of Fc receptors on the effector cells with a mechanism highly reminiscent of ADCC [93, 94, 96]. The pathogenic relevance of these observations in determining endothelial dysfunction *in vivo* in patients with SSc is still not known; as discussed previously, only a minority of SSc sera display cytotoxic activity *in vitro*.

Similarly, Del Papa *et al.* [98] showed that IgG AECA from patients with WG, can be cytotoxic for ECs in the presence of human normal PBMC but not in the presence of polymorphonuclear leukocytes or adherent mononuclear cells. Nevertheless, it is well known that AECA represent an extremely heterogeneous family of autoantibodies, not only because of the variety of their target antigens, but also for the heterogeneity of their effects [99].

Together with cytotoxicity, apoptosis of ECs mediated by binding of AECA has emerged as a novel mechanism by which AECA may exert a pathogenic role in autoimmune conditions (Fig. 2B). In particular, studies have focused on the apoptotic effect of AECA from SSc patients, since Sgonc et al. [100] provided evidence that ECs in the deeper dermis of patients with SSc undergo apoptosis at very early stages in the course of the disease process, and only in areas characterized by early or prefibrotic skin lesions. These results were also confirmed in an avian model of SSc, University of California at Davis (UCD) lines 200/206 chicken, which spontaneously developed a scleroderma-like disease characterized by perivascular lymphocytic infiltration of the dermis with fibrosis of skin and internal organs and the presence of serum autoantibodies such as antinuclear antibodies, aCL antibodies, and AECA [100-103]. In addition, in most cases, endothelial cell apoptosis was associated with positive staining of microvessels for Ig, supporting the hypothesis that AECA could mediate vascular damage. Sgonc et al. [104] later demonstrated that AECA from a subset of patients with SSc were able to induce apoptosis of HDMECs, but not HUVECs, through ADCC via CD95/Fas, as demonstrated by in vitro inhibition of NK-mediated EC apoptosis with the use of blocking anti-FasL antibodies.





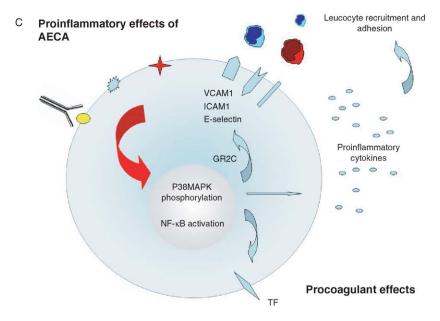


FIG. 2. Pathogenic mechanisms of AECA. The binding of AECA to ECs might be able to induce several effects: (A) cytotoxicity through a mechanism resembling ADCC or mediated by complement fixation; (B) apoptosis through interaction with endothelial adhering or inducible antigens (i.e., β_2 -GPI and HSP 60) indirectly mediated by Toll 4 and annexin II receptor. Moreover, EC apoptosis may be induced directly through interaction of AECA with CD 95/ FAS. As a consequence of EC apoptosis both constitutive antigens in apoptotic blebs and β_2 -GPI–anionic phospholipid complex are exposed to the immune system on the endothelial surface. Moreover, endothelial microparticles may be released from ECs; and (C) proinflammatory and procoagulant effects with overexpression or induction of several adhesion molecules, cytokines, and tissue factor, respectively. β_2 -GPI, β_2 -glycoprotein-I; TLR4, toll-like receptor 4; aPL, antiphospholipid antibodies; PS, phosphatidylserine; ADCC, antibody-dependent cellular cytotoxicity; PBMC, peripheral blood mononuclear cells; TF, tissue factor.

5.2. PROINFLAMMATORY EFFECTS OF AECA

There is convincing evidence to support the notion that AECA are able to induce overexpression of endothelial adhesion molecules, such as E-selectin, ICAM-1, VCAM-1, as well as secretion of proinflammatory cytokines by ECs leading to an upregulation of leukocyte adhesion to ECs (Fig. 2C). This suggests an important role for AECA in contributing to vascular damage in autoimmune conditions such as SLE as well as in other form of vasculitis [105–108]. In particular, AECA from patients with WG induced the secretion of IL-1 β , IL-6, IL-8, and MCP-1 by ECs [109]. Several of these effects may be

related to an autocrine or paracrine action of IL-1 generated by ECs in response to AECA [110]. Moreover, IgM AECA may stimulate endothelin-1 release from HUVECs, thus increasing vascular damage [111] as suggested in preeclampsia [112]. Expression of potential antigens on ECs has been suggested because the treatment of these cells with tumor necrosis factor alpha increased IgG binding from sera of some patients with active SLE [113]. The heterogeneity of the AECA effects was observed by Bordron et al. [99] who clearly showed that AECA from different patients induced activation of ECs, while AECA from others induced apoptosis. The activation of ECs was further validated by Yazici et al. [114], who described proinflammatory effects on live ECs due to a monoclonal IgG AECA generated by hybridoma formation with human SLE B cells. In particular, this mAb bound to a 42-kDa EC cell membrane protein and induced an overexpression of E-selectin and ICAM-1 through NF- κ B activation. Similar findings were observed with mAb AECA from a patient with TA, which bound and activated macrovascular ECs but not microvascular ECs [115] and with mAb from a patient with WG [116]. The proinflammatory phenotype of ECs due to binding of AECA may also be dependent on the origin of ECs. Affinitypurified AECA F(ab)₂ from patients with thrombotic thrombocytopenic purpura (TTP) bound to and activated only microvascular ECs but not large vessel ECs [117]. On the contrary, in uremic patients undergoing hemodialysis AECA reactivity with HUVECs but not with microvascular or EaHy 929 EC lines was observed [118].

Carvalho et al. [110] were the first to report that AECA from SSc patients are able to functionally affect in vitro interactions between ECs and leukocytes by increasing adhesion of the myelomonocytic cell line U937 to cultured HUVECs. This effect was reproducibly mediated by both sera as well as purified IgG from AECA positive SSc patients binding to ECs and related to de novo increased expression of selectins and adhesion molecules (i.e., E-selectin, ICAM-1, and VCAM-1) by HUVECs. In this in vitro system, leukocyte-endothelial cell interactions were not dependent on Fc engagement on U937 cells since Fab fragments also induced leukocyte adhesion with a similar efficacy compared to whole IgG. However, as suggested by the kinetics of leukocyte adhesion and adhesion molecule expression, the effect of AECA was dependent on the direct stimulation of soluble mediator(s) from HUVECs since the ability of inducing leukocyte adhesion to ECs was maintained using IgG-depleted AECA-conditioned HUVEC medium. Inhibition experiments using a cocktail of blocking antibodies suggested that the effect AECA was, at least in part, mediated by stimulation of cytokine production, and in particular IL-1, from HUVECs in vitro. Although an exhaustive proof of the ability of AECA from SSc patients to directly induce cytokine production in HUVECs was missing in this study, AECA from other rheumatic conditions (i.e., WG as reported previously) have been shown to be to upregulate cytokine expression from ECs *in vitro* [109].

5.3. AECA AND THROMBOTIC VASCULAR INVOLVEMENT

The role of aPL and AECA in the pathogenesis of APS is now well investigated. Since the first description of AECA, it was evident that antibodies directed against negatively charged phospholipids can explain part of the AECA reactivity [119]. In fact, absorption with cardiolipin liposomes partially inhibited AECA reactivity [119, 120]. Thus, it is likely that AECA directed against aPL may be involved in thrombotic diathesis via more than one mechanism. First, these antibodies may activate ECs through apoptotic or nonapoptotic mechanisms [121] (Fig. 2B and C); second, these autoantibodies may induce significant increases in tissue factor (TF) transcription and expression by ECs [122], as well as by monocytes [123, 124].

Incubation of EC with IgG, from patients positive for aPL, increased the expression of VCAM-1, E-selectin, and MCP-1 as previously reported for AECA [125]. Studies have shown that upregulation of adhesion molecules on ECs by aPL correlates with an increased adhesion of leukocytes to ECs in mouse microcirculation and with enhanced thrombosis *in vivo* [126]. Pretreatment of ECs with fluvastatin further increased the expression of EC activation as well as TF expression [125], and pretreatment with aspirin decreased VCAM-1 at the cell surface of ECs [127]. On the contrary, a previous study showed that fluvastatin was able to inhibit the expression of TF expression on ECs induced by aPL from patients with APS as well as by affinity-purified polyclonal IgG-aPL and IgM human monoclonal anti- β_2 -GPI antibodies [128, 129]. Moreover, fluvastatin in a mouse model significantly diminishes aPL-mediated thrombosis and EC activation *in vivo* [130].

A novel pathway for EC activation induced by aPL/anti- β_2 -GPI antibodies may be due to the cross-linking or clustering of annexin A2 on the endothelial surface [121].

Dieude *et al.* [3] showed that AECA from SLE patients with APS bind to the surface of ECs and share reactivity against a 60-kDa EC surface polypeptide that was identified as human HSP60. Moreover, the incubation of ECs with purified anti-HSP60 antibodies induced apoptosis as demonstrated by the exposure of the phosphatidylserine on the plasma membrane [131]. Although AECA with this reactivity were not exclusive to SLE patients an intriguing association was observed between these antibodies and LAC. This may represent the first pathogenic hit for induction of AECA and for endothelial perturbations followed by a second hit represented by aPL antibodies binding to ECs. In line with these results anti-HSP60 purified from patients with coronary-artery disease may cause apoptosis of nonstressed ECs; this can be considered a primary event in the pathogenesis of atherosclerosis [132]. According to this hypothesis, Chen *et al.* [133] showed that aPL binding to ECs occurs only after activation of these cells. Worda *et al.* [134] reported evidence of *in vivo* binding of AECA to the microvascular endothelium associated with a significant increase in EC apoptosis. In line with these evidences, AECA might induce an endothelial perturbation with the release of endothelial microparticles that were described in sera from patients with APS [135]. Taken together, these findings support the idea that AECA may be pathogenic and may be able to induce aPL production. The effects of aPL on ECs may be mediated by an overexpression of TF. In line with this hypothesis, Vega-Ostertag *et al.* [122] showed that treatment with IgG from patients with aPL induced significant increases in TF transcription, expression, and activity in HUVECs and was mediated by the phosphorylation of p38 MAPK and activation of NF- κ B.

AECA represent a heterogeneous family of autoantibodies directed to several antigens, including partially overlapping specificities. Several findings suggest β_2 -GPI as the most relevant antigen of APS also showing AECA reactivity [136]. β_2 -GPI is a plasma protein able to bind to anionic phospholipids; this apolipoprotein represents a cofactor required for the generation of the antigenic epitopes recognized by some aPL. Pure anti- β_2 -GPI antibodies have been also detected [137–139]. The binding of β_2 -GPI to several cells, such as apoptotic thymocytes, trophoblast cells, macrophages, platelets, and ECs, has been previously reported [140]. In particular, the immunoreactivity of β_2 -GPI has been largely investigated in ECs but whether this reactivity is due to plasma adhesion or to a direct intracellular synthesis of the glycoprotein by ECs is still debated. The disappearance of β_2 -GPI immunoreactivity has been described following extensive washing of HUVECs or culture in serum-free medium [141]. Interestingly, such a phenomenon was not found using primary human EC culture from skin or brain microcirculation suggesting the capability of these cell types to synthesize β_2 -GPI [141]. In this regard, our group clearly demonstrated β_2 -GPI mRNA expression in ECs cultured both in the absence and presence of β_2 -GPI in the culture medium [140]. It is conceivable that ECs constitutively express β_2 -GPI mRNA, since culture in the absence of β_2 -GPI for several days does not influence β_2 -GPI mRNA synthesis in ECs. This finding is also supported by the observation that in ECs β_2 -GPI is located and accumulates in late endosome [142, 143]. The same results were observed for astrocytes, neurons, and human monocytes [124]. There is evidence that β_2 -GPI is also expressed in vivo on trophoblast vessels in term placentas as well as in circulating monocytes [124]. The immunoreactivity of β_2 -GPI expressed by

several cells involved in the photogenic scenario of APS may be due, in part, to a direct β_2 -GPI synthesis. On the other hand, it is possible to speculate that the β_2 -GPI synthesis may be increased *in vitro* by stress conditions, such as serum depletion, from the culture medium. In fact, it is important to point out that plasma protein, such as β_2 -GPI may also be absorbed on the cell membrane directly or through an endothelial receptor(s). In line with such a hypothesis, it has been suggested that β_2 -GPI might interact with anionic EC membrane structures such as heparan sulfate (HS), annexin A2, and apolipoprotein E receptor 2 (reviewed in Ref. [144]). Although a previous study showed a minimal influence of β_2 -GPI for AECA reactivity [120], to date it is well accepted that β_2 -GPI on ECs may be a target for AECA. Raschi et al. [145] clearly demonstrated that both human monoclonal IgM antibodies anti- β_2 -GPI as well as affinity-purified polyclonal anti- β_2 -GPI induce phosphorylation of the IL-1 receptor activated kinase (IRAK) and suggest that β_2 -GPI might interact with the TLR4 because of his homology with microbial structures.

6. Conclusions

AECA represent a heterogeneous family of autoantibodies directed to several antigens, including partially overlapping specificities. Despite the fact that most studies have shown a higher prevalence of AECA in autoimmune diseases, the clinical relevance of these autoantibodies is still a matter of debate and need to be confirmed by longitudinal studies on a large number of patients. Nevertheless, there are many evidences about the pathogenic role of AECA in SLE, APS, SSc, and vasculitis. Moreover, in the future, evaluation of AECA using as substrates ECs from macro- and microvasculature may be important to assess more precisely AECA reactivity in the various forms of autoimmune diseases. For these reasons, we believe that a more precise definition of the AECA role in autoimmune diseases has to be included in the research agenda.

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