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Anti-Endothelial Cell Antibodies in Systemic Sclerosis

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Systemic sclerosis (SSc), or scleroderma, is characterized by early vascular endothelial cell (EC) damage, followed by cutaneous and visceral fibrosis. There is substantial interest in the role of anti-EC antibodies (AECA) in SSc as recent findings have brought new insights into the understanding of the pathogenicity of such autoantibodies.

A number of methods have been used to detect AECA including indirect immunofluorescence (IIF), cell enzyme-linked immunosorbent assay (ELISA), radioimmunoassay, and Western blotting (WB). The cell substrates used include EC derived from different origins, viz arteries and human umbilical veins and microvessels, as well as endothelial lineage cells (the prevalence of AECA detected in SSc varies from 28 to 85% of the samples tested). Given the differences in the tests and cell origins, the results vary from one study to another. These autoantibodies are not specific for SSc. Yet, assuming that SSc affects mainly microvessels, one may predict that microvascular ECs reflect the in vivo situation more reliably than other cell substrates.

Some AECA-containing sera from patients with SSc generate complement-mediated or antibody-dependent cellular cytotoxicity (ADCC). Incubation of EC with AECA also makes some changes in the functions of the cells. These include increased production of cytokines, such as interleukin-1 (IL-1) and IL-6; enhanced expression of adhesion molecules, and induction of a procoagulant stage. Furthermore, EC-reactive antibodies sustain leukocyte adherence to ECs, and an antibody subset may be capable of initiating apoptosis of ECs.

In addition, apoptosis of ECs is an early finding in animal models of spontaneous disease. As demonstrated with humans (but not with mice), this apoptosis may be initiated by AECA. Clearly, the detection of AECA deserves much attention in the diagnosis and treatment of SSc. Identification of the epitopes targeted by these antibodies is currently in progress. Such studies would probably be helpful for monitoring patients with SSc.

SSc occurs as a continuum from benign primary Raynaud's phenomenon through severe diffuse disease. The disease is thus multifactorial in nature, and indeed the pathophysiology of such a nonorgan-specific autoimmune condition, referred to as "the mosaic of autommunity" (51), involves genetic, environmental, hormonal, and immunologic features (8). However, the precise mechanism of the initial microvascular injury remains largely unknown. Unequivocally, the earliest changes consist of EC damage. Then, intimal proliferation and vascular wall thickening occur to different organs, along with mast cell infiltration and cutaneous as well as visceral fibrosis (44).

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Over the recent years, several investigators have claimed that the production of AECA is relatively common in patients with SSc, although the evidence has long been lacking that such autoantibodies are pathogenic (36). Patients with SSc have also been found to produce antibodies that react with two specific cellular components: the kinetochore, targeted by anticentromere antibodies in a number of patients with limited SSc, including the CREST syndrome (calcinosis, Raynaud's phenomenon, esophagal dysmotility, sclerodactyly, and telangiectasias), and the topoisomerase I recognized by anti-Scl70 antibodies in certain patients with diffuse SSc (8). It is noteworthy that AECA are significantly associated with antiphospholipid (PL) antibodies (aPL) in a number of connective tissue diseases.

This review summarizes the current knowledge on the association of AECA with SSc and analyzes the potential role of these antibodies in the development of the disease among a subgroup of patients. The vascular endothelium (which presents as a continuous monolayer) is in close contact with flowing blood components and is endowed with numerous functions, such as inflammation, thrombosis-hemostasis, lymphocyte migration, and wound healing (29). In this respect, several effects of AECA are now being unraveled with the benefit of very recent data.

THE PREVALENCE OF AECA IN SSc

The initial discovery that led to the characterization of AECA was the demonstration that systemic rheumatic disease sera were reactive with capillaries (33, 54). Such autoantibodies were described by IIF of EC with mouse and rat kidney and liver as substrates. Subsequent reports (reviewed in reference 37) were based on the use of human umbilical vein EC (HUVEC) in culture or HUVEC membranes to develop specific ELISAs. These reports confirmed the results of the pioneering IIF studies. The presence of AECA can also be demonstrated by flow cytometric analysis but this method requires a large number of EC in suspension (58).

Several investigators have reported detecting AECA in cases of SSc. The prevalence of the antibodies reported in these studies (Table 1) varies from 28 to 85% of the samples tested (6, 22, 26, 34, 36, 47, 48). The discrepancies in the detection of AECA may be ascribed to differences in patient selection and in the tests used in different laboratories. A crucial need for standardization of the AECA test has therefore been acknowledged (60). Though a consensus on the prevalence of AECA has not been reached, there is no disease specificity associated with AECA (reviewed in references 1 and 61). The proportion of AECA-positive sera ranges from 15 to 88% in systemic lupus erythematosus (SLE), from 0 to 87% in rheumatoid arthritis (RA), and from 19 to 81% in Wegener's granulomatosis (WG). AECA can also be found in cases of other diseases, such as diabetes mellitus, coronary artery disease following cardiac transplantation, and Kawasaki's disease. In our previ-

TABLE 1. Prevalence of AECA in SSc

Assay, cell substrate	% Serum samples positive (no. of serum samples tested)	Reference
Cell-ELISA		
HUVEC	43 (57)	22
HUVEC	30 (30)	47
HUVEC	71 (42)	6
HUVEC	40 (50)	43
HUVEC	28 (76)	39
EA.hy 929	55 (81)	48
Immunoblot		
HUVEC	85 (20)	26

ous study of SSc patients, we assumed that the cutoff value was the mean of normal sera plus three standard deviations, and there were virtually no positive results in the controls (48). This lack of disease specificity suggests that several autoantibody subsets are identified by the AECA test. One or several of these subsets may be specific for SSc. Alternatively, AECA may characterize a subgroup of SSc patients at higher risk of developing vascular crises.

Importantly, major differences in functional and antigenic characteristics are associated with EC derived from various organs. Page et al. (40) have shown that vascular EC in different anatomic compartments of the liver, lung, and kidney express different patterns of surface antigens. Furthermore, capillary endothelium is phenotypically different from the EC lining large vessels, suggesting that it may be more efficient at antigen presentation and more susceptible to immune attack in vivo. EC have also been eluted from arteries, umbilical veins, and capillaries from different organs and have been used as the substrate to capture the related autoantibodies. To overcome the variation in EC phenotype and numbers, permanent cell lines have been established: for example, EA.hy 929 cells result from a fusion between HUVEC and the permanent epithelial cell line A 549/8 (19). Another attractive project aims at establishing EC clones in the hope that these would differentially express specific cell surface markers as potential targets for AECA. We have previously reported the successful establishment of several clones that appeared to be slightly different from each other (32).

To determine the importance of the EC origin in the detection of AECA in SSc, we have tested nonselected sera from SSc patients by using cellular ELISAs. The prevalence of antibodies against microvascular EC was significantly higher than that of the antibodies against HUVEC. The respective AECA were not completely overlapping populations because the global autoantibody prevalence was increased when the results obtained with microvascular EC were combined with those obtained with HUVEC (121 and 74 of 477 serum samples from patients with SSc were respectively found to be positive, i.e., 25 and 16%). This finding suggests that the variety of AECA reflects the heterogeneity of the disease presentations (46a).

AUTOANTIGENS

WB of EC extracts or immunoprecipitated membrane structures was developed to identify antibodies reacting with electrophoresed preparations of EC. AECA were claimed to recognize antigens that appeared as multiple bands ranging in molecular mass from 20 to 200 kDa, and the nature of these target antigens awaits elucidation (11, 56, 57). WB patterns have been claimed to be disease specific for SLE (56), RA (57), and WG (37, 60), but the results vary from one study to another. Identification of target antigens deserves further study.

A variety of autoantibodies may, in fact, bind to EC through electric charges but their contribution to the EC reactivity is considered negligible. These include anti-double-stranded (ds) DNA (7), aPL (13, 18), and anti-heparan sulfate antibodies (46). AECA may also react with the extracellular matrix or the glycosaminoglycan part of extracellular receptors (16). Most investigators agree that AECA and antineutrophil cytoplasmic antibodies (ANCA) are two distinct antibody populations, and indeed, after depletion of ANCA, Damianovich et al. (10) were able to show in a murine model that the immunoglobulin G (IgG) fraction from a patient with WG could display AECA but not ANCA. All of these autoantibodies may have EC-binding properties, though there are no overlapping antigenic targets.

PATHOGENIC ROLE OF AECA IN SSc

Clinical and biological associations. The pathogenicity of AECA in SSc remains uncertain. Some autoantibodies might merely be an epiphenomenon of vascular injury (low-affinity AECA), while others might be pathogenic (high-affinity AECA). Although there are no follow-up studies showing that AECA levels fluctuate with disease activity in individual patients, the likelihood of such pathogenicity of AECA is suggested by the relationship between the autoantibodies and disease severity. Clinically, AECA are correlated with the extent of vascular involvement and subsequent complications: 23% of the serum samples tested were thus found to be AECA positive in primary Raynaud's phenomenon, 44% were positive in limited SSc, and 84% were positive in diffuse SSc (48). A significant association was also found between AECA and the parameters reflecting alveolo-capillary impairment (43). IgG-AECA has recently been confirmed to be an important marker for disease severity in SSc (39), since patients with AECA had significantly higher incidences of digital infarcts, gangrene, and pulmonary arterial hypertension than those without AECA. More interestingly, in the CREST syndrome, a subset of AECA binding to an unidentified component was shown to be associated with anticentromere activity (26). Hill and colleagues were able to find antibodies to an 18- to 19-kDa membrane epitope in 11 of 20 SSc patients but not in the controls. These autoantibodies were associated with CREST syndrome (nine of nine serum samples were positive) and, after elution, were shown to possess anticentromere activity. Hill et al.'s interpretation of these results was that membrane-reactive antibodies, including anticentromeric antibodies, may play a central role in the pathogenesis of SSc through their ability to react with EC (26). This binding has not been confirmed by other investigators.

Cytotoxicity. Nineteen to 75% of the serum samples from SSc patients has been shown to be cytotoxic for EC (Table 2). Intriguingly, this cytotoxic effect was not restricted to EC, inasmuch as some studies (34), but not all (31), reported that fibroblasts were also susceptible. Within the population of AECA, a subset of autoantibodies might recognize fibroblasts. An alternative view is that AECA are not directed against endothelial-specific antigens. Cytotoxicity in SSc was attributed by early investigators (31) to a protease-like factor but this mechanism has not been confirmed. Several mechanisms of AECA cytotoxicity have been established. AECA have been reported to fix complement in vitro in SLE and WG patients but not in patients with SSc (reviewed in references 1, 36, and 37). In vitro, AECA induce secretion of chemotactic cytokines and expression of adhesion molecules which encourage the recruit-

TABLE 2. Serum-mediated endothelial cell cytotoxicity in SSc

Target cells	% Serum samples positive (no. of serum samples tested)	Reference
HUVEC	50 (52)	31
HUVEC	39 (36)	9
Lung artery EC	41 (36)	9
HUVEC	23 (30)	38
HUVEC	20 (39)	41
HUVEC	23 (43)	17
Dermal microvascular EC	19 (26)	34
Aortic EC	75 (4)	34
HUVEC	20 (48)	27
Umbilical artery	27 (28)	27

ment and adherence of leukocytes (6). Alternatively, in vitro studies (12) have shown the capacity of patient sera to induce ADCC on human endothelial monolayers. However, it is noteworthy that AECA-mediated ADCC has also been described in SLE (42) and WG (14). Control studies using sera from normal volunteers and patients with either diabetes or extensive athero-sclerotic vascular disease failed to reveal any similar cytotoxicity (41).

Production of cytokines and expression of adhesion molecules. Serum samples from patients with SSc have been reported to contain elevated amounts of cytokines, including IL-1, IL-2, IL-4, and IL-6. Given that HUVEC produce proinflammatory and chemoattractant cytokines (such as IL-1β and IL-8 and monocyte chemotactic protein 1, respectively), in the presence of AECA, their release may reflect activation of EC by AECA, at least in WG, and it may be speculated that, in return, these cytokines amplify the activation of EC (12). Furthermore, cytokines stimulate fibroblast proliferation and collagen synthesis, which is the hallmark of the disease. Curiously enough, anticytokine autoantibodies have been described in SSc, e.g., anti-IL-6 and anti-IL-8 antibodies. However, the link between these autoantibodies and AECA remains a matter for speculation (45).

The expression of adhesion molecules is elevated in the skin of patients with SSc of recent onset (52). The adhesion molecules are also shed in the circulation (21, 24) of patients with SSc. Adhesion molecules such as intercellular adhesion molecules (ICAM)-1, -2, and -3, vascular cell adhesion molecules (VCAM)-1, and E-selectin are involved in the binding of leukocytes and their diapedesis to the perivascular area during any inflammatory response. This binding of leukocytes may be reproduced in vitro since pretreatment of HUVEC with AECA leads to an increased expression of adhesion molecules (12). The view that this is, in part, due to an autocrine action of IL-1 is supported by the fact that anti-IL-1 antibodies reduce the adhesion to EC (6).

Interaction of EC with fibroblasts. In culture with EC the function of fibroblasts is downregulated, as substantiated by the reduction of collagen synthesis (15). Fibroblast dysfunction may be incriminated in abnormal fibrosis and collagen production. Interaction between EC and fibroblasts might be mediated by soluble factors produced by EC, e.g., transforming growth factor β, which is elevated in the circulation of SSc patients (30), or, given that some target antigens are shared by EC and fibroblasts, this interaction may be due to activation of EC and fibroblasts by AECA (26). As suggested by Marks et al. (34), AECA-induced activation may result in pathologic changes such as fibrosis, which has been attributed to abnormalities of fibroblast numbers and function. The possible role of AECA in this interaction is far from being understood.

Synthesis of coagulation factors. Increased concentrations of von Willebrand factor (vWf), thrombomodulin (TM), and tissue plasminogen activator as well as reduced activity of the angiotensin-converting enzyme reflect an endothelial injury (25, 35). Owing to their release by EC, plasma levels of vWF and TM correlate to the extent of visceral involvement and, therefore, to the disease prognosis (2). Electron microscopy study of skin biopsies has shown that the number of vWf-containing Weibel-Palade bodies is reduced (44). The view that AECA are involved in this process is supported by the emergence of procoagulant characteristics following activation of EC with AECA (23). For example, IgG from patients with thrombosis affects prostacyclin production by EC, suggesting that the thrombophilic diathesis might be due to an imbalance between the production of prostacyclin by EC and that of thromboxane by platelets (5). Some mechanisms of EC activation have, however, been observed only in vitro or in animals.

Conceivably, intensified coagulation and fibrin clot formation in SSc is due to tissue factor (TF) exposure by activated EC. TF interacts with factor VII on the surface of EC, and the resulting complex activates factor X. In the presence of factor Va, this complex cleaves prothrombin into thrombin. The latest factor is the primary serine protease that activates platelets, changes fibrinogen into fibrin, and favors the formation of the fibrin clot. Expression of TF by EC is increased by several factors such as IL-1, tumor necrosis factor alpha (TNF-α), and endotoxin but also by sera from patients with AECA. Antibody and immune complexes induce TF production by human EC (55). Although other investigators (23) failed to detect any direct effect of purified IgG AECA on TF expression, they reported that purified IgG from AECA-positive SLE patients induced the expression of TF by HUVEC preincubated with low doses of TNF-α.

Induction of apoptosis. The recent finding that EC apoptosis is a primary pathogenic event underlying skin lesions in SSc (49) might be relevant to the pathogenesis of AECA. We previously reported the first evidence that six of eight AECA-positive serum samples from patients with SSc were capable of initiating programmed cell death (3). The exposure of anionic PL, such as phosphatidylserine (PS), is one of the early characteristic of cells undergoing apoptosis. This was not observed with sera which were negative for AECA or with sera with aPL, anti-dsDNA, anti-ribonucleoprotein, and rheumatoid factor (RF) antibodies. In addition, antibodies to surface molecules on EC, including protein S, ICAM-1, lymphocyte function antigen 3, VCAM-1, major histocompatibility class I, and TM, failed to induce apoptosis. The apoptosis triggered by AECA was both concentration and time dependent and did not rely on Fc gamma receptor interactions. Assuming this to be the case with AECA-treated EC, β_2 glycoprotein (β_2GPI) would be expected to interact with the cells. By using two mouse AECA monoclonal antibodies, human β_2GPI and aPL, we have obtained data (4) which indicate a mechanism by which some AECA may be pathogenic, and we hypothesize that AECA may even have the potential to induce the production of aPL. Twenty to 36% of the cells expressed anionic PL following incubation with AECA, as revealed by the binding of annexin V and β_2GPI to PS. Interestingly, bound aPL resided exclusively within the AECA-positive EC population and PS-positive cells were restricted to those targeted by AECA.

ANIMAL MODELS OF SSc

Animal models are invaluable for monitoring the sequence of pathological changes of a disease. They are also useful for assessing the effects of new drug regimens. Since no ideal

animal model for SSc is currently available, a few experimental systems have been proposed. These models are for either spontaneous or induced disease.

Spontaneous disease models. Several animal models of spontaneous scleroderma exist. Skin biopsies from the tight-skin mouse display excessive collagen deposition. However, inflammatory and immunological features, which are prominent in patients, have not been demonstrated in this mouse model.

The chicken strains UCD-200 and 206 spontaneously develop a scleroderma-like disease after hatching, with abnormalities in their comb at the age of 1 to 2 weeks followed by peripheral polyarthritis and dermal lesions by 6 weeks of age. These chicken generally die from cutaneous infection at 2 to 4 months of age. Serological studies reveal RF, antinuclear, and anti-collagen II antibodies. Using this model, Sgonc et al. (49) have demonstrated that EC were the first cells to undergo apoptosis in situ and hypothesized that AECA might have triggered apoptosis of these EC. Subsequent to this interaction, lymphocytes infiltrate the dermal layer and interact with EC, while the level of vWF is raised. Thus, lymphocyte-EC interaction is probably not a major initiating event in SSc.

Induced disease models. A handful of animal models for induction of SSc were established by injection of lymphoid cells into rats (53) and injection of urine glycosaminoglycan from SSc patients (28), bleomycin (20), or organic solvents into mice (59). All these animals suffer, in fact, from limited manifestations of the disease.

To the best of our knowledge, AECA-induced disease has not been documented in SSc thus far. Idiotypic manipulations could, therefore, be a fruitful tool, inasmuch as different autoimmune diseases have previously been induced that way. These diseases include SLE and the aPL syndrome (50). Human AECA have been utilized to induce vascular injury in mice (10). This result was the first direct proof for the pathogenicity of AECA as vasculitis-like lesions were produced in mouse lungs and kidneys. The interpretation of these findings was that AECA bound to EC induced a series of cellular modifications that were essential for lymphocyte activation and for induction and/or maintenance of the inflammatory process seen in vasculitis.

In conclusion, the vasculitic process observed in SSc involves at least three cell types: EC, fibroblasts, and lymphocytes. In vivo, EC are activated, as suggested by augmented levels of cytokines, increased expression of adhesion molecules, abnormal synthesis and release of coagulation factors, and noticeable fibrotic reactions. These phenomena can be reproduced in vitro by using AECA-positive sera. Early on, activation is associated with the presence of AECA, the titer of which correlates with the severity of the disease. Given that AECA are not found in all SSc patients, these autoantibodies may be operative only in a distinctive subgroup of patients and/or at a particular stage in the disease process, presumably the early stage.

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