Putative functional pathogenic autoantibodies in systemic sclerosis

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Abstract

Systemic sclerosis (scleroderma, SSc) is a systemic disease characterized by vascular lesions, fibrosis, and circulating autoantibodies. A complex interplay between innate and adaptive immunity, and with regard to the latter, between humoral and cellular immunity, is believed to be involved in SSc pathogenesis. Lately, close attention has been paid to the role of B cells which, once activated, release profibrotic cytokines, promote profibrotic Th2 differentiation, and produce autoantibodies. Several novel interesting autoantibodies, targeting antigens within the extracellular matrix or on the cell surface, rather than the nuclear antigens of canonical SSc-autoantibodies, have been recently described in patients with SSc. As they show stimulatory or inhibitory activity or react with structures involved in the pathogenesis of SSc lesions, they can be considered as potentially pathogenic. In this paper, we will review those which have been better characterized.

Keywords: Systemic sclerosis, autoantibodies, scleroderma, platelet-derived growth factor, endothelial cells

Introduction

Systemic sclerosis (SSc) is a chronic autoimmune disease characterized by vascular alterations, progressive and extensive fibrosis, and the presence of circulating autoantibodies directed to several cellular and extracellular autoantigens (1). The pathogenic mechanisms involved in SSc are unclear, despite the recent progress in the treatment of its complications (2). Several genetic susceptibility loci, encoding cytokines, cytokine receptors, chemokines, and extracellular proteins have been reported to be associated with SSc (3-6), as well as environmental factors (7) and infectious agents (8). The presence of autoantibodies is one of the most common manifestations in SSc, being observed in more than 90% of patients (9). Several autoantibodies have been recognized for their value in the diagnosis of SSc, clinical subset classification, and for predicting organ involvement. Antibodies to DNA topoisomerase I (ATA), also known as anti-Scl-70, are associated with diffuse cutaneous SSc (dcSSc), severe fibrosis, interstitial lung disease, and digital ulcerations.

Anti-centromere antibodies (ACA) are most commonly associated with limited cutaneous SSc (lcSSc), and may be associated with pulmonary arterial hypertension (PAH). Anti-RNA polymerase III is also an important biomarker, associated with severe accelerated dcSSc, risk of scleroderma renal crisis, gastric antral vascular ectasias and malignancy (10, 11).

Other autoantibodies, including anti-polyomysis scleroderma (PM-Scl), anti-fibrillar (U3 RNP), and anti-Th/To ribonucleoprotein (Th/To) are sometimes associated with overlap myositis and severe lung disease characterized by both fibrosis and vascular diseases.

Although the diagnostic and prognostic value of ATA, ACA, and other antibodies is clear (12), their role in the pathophysiology of vasculopathy, tissue fibrosis, and organ dysfunction has not been completely elucidated.

Autoantibodies can be considered pathogenic when they contribute to the development of an autoimmune disease and mediate the disease manifestations (13). Functional autoantibodies are pathogenic when they bind to their cognate autoantigen, stimulating (agonistic effect) or inhibiting (antagonistic effect) a specific molecular pathway.
Several criteria that should be fulfilled in order to demonstrate the putative pathogenicity of autoantibodies have been proposed (14). In general, the experimental evidence should derive from both in vitro and in vivo studies. The autoantibodies in question should be specific to the disease, precede its development, and cause disease manifestations when introduced into a healthy subject (or an experimental animal model).

In this review, we will discuss the existing evidence regarding the putative pathogenicity of autoantibodies in SSc.

Autoantibodies against endothelial cells

Anti-endothelial cell antibodies (AECA) have been identified in the sera of patients with autoimmune and connective tissue diseases as well as in patients with diabetes mellitus, multiple sclerosis, and pre-eclampsia (15). Although AECA are not specific to SSc, several studies have reported their association with lung and vascular involvement in patients with SSc (16-18), and the capacity of immunoglobulin G (IgG) from AECA-positive patients with SSc with PAH to induce activation in vitro of human umbilical vein endothelial cell (HUVEC) with higher expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin; and the production of interleukin (IL)-6, IL-8, and C-C motif chemokine ligand 2 was considered as evidence for the role of AECA to cause vascular damage and inflammation. In vivo, transfer of AECA-positive serum from University of California at Davis Line 200 chickens, an animal model of human SSc, but not AECA-negative serum, resulted in induction of endothelial cell apoptosis in healthy chicken embryos (19).

The interpretation of this study, as of others discussed below, is not immediate because of the experimental use of total serum IgG that includes a multitude of different antibodies targeting many different antigens.

The exact nature of the antigen targeted by AECA has been elusive, preventing a precise identification of the mechanisms involved in the vascular damage occurring in SSc. Hill et al. (20) reported that the antibody reacting with the HUVEC membrane possesses anti-cen tromere activity, and Servettaz et al. (21), using a quantitative immunoblotting technique, showed that centromere protein B may be the main target of AECA in patients with lcSSc. Interestingly, Lunardi et al. (22) reported the presence of circulating antibodies recognizing the human cytomegalovirus (CMV) late protein UL94 in patients with SSc. These antibodies induced endothelial cell apoptosis in vitro by cross reacting with the cell surface tetraspan in transmembrane 4 superfamily member 7 (Nag-2) molecule, suggesting a link between CMV infection and anti-EC humoral immunity. The same group subsequently showed that Nag-2 is also expressed on dermal fibroblasts and that anti-Nag-2 antibodies, upon binding to fibroblasts, induced upregulation of 989 transcripts including genes involved in extracellular matrix (ECM) deposition and encoding growth factors, chemokines and cytokines (23). Vascular damage, fibrosis, and autoantibodies were, thus, connected. No evidence has been so far provided in experimental animals that these mechanisms are active in vivo.

Another potential pathogenic role of AECA is their ability to induce apoptosis in human dermal microvascular endothelial cells, but not in HUVEC, in the presence of activated NK cells via the Fas pathway (24).

It is also worth recollecting the report on anti-ICAM-1 antibodies in 32% of patients with dcSSc and 39% of patients with lcSSc by enzyme-linked immunosorbent assay. Interest ingly, the exposure of HUVEC to anti-ICAM-1 antibodies induced increased generation of reactive oxygen species (ROS) and VCAM-1 expression (25). All these findings suggest that AECA also include antibodies targeting ICAM-1, which are responsible for pro-inflammatory activation of HUVEC, and thus may contribute to SSc vascular lesions.

A general mechanism by which AECA may cause SSc lesions linking endothelial cell apoptosis and fibrosis has been proposed (26). Apoptotic endothelial cells would release soluble mediators responsible for the induction of an anti-apoptotic phenotype in fibroblasts. Besides resistance to apoptosis, dermal fibroblasts would acquire a myofibroblast phenotype that constitutes the cellular basis of a persistent pro-fibrotic response. Interestingly, in the same study, human fibroblasts derived from SSc skin lesions were found to be more sensitive to the anti-apoptotic activities of mediators produced by apoptotic endothelial cells than normal fibroblasts. The molecular pattern of resistance to apoptosis in fibroblasts was reproduced by a synthetic peptide containing an endothelial growth factor (EGF) motif present on the C-terminal fragment of perlec an. Thus, persistent apoptosis of endothelial cells would induce and maintain fibrosis in SSc.

Autoantibodies against angiotensin II type 1 receptor and endothelin-1 type A receptor

Angiotensin II type 1 receptor (AT1R) and endothelin-1 type A receptor (ETAR) are widely expressed on cells of the vascular system and on immune cells. Functional anti-AT1R autoantibodies were first isolated from the sera of women with pre-eclampsia, and their agonistic activity was demonstrated by the chronotropic effect induced on cultured, spontaneously beating, neonatal rat cardiomyocytes, which was completely inhibited by the selective receptor antagonist losartan (27, 28). Subsequent studies showed that anti-AT1R autoantibodies stimulate AT1R on several cell types, inducing biological responses relevant to the pathophysiology of vascular diseases, such as malignant hypertension, renovascular diseases, and renal allograft rejection (29, 30). Anti-ETAR autoantibodies were first identified in sera from patients with idiopathic PAH (31).

Later on, anti-AT1R and anti-ETAR were also identified in patients with SSc. Using a solid-phase assay, Riemekasten et al. (32) found anti-AT1R and anti-ETAR antibodies in about 85% of patients with SSc. The antibody levels strongly correlated with each other and showed cross-reactivity for both receptors. At the clinical level, higher levels of anti-AT1R and anti-ETAR antibodies were associated with severe SSc vascular manifestations such as digital ulcers and PAH (32). Becker et al. (33) subsequently reported that anti-AT1R and anti-ETAR antibodies were more frequent in PAH associated with SSc or other connective tissue diseases compared with other forms of pulmonary hypertension and might serve as prognostic and predictive biomarkers for cardiovascular complications and mortality.

Interestingly, these autoantibodies-induced extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) phosphorylation and increased the expression of transforming growth factor beta (TGFβ) in human dermal microvascular endothelial cells (32). Furthermore, when exposed to anti-AT1R and anti-ETAR antibodies, human microvascular endothelial cells showed evidence of increased expression of IL-18 and
VCAM-1 (34). In the subsequent studies, the presence of AT1R and ETAR was also described on human peripheral T cells, B cells, and monocytes, and their interaction with IgG from patients with SSc was responsible for increased production of IL-8 and the CC-chemokine ligand 18 (35).

In vivo, repeated intravenous administrations of total IgG from patients with SSc (positive for anti-AT1R and anti-ETAR antibodies) induced thickening of airway vessels and elevated the cell density in interstitial tissue in healthy mice. Moreover, increased neutrophil count was found in the bronchoalveolar lavage of SSc IgG-treated mice as compared with HC IgG-treated mice, whereas no differences were observed for macrophages or lymphocytes (34).

Although interesting, these studies did not evaluate collagen expression and, more importantly, the total SSc IgG preparation that was administered might contain other agonistic antibody specificities in addition to the anti-AT1R and anti-ETAR.

Autoantibodies against muscarinic-3 receptor
Gastrointestinal (GI) involvement leading to dysmotility is frequent in SSc as a result of disturbance of cholinergic neurotransmission and smooth muscle atrophy. Acetylcholine secreted after stimulation of the muscarinic-3 receptor (M3R) is the principal excitatory mediator of GI tract motility acting on intrinsic neurons in the myenteric plexus. Antibodies blocking M3R would, therefore, inhibit excitatory enteric neurotransmission causing dysmotility.

Following this hypothesis, preliminary studies found a high prevalence of anti-myenteric neuronal antibodies in the sera of patients with SSc with GI symptoms (36) and demonstrated that passive transfer of these antibodies into a rat model significantly disrupted intestinal myoelectric activity (37). Finally, IgG from patients with SSc attenuated the M3R activation in smooth muscle cells of a rat internal anal sphincter (38).

Moreover, the administration of IgG fractions from patients with SSc (and also from patients with Sjögren’s syndrome) into mice inhibited the contraction of the colonic smooth muscle caused by carbachol-induced activation of M3R in a concentration dependent fashion, further supporting earlier observations on the possible antagonistic function of anti-M3R autoantibodies (39). Similar results have been reported by Kawaguchi et al. (40) using an enzyme immunoassay. In this study, anti-M3R antibodies were found in 9/14 early-onset patients with SSc with severe GI tract involvement compared with only 3 positive out of 62 early-onset patients with SSc without severe GI tract involvement.

However, 43 patients had GI tract involvement but no circulating auto-anti-M3R autoantibodies, implying that other mechanisms might be responsible for intestinal involvement in patients with SSc. Interestingly, Kumar et al. (41) demonstrated that in the early stage of the disease, patients with SSc had antibodies blocking the ganglionic cholinergic neurotransmission but as the disease progressed, inhibition of acetylcholine action occurred at smooth muscle cell membrane. How these events lead then to fibrosis remains to be elucidated.

Interestingly, using intact rat colon smooth muscle strips, SSc-associated GI dysfunction at both the neuropathic and myopathic stages may be potentially reversible with the administration of intravenous immunoglobulin (41, 42).

However, using a novel luminescence-based assay to detect functionally active antibodies to M3R failed to find antibodies inhibiting carbachol-induced activation of M3R in a cohort of 47 patients with SSc (43).

Autoantibodies against platelet-derived-growth factor receptor
Platelet-derived growth factor receptor (PDGFR) is a cell surface tyrosine kinase receptor mediating the activation of different cell types that are involved in SSc pathogenesis, including fibroblasts and smooth muscle cells. In normal fibroblasts, the activation of PDGFR by PDGF triggers an increased production of ROS, which, in turn, activates ERK1/2 pathway and the downstream viral Harvey rat sarcoma (Ha-Ras) gene. Activation of ERK1/2 and high ROS levels stabilize the Ha-Ras protein by inhibiting proteasomal degradation. As compared with normal fibroblasts, fibroblasts in SSc are characterized by an amplified and persistent ROS-ERK1/2-Ha-Ras signaling loop, which stimulates excessive collagen gene transcription (44, 45). Moreover, the accumulation of collagen I consequent to the activation of the Ha-Ras pathway in SSc fibroblasts is independent of TGFβ stimulation (46).

Thus, it was hypothesized that the presence of a factor unrelated to PDGF or TGFβ, such as an agonistic autoantibody against PDGFR, could sustain the profibrotic phenotype of SSc fibroblasts.

Anti-PDGFR antibodies were actually detected in total serum IgG purified from the sera of 46 patients with SSc (47). These autoantibodies were able to immunoprecipitate PDGFR from human fibroblasts, stimulate production of ROS, and activate the ROS-ERK1/2-Ha-Ras loop in mouse embryo fibroblasts expressing human PDGFRα (Fa) but not in PDGFRα negative cells (F-/−). Importantly, IgG purified from the serum of patients with other connective tissue diseases, used as negative controls, did not display these binding and stimulatory activities.

As other groups failed to replicate these data (48, 49), mostly because of the use of different read-out methods based on different cell lines (50), a more reliable characterization of these anti-PDGFR antibodies was necessary.

Indeed, the generation of human monoclonal antibodies using memory B cells from a single patient with SSc allowed the identification of the major epitopes recognized by anti-PDGFRα antibodies and the development of a specific enzyme immunoassay (51). Surprisingly, four different monoclonal antibodies were identified, with similar heavy chains but different light chains. Each monoclonal antibody showed a peculiar binding and functional activity toward PDGFRα, ranging from low affinity binding without agonistic activity toward fibroblasts to high affinity binding with induction of the ROS-ERK1/2-Ha-Ras loop and increased collagen gene transcription in human fibroblasts in vitro (51, 52). Using a large PDGFRα peptide library, it was possible to identify the conformational PDGFRα epitope recognized by the antibody with agonistic activity, i.e., a discontinuous motif encompassing three distinct sequences across the second and third extracellular PDGFRα domains, largely overlapping the PDGFR binding region. On the contrary, the non-agonistic antibodies targeted one linear aminoacidic sequence in the first extracellular PDGFRα domain (52).

In addition, anti-PDGFRα antibodies with receptor affinity as high as that of the collagen-inducing monoclonal autoantibody can be detected by ELISA in the sera of patients with SSc. Using this technique, anti-PDGFRα antibodies were detected in 66 of 70 patients with SSc (94.3%), 63 of 130 healthy controls (48.5%), 11 of 26 patients with primary Raynaud’s phenomenon (42.3%), and 11 of 29 patients with SLE (37.9%). Importantly, IgG purified from ELISA-positive SSc serum samples turned out to be positive in the ROS bioassay also, whereas IgG purified from ELISA-negative healthy controls serum samples did not (51).

Overall, these findings indicate that both agonistic and non-agonistic anti-PDGFRα autoan-
tibodies may be produced, and even coexist, in the same patient with SSc. Moreover, the anti-PDGFRα autoantibodies that can also be detected in healthy subjects or patients affected by other connective tissue diseases are non-agonistic, and thus they should be considered as natural autoantibodies (53). Conversely, agonistic anti-PDGFRα autoantibodies recognize specific conformational epitopes, largely overlapping the PDGF binding region, suggesting their pathogenic role in the SSc-specific, unbalanced autoimmune response against cellular antigens.

A small clinical study provided the first indirect evidence of anti-PDGFRα antibody pathogenicity in vivo. Six patients with SSc with severe skin fibrosis, unresponsive to canonical immuno-suppressive therapies, were treated with 375 mg/m² per week of rituximab for a total of four doses. A good clinical response, evaluated as decrease of the skin score and improvement of the disability indices observed in all patients, was observed, as well as a significant reduction of ROS stimulatory activity in vitro by IgG purified from the patients’ sera. Furthermore, fibroblasts derived from skin biopsies performed at baseline and after 3 and 6 months showed downregulation of specific intracellular signaling pathways and type I collagen gene expression (54).

A subsequent study provided the first direct in vivo evidence of anti-PDGFR antibody pathogenicity. Three-dimensional bioengineered skin samples containing human keratinocytes and fibroblasts isolated from skin biopsies of healthy donors were generated and grafted onto the back of severe combined immunodeficiency mice. The dermis of the skin grafts was then injected with total IgG purified from the serum of either patients with SSc (SSc IgG) or healthy controls (HC IgG), i.e., either with the agonistic, collagen-inducing anti-PDGFRα monoclonal antibody or with the non-agonistic one. Strikingly, the injection of SSc IgG, but not of HC IgG, induced increased deposition of type I collagen and upregulation of fibroblast activation markers in healthy donor skin grafts. These findings demonstrated that the agonistic anti-PDGFRα antibodies, and not the non-agonistic ones, are profibrotic in vivo (55).

Agonistic anti-PDGFRα autoantibodies (both total IgG and the collagen-inducing monoclonal antibody mentioned earlier) have also been recently demonstrated to induce proliferation and migration of human pulmonary vascular smooth muscle cells (VSMCs) in vitro (56). The activation of PDGFR by SSc IgG was both selective and ROS dependent. Similar findings showing that serum IgG from patients with SSc induces contraction of VSMCs in a collagen matrix, in contrast with IgG from healthy controls, had been previously reported (57, 58), although in the latter study, SSc IgG, even if engaging PDGFR, leads to the activation of the EGFR through a PDGFR-independent pathway (58). These findings are important as they further corroborate the hypothesis that anti-PDGFRα antibodies have agonistic activity and, therefore, they may contribute to the pathogenesis of SSc and, potentially, of SSc-associated vasculopathy.

Other autoantibodies
Several additional putative pathogenic autoantibodies have been described, but evidence is currently limited to clinical associations and in vitro experimental studies.

Anti-fibrillin antibodies have been investigated in several studies. In 1999, Tan et al. (59) reported a high prevalence of anti-fibrillin antibodies in Japanese patients with SSc; however, their presence was much lower in Caucasian and African-American subjects. Ethnic differences in epitope specificity of anti-fibrillin antibodies were later reported (60), but the presence of these autoantibodies was not associated with specific subsets of the disease or clinical manifestations. Experimental data in vitro showed that affinity-purified anti-fibrillin antibodies from patients with SSc induced increased expression of collagen and several other ECM components in normal human fibroblasts, and neutralization of TGF-β1 significantly diminished their activation (61). Other groups failed to replicate these findings in the Caucasian patients with SSc (62).

Weigold et al. (63) first reported the detection of anti-CXCR3 and anti-CXCR4 antibodies in patients with SSc. These autoantibodies were associated with dcSSC and ATA positivity and disease activity (65). In vitro, the percentage of activated T regulatory cells was significantly higher in anti-ER positive than in anti-ER negative patients, suggesting that these autoantibodies may act as functional modulators of the immune system.

Functional anti-CD22 antibodies have been detected by the ELISA assay in 22% of patients with SSc, although they are not specific to SSc (66). In vitro assays suggested that anti-CD22 antibodies were able to stimulate B cell response in both patients with SSc and SLE.

Finally, Sato et al. (67) first reported the presence of anti-matrix metalloproteinase-1 (MMP-1) and anti-MMP-3 antibodies in patients with SSc (68). In vitro studies showed that total serum IgG from anti-MMP-1 or anti-MMP-3 positive patients with SSc inhibited the activity of MMP-1 and MMP-3, respectively, suggesting their potential role in the impaired ECM turnover in the pathogenesis of SSc.

Conclusion
The detection of several putative pathogenic serum autoantibodies in patients with SSc supports the hypothesis that targeting the production of autoantibodies or preventing their functional activity may provide advantages in the management of this heterogeneous condition lacking disease-modifying therapies (69-71).

The identification of autoantibodies with specific functional properties may help identify different clinical variants of the disease as well as shed light on SSc pathogenesis. Unraveling the pathways triggered or inhibited by functional autoantibodies may, in turn, pave the way for novel therapeutic approaches. These therapeutic strategies should preferably rely on selective targeting of the receptors rather than on an unselective block of total receptor activity, possibly burdened with significant adverse effects.

Further studies with robust animal models are needed before definitive conclusions on the putative pathogenicity of these autoantibodies can be drawn.

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