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Pathogenic roles of autoantibodies in systemic sclerosis: Current understandings in pathogenesis

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Abstract

The potential pathogenic role for autoantibodies in systemic sclerosis has captivated researchers for the past 40 years. This review answers the question whether there is yet sufficient knowledge to conclude that certain serum autoantibodies associated with systemic sclerosis contribute to its pathogenesis. Definitions for pathogenic, pathogenetic and functional autoantibodies are formulated, and the need to differentiate these autoantibodies from natural autoantibodies is emphasized. In addition, seven criteria for the identification of pathogenic autoantibodies are proposed. Experimental evidence is reviewed relevant to the classic systemic sclerosis antinuclear autoantibodies, anti-topoisomerase I and anticentromere, and to functional autoantibodies to endothelin 1 type A receptor, angiotensin II type 1 receptor, muscarinic receptor 3, platelet-derived growth factor receptor, chemokine receptors CXCR3 and CXCR4, estrogen receptor α, and CD22. Pathogenic evidence is also reviewed for anti-matrix metalloproteinases 1 and 3, anti-fibrillin 1, anti-IFI16, anti-eIF2B, anti-ICAM-1, and anti-RuvBL1/RuvBL2 autoantibodies. For each autoantibody, objective evidence for a pathogenic role is scored qualitatively according to the seven pathogenicity criteria. It is concluded that anti-topoisomerase I is the single autoantibody specificity with the most evidence in favor of a pathogenic role in systemic sclerosis, followed by anticentromere. However, these autoantibodies have not been demonstrated yet to fulfill completely the seven proposed criteria for pathogenicity. Their contributory roles to the pathogenesis of systemic sclerosis remain possible but not yet conclusively demonstrated. With respect to functional autoantibodies and other autoantibodies, only a few criteria for pathogenicity are fulfilled. Their common presence in healthy and disease controls suggests that major subsets of these immunoglobulins are natural autoantibodies. While some of these autoantibodies may be *pathogenetic* in systemic sclerosis, establishing that they are truly *pathogenic* is a work in progress. Experimental data are difficult to interpret because high serum autoantibody levels may be due to polyclonal B-cell activation. Other limitations in experimental design are the use of total serum immunoglobulin G rather than affinity-purified autoantibodies, the confounding effect of other systemic sclerosis autoantibodies present in total immunoglobulin G and the lack of longitudinal studies to determine if autoantibody titers fluctuate with systemic sclerosis activity and severity. These intriguing new specificities expand the spectrum of autoantibodies observed in systemic sclerosis. Continuing elucidation of their potential mechanistic roles raises hope of a better understanding of systemic sclerosis pathogenesis leading to improved therapies.

Keywords

Autoantibody, centromere protein B, cytokine, endothelial cell, G-protein-coupled receptor, fibroblast, pathogenicity, receptor, smooth muscle cell, topoisomerase I

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Introduction

For the past 40 years, the question of a potential immunopathogenic role for autoantibodies (aAbs) in systemic sclerosis (SSc) and other systemic autoimmune rheumatic diseases (SARDs) has mesmerized clinician researchers as well as basic researchers.[1–6] As pointed out by Fritzler and Choi,[5] this scientific question was fuelled by definitive evidence of the pathogenic role of aAbs in organ-specific autoimmune diseases such as myasthenia gravis and Graves' disease, as well as the demonstration that certain antinuclear autoantibodies (ANAs) bound to the "active site" of their cognate antigen and inhibited their physiologic activity.[5,7–9]

In SSc specifically, this perennial question was initiated by the seminal discoveries of two major ANAs in patient sera. First, in 1979 Douvas et al.[10] reported that serum aAbs directed to a 70 kD nuclear autoantigen (aAg) were highly specific for the diagnosis of SSc, and named these aAbs as anti-Scl-70 (anti-topoisomerase I). Subsequently, the molecular identity of the Scl-70 aAg was shown by Earnshaw and Rothfield[11] to correspond to DNA topoisomerase I (topo); hence nowadays, the preferred terminology of anti-topoisomerase I (anti-topo).

Second, in 1980, anticentromere (anti-CENP-B) antibodies (ACAs) in sera from SSc patients were discovered in Dr Tan's laboratory, using cultured human epithelial type-2 (HEp-2) cells to characterize ANAs by indirect immunofluorescence (instead of the rodent tissue substrates routinely used until then for ANA detection),[12] and Fritzler and Kinsella[13,14] reported their high specificity for the limited cutaneous systemic sclerosis (lcSSc) subset (formerly the CREST syndrome). Later, using sera with ACAs from patients with SSc to probe chromosomal extracts, Earnshaw and colleagues[15,16] discovered the three major centromere proteins (CENPs), named CENP-A, -B, and -C. Subsequently, a cDNA was cloned for CENP-B, the major human centromere aAg recognized by human ACA, that is, anti-CENP-B, thus opening the way to clinical immunoassays using the cloned aAg to detect ACAs.[17,18]

Thereafter, anti-RNA polymerase III (anti-RNAPIII) and anti-Th/To aAbs were described respectively as the third and fourth classic major ANAs that are SSc specific.[19–23] These associations were confirmed, although not always in the same frequency, in various ethnogeographic SSc patient groups worldwide. Therefore international classification criteria for SSc now include three of the four classic ANAs strongly associated with SSc, that is, ACA, anti-topo, and anti-RNAPIII.[24–26] In clinical practice anti-CENP-B, anti-topo, and, where available, anti-RNAPIII and anti-Th/To, are now routinely used as diagnostic markers for SSc.

The strong link between these four, usually mutually exclusive, aAbs and SSc, altogether with subsequent novel important data on their remarkable phenotypical associations within the SSc spectrum (as discussed below), logically led to the question of whether SSc aAbs were more than diagnostic and phenotypic markers, that is, could they contribute to the pathogenesis of SSc? At present, it is generally stated that their direct pathogenic roles remain to be documented.[27]

In addition, several novel intriguing aAbs in SSc sera have been reported in recent years that are directed not to nuclear aAgs but rather to cell-surface receptors or to extracellular aAgs. Some of these aAbs have functional agonistic properties, as defined in the following sections. These aAbs have been recently reviewed in-depth,[28–31] and experimental investigations for potential pathophysiological roles in SSc are of great interest.[27] Given that SSc is an incurable orphan disease with high morbidity and mortality for which novel effective therapeutic approaches are needed,[32] one major objective of these investigations is to identify new potential therapeutic targets.[28,30]

In this article, we focus on reviewing objective scientific evidence in favor of, or against, a pathogenic role for various aAbs in SSc.

Caveats

From the outset, some cautionary remarks are necessary. First, it is acknowledged that the pathogenesis of SSc, encompassing autoimmunity, early inflammation, microvasculopathy and fibrosis, is complex, multifactorial, and incompletely understood.[6,27,33–39] Moreover, multiple components of the innate and adaptive immune systems are recruited in SSc pathogenesis and adaptive humoral immune responses, to which this review is confined, are but one facet of that complexity.

Second, the *origins* of SSc aAbs, that is, the molecular events that initiate their production and the subsequent mechanisms that perpetuate their biosynthesis during the disease course, are important but separate issues that are not addressed herein.[34,40,41]

Third, this review does not encompass several other aAbs associated with SSc manifestations such as anti-U3RNP and anti-U11/12RNP or occurring primarily in the setting of an overlap connective tissue disease.[42,43] In addition, this review focuses on aAbs with a well-established molecular specificity rather than using broad generic terms such as anti-endothelial cell (EC) aAbs.

Last, it is acknowledged that rare patients with SSc apparently do not have in their circulation one of the major SSc aAbs,[44,45] which could be used as an argument against a pathogenic role for aAbs in this disease. However, as new ANA aAb specificities are being discovered in SSc,[46,47] we suspect that some of these patients may have a hitherto undiscovered SSc aAb. In addition, given that the titer of certain SSc ANAs may fluctuate and become negative over time,[48] as discussed in the following sections, the apparent absence of a classic SSc ANA at a given time

point in an SSc patient does not preclude its presence at an earlier or a later time point.

Definitions for pathogenic, pathogenetic, natural, and functional aAbs

Pathogenic and pathogenetic aAbs

When SSc aAbs are analyzed, the demarcation between pathogenicity and pathogenesis is often unclear. Therefore, clear definitions are necessary to better interpret current mechanistic evidence.

Pathogenicity is defined as the ability to *produce* pathologic changes or disease,[49] or the ability to *cause* disease.[50] For example, the pathogenicity of a microorganism is its ability to *cause* disease.[51] *Pathogenic*, the adjective corresponding to pathogenicity, is defined as: *causing* disease or disease symptom, or as causing disease or abnormality.[50]

Pathogenesis is the *development* of morbid conditions or of disease. More specifically, it designates the *cellular events* and reactions and other pathologic mechanisms occurring in the development of disease.[49] *Pathogenetic* is the adjective corresponding or relating to pathogenesis.[49]

Thus, although *pathogenic* and *pathogenetic* are sometimes used interchangeably, there is an important mechanistic difference: *pathogenic* implies a definitive *causal*, active contributory role in the disease process, whereas a *pathogenetic* phenomenon is not necessarily causal but rather it occurs in the course of the disease as a consequence of its mechanisms, including a *preceding pathogenic event*. Two definitions are therefore used herein (Table 1):

- *Pathogenic aAbs* are defined as immunoglobulins contributing to the development of an autoimmune disease and its organ and/or systemic manifestations.[2,4,28] For example, certain anti-native DNA aAbs cross-reactive with NR2 glutamate receptors are pathogenic.[52] If the lack of pathogenicity is demonstrated for an aAb, it is referred to as *non-pathogenic*.
- *Pathogenetic aAbs* are immunoglobulins present during the development of an autoimmune disease and its organ and/or systemic manifestations but not known, or demonstrated to, contribute to the pathophysiological process. For example, anti-RNAPIII aAbs may originate as an anti-cancer immune response to mutated cancer epitopes, thus providing an important clue to the origin of these immunoglobulins.[53] Yet, no experimental evidence suggests that anti-RNAPIII are pathogenic per se and therefore, in the current state of knowledge, they are best classified as *pathogenetic aAbs*.

Functional aAbs

Functional aAbs are a subset of pathogenic aAbs which, by binding to their cognate aAg, directly activate or stimulate (agonistic effect) or inhibit (antagonistic effect) a molecular pathway (Table 1).[28,29] As specified by Riemekasten[28], designating an aAb as functional requires that its direct interaction with an identified target antigen leads to a molecular pathway activation or inhibition that can be replicated in an experimental setting. The archetype of functional aAbs are anti-thyroid aAbs to the thyroid-stimulating hormone receptor, a G-protein-coupled receptor (GPCR), which in Graves' disease stimulate excessive production of thyroid hormone, leading to hyperthyroidism.[54] Both thyroid-stimulating harmone (TSH) and thyroid-stimulating aAb of Graves' disease bind to an Mr 197,000 holoreceptor.[54–56]

Natural aAbs

Natural aAbs are immunoglobulins that are present in serum from healthy individuals and that react with self-molecules, including DNA, nuclear and cytoskeletal proteins, and cell-surface aAgs (Table 1). Although emphasis has been on IgM natural aAbs, isotypes of natural aAbs encompass immunoglobulin G (IgG) and immunoglobulin A (IgA) as well.[57–59] In fact, natural IgG and IgA are predominant serum and mucosal natural aAbs.[57–60] They are encoded by unmutated V(D)J genes and display weak to moderate affinity for self-antigens. Natural antibodies, notably of IgM isotype, which represent a considerable amount of the total IgM circulating in humans, provide a first line of defense against infections, serve housekeeping functions, contribute to the homeostasis of the immune system,[5,61,62] and act as protective aAbs notably by being anti-apoptotic.[63–65]

Although deciphering the properties and physiological function of IgG natural aAbs is a work in progress,[59] it is important to differentiate natural aAbs from pathogenic and pathogenetic aAbs because baseline titers of natural aAbs, including of IgG and IgM isotypes, can increase non-specifically in chronic inflammatory diseases and SARDs such as systemic lupus erythematosus (SLE) and SSc that are associated with polyclonal B-cell activation and hypergammaglobulinemia. Therefore, claims of specific association between SSc and novel aAbs must be validated by the comparison of aAb isotype, titers, and frequency in normal sera and in various non-autoimmune and autoimmune diseases (including SLE as the prototypical SARD associated with multiple aAbs), and also by determining whether there is a linear relationship between total serum IgG and the titer of the aAbs under study. Such a linear relationship suggests that increased aAb titers are due to non-specific polyclonal B-cell activation rather than to a specific aAg-driven autoimmune response.

Table 1. Definitions of pathogenic, pathogenetic, functional, and natural autoantibodies.

Names	References
Pathogenic autoantibodies	
Immunoglobulins that contribute to the development of an autoimmune disease and its organ and/or systemic manifestations	2,4,28,52
Pathogenetic autoantibodies	
Immunoglobulins that are present during the development of an autoimmune disease and its organ and/or systemic manifestations but not known, or demonstrated to, contribute to the pathophysiological process	53
Functional autoantibodies	
A subset of pathogenic autoantibodies which, by binding to their cognate autoantigen, directly activate or stimulate (agonistic effect) or inhibit (antagonistic effect) a molecular pathway	28,30,54–56
Natural autoantibodies	
Immunoglobulins of IgG, IgM, and IgA isotypes that are present in serum from healthy individuals and that react with self-molecules, including DNA, nuclear proteins, cytoskeletal proteins, and cell-surface molecules	57–65

Specific criteria defining pathogenic aAbs

To evaluate whether an aAb in SSc is truly pathogenic, scientific criteria for pathogenicity are needed. In 1993, Naparstek and Plotz[1] proposed such criteria on the premise that: "*To establish that an autoantibody is pathogenic, one must be able to construct a plausible picture of how it might act and to reproduce it experimentally*."

In Table 2, seven criteria for the definition of pathogenic aAbs in SARDs are proposed. These criteria are updated from the original five criteria,[1,66] notably by the addition of novel criteria nos. 1 and 2. In-keeping with Hill's[67] criteria of causation/causality, these new criteria emphasize disease specificity and temporality. The first four criteria are mostly based on clinical evidence, whereas the last three are based on experimental evidence (Table 2).

Pathogenicity criterion no. 1

The first criterion for a potential pathogenic role requires the aAb to be disease specific, as is the case in SSc for its hallmark aAbs anti-CENP-B, anti-topo, anti-RNAPIII, and anti-Th/To. As a corollary, this requirement draws attention to the necessity of excluding natural aAbs from the SSc-specific aAb repertoire.

In addition to the disease specificity of the four classic SSc aAbs, each one of them clusters with a distinct clinical phenotype, in terms of both disease subsets and selective visceral involvements.[68] Such phenotypic specificity strengthens the first pathogenicity criterion (Table 2). For example, anti-CENP-B and anti-Th/To are associated with lcSSc, pulmonary arterial hypertension (PAH), and severe digital ischemia. In contrast, anti-topo associates with pulmonary fibrosis (and often with diffuse cutaneous

Table 2. Pathogenicity criteria for the definition of pathogenic autoantibodies in systemic sclerosis and other systemic autoimmune rheumatic diseases*.

Clinical pathogenicity criteria
Criterion 1. The autoantibody should be specific to the disease
An even greater pathogenic value is suggested when the autoantibody is phenotype specific, that is, within the disease spectrum, it associates with a particular set of clinical and laboratory manifestations
Criterion 2. The autoantibody is serologically present before the onset of clinical manifestations
Criterion 3. Autoantibody levels and disease activity/severity should, in general, correlate
Criterion 4. Removal of the autoantibody, or blocking its functional effects, should ameliorate the disease process (e.g. by immunosuppression, plasma exchange, biological agent, immunotherapy, or other means)
Experimental pathogenicity criteria
Criterion 5. The autoantibody should be capable of causing in experimental systems the lesions attributed to it (e.g. in living cells or in an experimental animal model)
Criterion 6. A suitable immunization that leads to the production of similar autoantibodies should lead to a similar disease process
Criterion 7. The autoantibody should be found along with a plausible target antigen at the site of tissue damage

*Modified from Naparstek and Plotz[1].

systemic sclerosis (dcSSc) but not with renal crisis, whereas anti-RNAPIII are linked to dcSSc, renal crisis, and cancer, but not to pulmonary fibrosis.[6,68–70] In clinical practice, these disease-specific aAbs are exceptionally useful as clinical tools to stratify patients and anticipate particular disease complications.[26,70,71] Thus, these phenotypic associations further strengthen the link between aAbs and pathogenesis.

Pathogenicity criterion no. 2

If an aAb is pathogenic, its presence in patient sera should logically not only accompany but precede clinical manifestations and diagnosis (Table 2). In keeping with Hill's temporality criterion of causality, the cause should precede the effect.[67] In addition, given the protracted prodrome before diagnosis in SSc and in other SARDs, the aAb could be expected to be serologically present as far back as can be tested, as shown for SLE.[13,72,73] Indeed, this is also the case for SSc, as shown in a 20-year prospective study of 586 patients with isolated Raynaud phenomenon (RP), where all four classic SSc aAbs, including anti-CENP-B, and anti-topo were independently highly predictive for the development of SSc.[74] Importantly, in the study by Koenig, aAbs typically preceded or accompanied microvascular damage as evaluated by nailfold capillary microscopy. By multivariable Cox proportional hazards models, ACAs and anti-Th/To aAbs predicted enlarged capillaries and, along with anti-RNAPIII, they also predicted capillary loss whereas anti-CENP-B predicted capillary telangiectases.[74] In addition, each aAb was associated with a distinct time course of microvascular damage. Taken altogether, these diagnostic and phenotypic associations as well as the strong association between each SSc aAb and the type and time course of microvascular damage suggest that the aAbs are not merely pathogenetic, that is a reflection of the disease, but that they may actually contribute to it, that is, they may be pathogenic.

However, these data are only *indirectly* suggestive of causality, as they do not prove per se that these aAbs are pathogenic. As pointed out by Rosen, "*the data demonstrating that aAbs are serological markers of a specific disease phenotype in SSc are extremely strong, but the mechanistic implications of such observations remain uncertain*."[6] Nevertheless, the respective phenotypes suggest that, depending on the specific SSc aAb present, distinct pathophysiological pathways are exerted preferentially in certain organs and tissues and via different mechanistic pathways and at different biological rates. Overall, the four classic SSc aAbs are among the best pathophysiological clues available and they should continue to be the focus of intense research to decipher their pathogenic significance.

Pathogenicity criteria nos. 3 and 4

A third criterion to define pathogenic aAbs is that aAb levels and disease activity/severity should, in general, correlate (Table 2).[1] In the SARDs, clinicians are already aware that serum levels of several ANAs and other aAbs may fluctuate with disease activity, including aAbs to DNA, U1RNP, Jo-1, MDA-5, signal-recognition particle (SRP), and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR).[75] In SSc specifically, longitudinal serum levels of anti-topo do correlate positively with disease activity and severity.[76] In a study of 59 patients with dcSSc, IgG anti-topo titers determined by enzyme-linked immunosorbent assay (ELISA) using recombinant topo correlated strongly with disease severity, as assessed by total skin score (TSS) measurements ($r = 0.61$, $p < 0.001$).

Moreover, mean anti-topo titers were higher in active versus inactive disease ($p < 0.001$) as determined from clinical examination and laboratory data.[76] Strikingly, in 8 of 11 patients analyzed longitudinally, anti-topo titers fluctuated in parallel with the TSS; in some patients, increasing titers actually preceded increases in TSS.[76] These data expanded a previous study by Kuwana et al.,[48] where SSc patients, in whom anti-topo turned negative at follow-up, experienced significant improvement in pulmonary function and survival, in comparison with patients with persistent anti-topo. Interestingly, in a patient with dcSSc and increasingly severe pulmonary fibrosis treated with monthly intravenous cyclophosphamide, we have observed a progressive fall in anti-topo titers measured over the course of 1 year in parallel with pulmonary improvement (unpublished observation).

Taken altogether, these data suggest that anti-topo fulfill not only the third pathogenicity criterion (Table 2) but may also fulfill in part the fourth criterion stating that removal of the aAbs should ameliorate the disease process.

It should be emphasized that ACAs that appear at disease outset typically persist in sera in high titers and usually do not fluctuate markedly over a disease course that may span decades. However, in our view, this does not contradict pathogenicity criterion no. 3 and is consistent with the mechanistic concept that slowly progressive microvascular abnormaties occurring in lcSSc with persistent circulating anti-CENP-B are the result of a persistent low-grade, unremitting, vascular obliterative pathogenic process ultimately leading to ischemic complications and PAH, and possibly mediated by ACAs, as discussed below.[4,74,77]

Pathogenicity criteria nos. 5 to 7

The archetypal example of a pathogenic aAb was provided by the late William J. Harrington et al.[78] when he became acutely thrombocytopenic after infusing himself with plasma from a patient with idiopathic thrombocytopenic purpura (ITP). Subsequent studies revealed that IgG antiplatelet aAbs were responsible for the thrombocytopenia of ITP. Similar in vivo studies cannot be done nowadays in humans and the demonstration that an aAb is pathogenic must rely in part on experimental evidence outlined in criteria nos. 5 to 7 (Table 2).

Most importantly, the aAb should be capable of causing the lesions attributed to it in experimental systems (e.g. in living cells or in an experimental animal model) (criterion no. 5), and a suitable immunization that leads to the production of similar aAbs should lead to a similar disease process (criterion no. 6). And last, but difficult to demonstrate in a chronic disease with a protracted course such as SSc, the aAb should be found along with a plausible target antigen at the site of tissue damage (criterion no. 7).

Proving beyond doubt that an aAb is pathogenic remains scientifically challenging and onerous.[52,79,80] Therefore, it is not surprising that progress has been slow. However, scientific advances are bringing new building blocks that strengthen pathogenic roles for some SSc aAbs.

Autoantigens released from apoptotic cells are biologically bifunctional molecules

Nuclear aAgs were long thought to be inaccessible to circulating ANAs because their cognate antigens are encased by the nuclear envelope and the plasma membrane and are therefore sequestered intracellularly. But this belief was disproved by the seminal work of Casciola-Rosen et al.,[81] demonstrating that during cell death by apoptosis, intranuclear aAgs, such as DNA, Ro, and La, translocated to the surface of human epidermal keratinocytes, where they localized in blebs and apoptotic bodies.

Subsequently, other aAgs such as Jo-1 (histidyl-tRNA synthetase) and Tyr (tyrosyl-tRNA synthetase) were found to be released from apoptotic cells.[82,83] Thus, during apoptosis, aAgs sequestered intracellularly may gain access to the extracellular microenvironment. Most intriguingly, such released aAgs display a second biological function, distinct from their intracellular roles: they act as tissue-specific chemoattractants by interacting with cell membrane receptors. Thus, extracellular Jo-1 is chemoattractant to naïve T lymphocytes and immature dendritic cells through CCR5-mediated interactions.[83] Bifunctional aAgs act via chemokine receptors, which are a subfamily of the G protein-coupled receptors (GPCRs).[83]

Matzinger[84] proposed that, in the normal host, aAgs may have a primary role in homeostasis by alerting the immune system to danger signals from invaded and damaged tissues in order to facilitate repair[85]. Subsequently, autoimmune responses via recruitment of mononuclear cells that induce innate and adaptive immune responses develop only in subjects with impaired immunoregulatory function.[83,86]

Indeed, in patients with SARDs such as SLE, the normal homeostatic process is overwhelmed by defective clearance of apoptotic cells,[87] leading in turn to secondary necrosis and loss of plasma membrane integrity that result in the release of various aAgs in the tissue microenvironment. These aAg fragments engage damage-associated molecular patterns (DAMPs) or alarmins that further contribute to immune responses to self-antigens. Simultaneously, aAg fragments may also become accessible to circulating aAbs, such as anti-Ro.[88,89] Moreover, although apoptosis is considered the major source of extracellular nuclear material, other cell death pathways such as necroptosis, NETosis, and pyroptosis can contribute as well to the extracellular release of nuclear molecules.[90,91]

In light of the bifunctional nature of these major aAgs, key questions are then: what about SSc aAgs such as topo and CENP-B? Can they be released as well in the extracellular microenvironment during cell death? Do they normally possess a second biological function? If so, could anti-topo and ACAs interfere with this second biological function, thereby contributing to SSc pathogenesis? Extensive experiments to address these questions with respect to topo[92–95] and CENP-B[96,97] were performed using sera from SSc patients with anti-topo or ACAs and results are presented in sequence in the following sections.

Classic SSc aAbs—anti-topo

Anti-topo is the single aAb specificity most studied experimentally for a pathogenic role in SSc.[98,99] Therefore, this review focuses more extensively on this aAb.

Anti-topo aAbs in SSc sera react with the surface of fibroblasts

In light of studies showing that antifibroblast autoantibodies (AFAs) present in the sera of SSc patients can induce a proadhesive and proinflammatory phenotype in fibroblasts,[100,101] characterization of the binding activity of aAbs from SSc sera on cultured fibroblasts as well as exploration of the association of these AFAs with major SSc ANAs was performed:[92]

- High titers of circulating AFAs were detected in 26% of patients with SSc;
- The mean IgG AFA levels in the SSc subset with high titers of AFA was higher than that in the other SSc patients or in any disease controls or normal controls ($p < 0.001$);
- Pulmonary fibrosis, a restrictive syndrome, and mortality were significantly more common in AFA-positive patients than in AFA-negative patients;
- IgG AFAs bound human lung and dermal fibroblast cell lines, but not to human primary ECs nor to pulmonary artery smooth muscle cells (SMCs), indicating a high specificity of AFAs for fibroblasts;

- All SSc-IgG AFAs strongly reacted with topo by ELISA and immunoblotting; the mean IgG anti-topo reactivity by ELISA was strikingly higher in AFA-positive sera than in AFA-negative sera ($p < 0.0001$);
- Binding intensities of IgG AFA and IgG anti-topo were strongly correlated ($r = 0.65$, $p < 0.0001$), suggesting that anti-topo IgG may be directly responsible for fibroblast binding;
- Affinity-purified anti-topo aAbs from AFA-positive SSc sera reacted with the surface of unpermeabilized fibroblasts by flow cytometry as well as by immunofluorescence and confocal microscopy;
- Last, competition with topo completely abrogated binding of purified anti-topo to the fibroblast cell surface, thus confirming the antifibroblast specificity of affinity-purified anti-topo.[92]

Altogether, these results demonstrated that AFA activity in SSc sera is directly mediated by anti-topo aAbs that are capable of recognizing a fibroblast surface antigen and, conversely, that anti-topo aAbs present in SSc sera are AFAs.[92]

Autoantigen topoisomerase I released from apoptotic ECs binds to the surface of fibroblasts and induces monocyte adhesion and activation in the presence of anti–topo aAbs

Further experiments were carried out to determine the molecular identity of the fibroblast surface antigen recognized by anti-topo and whether anti-topo binding to the fibroblast surface perturbs fibroblast function:[93]

- Topo itself, whether purified or released from apoptotic ECs, bound specifically to fibroblasts in a dose-dependent and saturable manner, where it was recognized by anti-topo purified from SSc patient sera;
- Fibroblast cell lines bound by topo and anti-topo complexes included primary human dermal fibroblasts isolated from biopsy samples obtained from lesional or nonlesional skin of patients with SSc and from normal controls;
- In coculture with monocytes, opsonization of fibroblasts by surface binding of topo/anti-topo complexes stimulated adhesion and activation of monocytes, thereby providing a source of growth factors that stimulate fibrosis.

Taken altogether, these data opened the door for a plausible model for the amplification of the fibrogenic cascade in anti-topo-positive SSc patients.[92,93] Therefore, additional mechanistic experiments were performed.

Topoisomerase I is a bifunctional aAg that interacts with CCR7 and exerts cytokine-like effects on dermal fibroblasts: a plausible model explaining the pathogenic effects of anti-topo aAbs

To understand better how anti-topo aAbs may contribute to SSc pathogenesis, the extracellular role of topo in the normal physiology of the fibroblast was investigated.[94] Using an in vitro wound-healing assay, evidence was found for a direct effect of topo in human fibroblast physiology, leading to the positive stimulation of migration that was accompanied by activation of intracellular signaling pathways. Evidence was also provided for a direct interaction of topo with CCR7 at the surface of normal human dermal fibroblasts. Interestingly, when expressed on non-immune cells, CCR7 is a chemokine receptor heavily involved in the development of fibrosis following stimulation by its ligands, CCL19, and CCL21.[94,102,103] Taken together,[92–94] these in vitro data fulfill in part pathogenicity criterion no. 5. An in vivo wound-healing assay would be of interest to further demonstrate that anti-topo I aAbs are profibrotic.

The normal role of topo in self-limited wound healing. These in vitro data[92–94] also suggest that, in normal physiology in vivo, topo is a bifunctional aAg which, when released from injured ECs, binds to bystander fibroblasts and stimulates a normal wound-healing process with self-limiting fibrogenesis. Topo also displays chemoattractant activity toward immature dendritic cells and human monocytes.[86] Overall, the results are in accordance with the concept that aAgs serve to alert the immune system to danger signals from damaged tissues in order to facilitate repair.[84,85]

Anti-topo may be pathogenic by promoting continuous healing and unremitting fibrosis. However, in the context of SSc pathophysiology and in the presence of anti-topo aAbs, a pathogenic cascade is activated (Table 3), as proposed in the following model where anti-topo exert their pathophysiologic effects by interfering with the normal role of topo (Figure 1, left panel):

- First, EC injury and apoptosis are likely primary pathogenetic events in SSc.[33,152,153] Apoptotic cell remnants should normally be rapidly cleared by phagocytes. However, in the event of a sudden increase in apoptotic cell numbers, the capacity of normal clearance mechanisms may be exceeded, resulting in the accumulation and progress of apoptotic cells toward a late apoptotic state (i.e. secondary necrosis), thereby allowing the release of apoptotic body contents to the tissue microenvironment. This may be the case in SSc, in which EC

apoptosis is detected simultaneously in several tissues, notably around small blood vessels.[152,153]

- Second, as topo is released from apoptotic ECs and given its specific affinity for fibroblast surfaces,[93] it binds to nearby cells in SSc tissues and particularly to fibroblasts, recruiting circulating anti-topo aAbs.
- Third, fibroblast surface binding of topo leads to the recruitment of monocytes[85] and opsonization of fibroblasts by topo-anti-topo complexes in turn leading to monocyte adhesion and activation.[93,94]

Ultimately, this chain of events could lead: (1) to amplification of the immune responses due to proinflammatory cytokines released by activated monocytes and (2) to fibrosis due to local secretion of profibrotic cytokines by activated fibroblasts. Thus, the presence of anti-topo in SSc patients would be directly associated with increased immune responses and fibrosis.

The pathogenic effects of anti-topo in vitro are blocked by heparin. Topo binds specifically to heparan sulfate proteoglycans on fibroblast surfaces and anti-topo aAbs from SSc patients amplify topo binding to heparan sulfate chains.[95] Unfractionated heparin and low molecular weight heparin inhibited the binding of topo/anti-topo immune complexes with heparan sulfate on dermal fibroblasts, suggesting a potential therapeutic role for heparin in SSc-associated fibrosis[95] (Figure 1, right panel). These data also further support that anti-topo fulfill pathogenicity criterion no. 4 (Table 3).

Immunization with topo peptide-loaded dendritic cells induces anti-topo aAb response and long-term fibrosis in an animal model

Given that anti-topo aAbs fulfill, as shown above, pathogenicity criteria nos. 1 to 4 and that criterion no. 5 is fulfilled in vitro, the next logical step was to investigate whether anti-topo are pathogenic in vivo, that is do they fulfill experimental pathogenicity criterion no. 6? In other words, does a suitable immunization that leads to the production of similar anti-topo aAbs in an experimental model also leads to a disease similar to SSc?

To answer that question, an immunization strategy was employed that uses the self-immune system and aAg topo. Since dendritic cells (DCs) loaded with self-peptide, as opposed to unpulsed DCs, induce several autoimmune disorders including experimental encephalomyelitis and autoimmune myocarditis,[154,155] it was hypothesized that immunization of Balb/c mice with DCs loaded with selected topo peptides may elicit and perpetuate experimental dcSSc-like disease.[156–158]

Table 3. Scientific evidence of a pathogenic role for autoantibodies in systemic sclerosis.

Autoantibodies	Strength of scientific evidence according to seven pathogenicity criteria*							Pathogenic role**	References
	1	2	3	4	5	6	7		
Classic antinuclear autoantibodies									
Anti-topoisomerase I (formerly anti-Scl-70)	+++	+++	+++	++	++	++	–	Possible	92–95
Anti-CENP-B (anticentromere)	+++	+++	++	–	++	–	–	Possible	96,97
Functional autoantibodies									
Anti-endothelin I type-A receptor (ETAR) and angiotensin II type-1 receptor (AT1R)	?	–	+	–	++	–	++	Possible	104–114
Anti-muscarinic type-3 receptor (M3R)	?	–	+	–	+	–	++	Possible	115–127
Anti-platelet-derived growth factor receptor (PDGFR)	?	–	–	–	++	–	–	Possible	128–135
Anti-chemokine receptors CXCR3 and CXCR4	?	–	?	–	–	–	–	Insufficient data	136,137
Anti-estrogen receptor α (ERα)	?	–	+	–	+	–	–	Insufficient data	138–140
Anti-CD22	?	–	?	–	++	–	–	Insufficient data	141
Miscellaneous autoantibodies									
Anti-matrix metalloproteinases 1 and 3	++	–	++	–	+	–	–	Possible	142,143
Anti-fibrillin 1	+	–	–	–	++	–	–	Insufficient data	144–147
Anti-interferon-inducible protein gene 16 (IFI16)	–	–	?	–	–	–	–	Insufficient data	148,149
Anti-eukaryotic initiation factor 2B (eIF2B)	+++	–	–	–	–	–	–	Insufficient data	47,150
Anti-ICAM-1	–	–	?	–	++	–	–	Insufficient data	151
Anti-RuvBL1/RuvBL2	+++	–	–	–	–	–	–	Insufficient data	46,150

*As described in Table 1. Grading: –, no evidence; ?, contradictory, inconclusive evidence; +, weak evidence; ++, some evidence; +++, strong definitive evidence.

**Grading: definitive, probable, possible, and insufficient data.

Two peptide epitopes of human topo protein (TOPOIA and TOPOIB) were selected to load DCs.[159,160] TOPOIA is located in the N-terminal region which elicits human aAb responses, preferentially by patients progressing to severe disease.[161,162] TOPOIB peptide region (known as DIDII) induces strong cellular responses by all SSc patients. In mice, both peptide regions elicit antibody responses[163] and the sequences of TOPOIA and TOPOIB are 100% identical in murine and human topo protein.

Mice were repeatedly immunized with unpulsed DCs or DCs loaded with either TOPOIA or TOPOIB peptides. At week 10, signs of perivascular, peribronchial, and parenchymal pulmonary inflammation were already observed in the TOPOIA DCs group, together with transient elevation in bronchoalveolar lavage cell counts, interleukin (IL)-17A expression, and CXCL4 production, a biomarker of early human dcSSc.[36] Thus, topo peptide-loaded DCs induced progressive anti-topo aAb response as well as the development of protracted skin and lung dcSSc-like disease. Pronounced lung inflammation, transient IL-17A, and CXCL4 expression preceded fibrosis development.[156] At week 12, TOPOIA DCs, but not TOPOIB DCs immunization, induced mixed inflammation and fibrosis in lungs (peribronchial, parenchymal as well as perivascular) and skin fibrosis with cutaneous thickening.[156] At a late time point (week 18), both TOPOIA DCs and TOPOIB DCs groups displayed increased α-smooth muscle actin (a marker for myofibroblasts) expression in lungs and dermis along with skin fibrosis distal from the site of injection when compared with unpulsed DCs.[156]

Importantly, both topo peptide-DC-immunized groups developed an IgG2a anti-topo aAb response. However, longitudinal analysis of anti-topo revealed that at week 12, IgG2a anti-topo levels in the TOPOIA DCs group (with prominent lung inflammation and fibrosis as well as skin thickening) were not significantly different from the unpulsed DCs control group, whereas anti-topo levels in the TOPOIB DCs group (with little lung inflammation and fibrosis or skin thickening) were significantly higher in comparison with the TOPOIA DCs group and unpulsed groups.[156] At week 18, IgG2a anti-topo levels in the TOPOIA DCs group had risen and, along with levels in the TOPOIB DCs group, were now significantly higher than in the unpulsed group.

In conclusion, DCs loaded with topo peptides elicit dcSSc-like disease. TOPOIA DCs were more inflammatory and fibrogenic, despite a late anti-topo response, whereas TOPOIB DCs were less inflammatory and

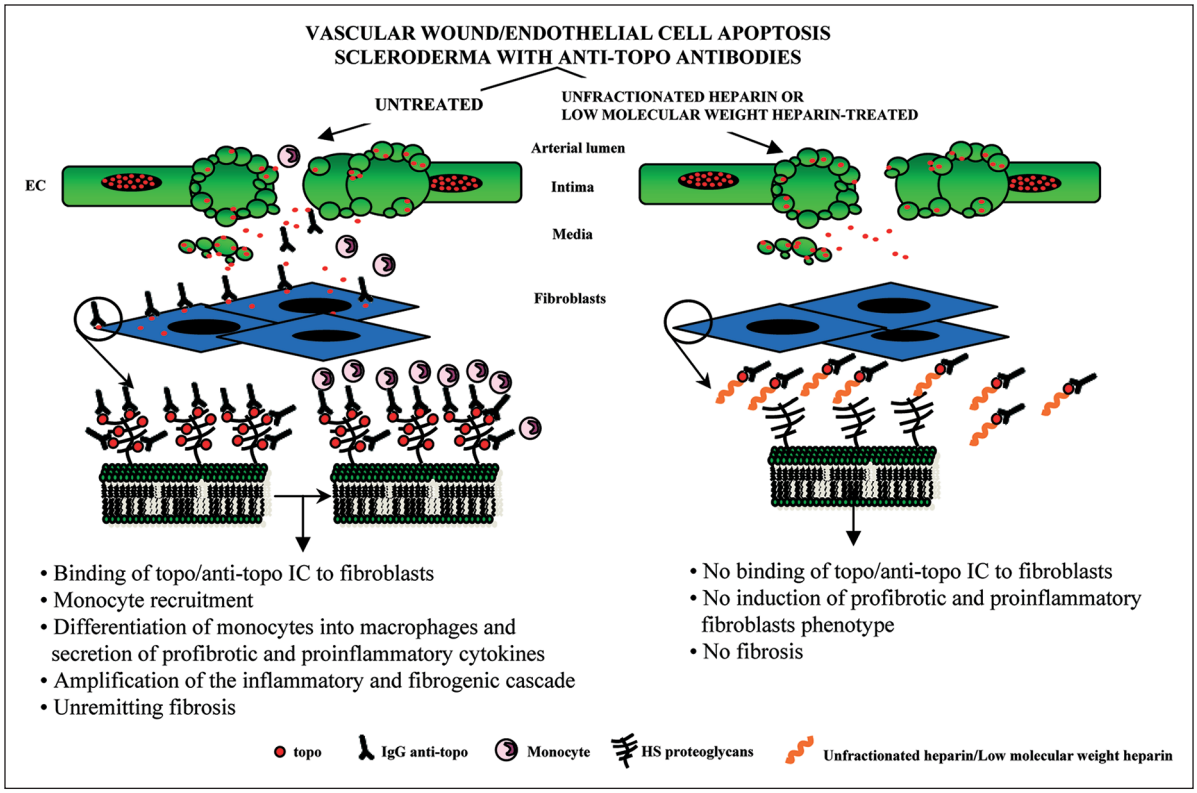

Figure 1. Signaling model illustrating the potential pathogenic role of topoisomerase I (topo I)/IgG anti-topo I immune complexes (ICs) on fibroblasts as a contributor to the initiation of fibrosis in systemic sclerosis (scleroderma) patients (left) as well as the effects of its perturbation by unfractionated heparin (UFH) or low molecular weight heparins (LMWH) treatments (right). Adapted from a study by Arcand et al.[94] with permission from the publisher.

fibrogenic despite eliciting an early anti-topo aAb response. Thus, as pointed out by Ho and Varga[158], "*although the mice mounted an anti-topo response similar to patients with SSc, fibrosis preceded the appearance of these aAbs (in the TOPOIA DCs group), therefore excluding their direct role in fibrosis initiation, though perhaps not in its persistence*."

Conclusion – anti-topo aAbs fulfill several pathogenicity criteria and may promote continuous wound healing and unremitting fibrosis

Four conclusions stem from the preceding data:[92–95,156–158]

- First, topo is a bifunctional aAg that has a normal role in self-limited wound healing.
- Second, anti-topo aAbs may be pathogenic in vitro by promoting continuous wound healing and unremitting fibrosis.
- Third, anti-topo aAbs do fulfill, at least in part, several pathogenicity criteria for SSc (Table 3). Criteria nos. 1 to 3 are completely fulfilled. Criterion no. 4 is fulfilled in part, according to clinical data reviewed[48,76] and by the in vitro blocking effects of heparin. Criterion no. 5 is also fulfilled in part.
- Fourth, however, criterion no. 6 was not clearly fulfilled in the experimental model presented, although the model did succeed in reproducing a protracted dcSSc-like disease and inducing anti-topo aAbs.[156]

At present, anti-topo are the single aAb specificity with the most cumulative evidence in favor of a pathogenic role in SSc fibrosis on the basis of several fulfilled pathogenicity criteria. These results will need to be confirmed by others. Yet, data presented in the experimental model suggest that the anti-TOPOIA aAbs are not pathogenic, at least in early SSc-like disease in mice. Whether this is also the case in human SSc is open to question since high titers of IgG anti-topo do precede clinical disease onset in patients.[74] Whether other topo epitopes may prove to be pathogenic remains to be determined in future studies and will require additional in vivo experiments. Thus, a contributory role of anti-topo to the pathogenesis of human SSc remains possible but not yet conclusively demonstrated.

Classic SSc aAbs—anti-CENP-B

As seen above, anti-CENP-B fulfills clinical pathogenicity criteria nos. 1 and 2. Older in vitro studies have shown that sera from SSc patients with ACA inhibited the organization of microtubules at the kinetochore and disrupted events required for chromosome movement at mitosis.[7,8] Since then, few studies have addressed how ACA or their target CENP aAgs may relate to SSc pathogenesis.[164,165] Thus, the question remains unanswered whether the major SSc aAg CENP-B and ACAs have a pathogenic role.

In light of the bifunctional nature of topo SSc aAg, key questions are asked: is CENP-B, like topo, released in the extracellular microenvironment during cell death? Is CENP-B a bifunctional aAg as well and, if so, what is its second biological function? In that case, could ACAs interfere with this second biological function and thus contribute to SSc pathogenesis? Experiments to address these questions were performed as follows.[96,97]

Autoantigen CENP-B released from apoptotic ECs binds to the surface of human pulmonary artery SMCs and stimulates their migration and secretion of IL-6 and IL-8

Purified CENP-B, or CENP-B released from apoptotic cells, was tested in vitro for surface binding to several human cell types relevant to SSc pathogenesis.[96] CENP-B binding was detected using affinity-purified anti-CENP-B from SSc patient sera. The biologic effects of CENP-B on cellular migration, IL secretion, and signaling pathways of its specific target cells were evaluated:

- CENP-B was found to bind specifically to the surface of human pulmonary artery SMCs and not to fibroblasts or ECs.[95] Furthermore, CENP-B bound preferentially to SMCs of the contractile type rather than the synthetic type;
- Binding of CENP-B to SMCs stimulated their migration during in vitro wound-healing assays, as well as their secretion of the proinflammatory cytokine IL-6 and chemokine IL-8 (CXCL8);
- The mechanism by which CENP-B mediated these effects involved the focal adhesion kinase, Src, ERK-1/2, and p38 MAPK pathways;
- CENP-B released from apoptotic ECs was found to bind to SMCs, thus indicating a plausible in vivo source of extracellular CENP-B.[96]

Human CENP-B is exclusively localized within heterochromatin in the central domain of the centromere. Therefore, CENP-B is not normally presented to cell-surface receptors. However, as shown, CENP-B is redistributed into apoptotic bodies during the course of EC apoptosis and can be released to the extracellular milieu, thus providing a source of extracellular CENP-B.[96] Given that patients with anti-CENP-B have a high frequency of PAH, which is caused notably by intimal migration and proliferation of vascular SMCs, the stimulation of migration induced by CENP-B is of particular interest. Overall, a second and novel biological role for CENP-B was identified, suggesting that this major SSc aAg may participate in normal and pathogenic mechanisms in which vascular SMCs are involved.

CENP-B is a bifunctional molecule that transactivates the epidermal growth factor receptor via chemokine receptor 3 in vascular SMCs and ACAs abolish this signaling pathway

Additional experiments were performed to determine whether CENP-B interacts with chemokine receptors on the surface of human pulmonary artery SMCs, to explore the relevant signaling pathways and to characterize the effects of anti-CENP-B binding on SMC stimulation:[97]

- CENP-B used chemokine receptor 3 (CCR3) to mediate human pulmonary artery SMC signaling. CENP-B binding subsequently stimulated the cross-talk between CCR3 and epidermal growth factor receptor (EGFR) via a matrix metalloprotease–dependent mechanism that involved the processing of heparin-binding EGF-like growth factor;[97]
- Transactivation of the EGFR through CCR3 was a critical pathway that elicited MAP kinase activation and secretion of cytokines such as IL-8;
- Anti-CENP-B aAbs abolished the effect of CENP-B on IL-8 production, thus preventing CENP-B from transactivating EGFR and exerting its cytokine-like activities toward vascular SMCs.[97]

Conclusion—Anti-CENP-B aAbs fulfill pathogenicity criteria nos. 1 and 2 and in part no. 5 and may lead to vascular complications in SSc by promoting low-grade but unremitting vascular repair

Taken together, the data reviewed herein indicate that anti-CENP-B fulfill pathogenicity criteria nos. 1 and 2 and in part no. 5(Table 3).[96,97] Given that persistent high titers of anti-CENP-B are statistically strongly associated with slowly progressive microvascular abnormalities that indicate a low-grade, unremitting, vascular obliterative pathogenic process,[74] this provides some evidence that anti-CENP-B fulfill pathogenicity criterion no. 3 as well.

The identification of CENP-B as a CCR3 ligand opens up new perspectives for the study of the pathogenic role of anti-CENP-B aAb, as proposed in Figure 2. Given that

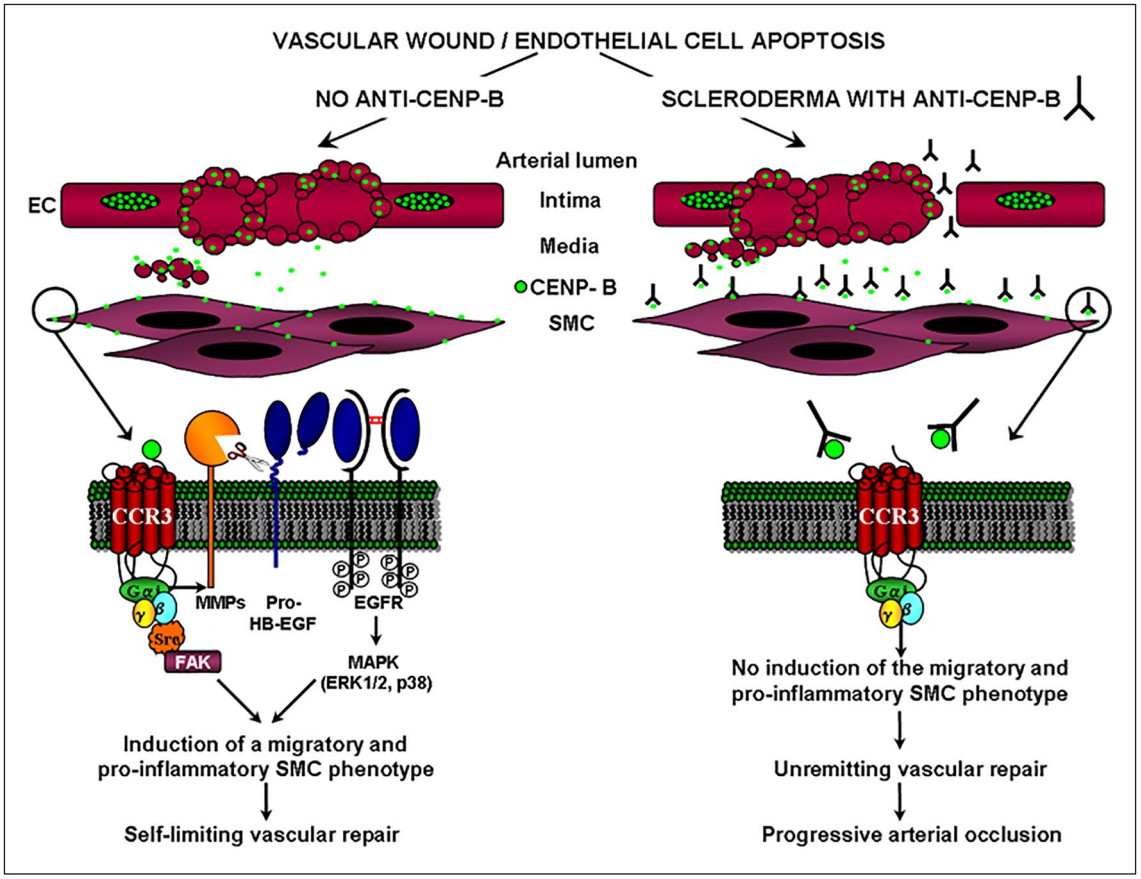

Figure 2. Proposed signaling mechanism illustrating the physiological role of centromere protein B (CENP-B) in vascular smooth muscle cells (SMC) as contributing to the initiation of wound-repair processes (left panel) and its perturbation by anti-CENP-B autoantibodies (right panel). CENP-B/anti-CENP-B immune complexes could perturb and/or prevent the normal tissue repair processes where CENP-B is involved, thus leading to a state of unremitting vascular repair and subsequently to progression of arterial occlusion. EC, endothelial cells. Adapted from a study by Robitaille et al.[96] with permission from the publisher.

CENP-B has the hallmarks of a bifunctional molecule that participates in normal mechanisms in which SMCs are involved, the chain of signaling events involving CENP-B could lead to the induction of a migratory and proinflammatory SMC phenotype and to a rapid and localized mobilization of SMCs, thus contributing to the initiation of wound-repair processes (Figure 2).

However, given that the presence of anti-CENP-B aAbs abolished the effects of CENP-B on IL-8 production, thus preventing CENP-B from transactivating EGFR and exerting its cytokine-like activities toward vascular SMCs,[97] we speculate that CENP-B/anti-CENP-B immune complexes could prevent and/or perturb the normal tissue repair processes in which CENP-B is involved, thus leading to a state of unremitting vascular remodeling (Figure 2). This hypothesis is consistent with the concept that the SSc pathophysiologic process resembles an incomplete and ever-ongoing repair process.[166] Thus, the presence of anti-CENP-B in lcSSc patients would be directly associated with vascular damage and would directly contribute to the pathogenesis of vasculopathy. This hypothesis might explain why anti-CENP-B are associated with a prominent vascular phenotype, including PAH and severe digital ischemia.

Functional aAbs to endothelin 1 type-A receptor and angiotensin II type-1 receptor

The endothelin and angiotensin II axis in SSc

Endothelin 1 (ET1) and angiotensin II (AngII) are both vasomodulatory peptides involved in the pathophysiology of SSc. ET1 is a potent vasoconstrictor produced by ECs, immune cells, and fibroblasts.[167] ET1 plasma levels are elevated in SSc patients, and expression of ET1 has also been documented in the skin of SSc patients.[168] ET1 signaling via endothelin receptor type A (ETAR) has critical roles in SSc vasculopathy and specific ETAR blockade is effective for the treatment of SSc-associated PAH and digital ulcers. ET1 also induces a fibrotic phenotype in normal fibroblasts, is expressed at higher levels by SSc fibroblasts relative to controls fibroblasts, and also has a number of proinflammatory effects.[168,169]

AngII is a vasoconstrictor peptide involved in hemodynamics regulation in kidney and heart. Serum AngII levels are elevated in dcSSc compared to lcSSc and healthy controls (HCs).[170] AngII is also present in skin from SSc patients but not in normal skin.[170] Blockade of AngII by angiotensin-converting enzyme inhibitors is effective for the treatment of SSc renal crisis. AngII signaling via type 1 angiotensin II receptor (AT1R) is implicated in the pathogenesis of fibrosis and may induce inflammatory response.[170–172] Based on that evidence, it has been hypothesized that functional agonistic anti-AT1R and anti-ETAR aAbs may contribute to the pathogenesis of SSc.

Clinical pathogenicity criteria

The presence of anti-AT1R and anti-ETAR IgG as measured by ELISA in sera from SSc patients was first reported by Riemekasten et al.[104] who investigated 478 patients from three independent cohorts, 372 HCs and 311 disease control subjects (rheumatoid arthritis (RA), n = 208; Sjögren syndrome (SjS) n = 38; primary RP, n = 32; morphea, n = 33). No SLE controls were included. A strong correlation between the presence of anti-AT1R and anti-ETAR was found ($r = 0.917$, $p < 0.01$). Although no aAb frequencies were reported, a significant proportion of HCs and disease controls expressed anti-AT1R and anti-ETAR aAbs and the frequencies of these aAbs in SSc patients could be estimated respectively as 35% (n = 65/186) and 59% (n = 110/186). In a subsequent study by the same investigators in SSc patients with PAH, and frequencies of anti-AT1R and anti-ETAR aAbs were 69.1% and 65.4%, respectively.[105] With respect to classical SSc aAbs, an association between anti-topo positivity and lower levels of anti-AT1R and anti-ETAR aAbs was noted in the former study, whereas no relationship was mentioned in the latter.

Although anti-AT1R and anti-ETAR are present in the majority of SSc patients, they are not specific for SSc. In the first study by Riemekasten et al.,[104] data analysis using the arbitrary cutoffs defined by the authors shows that these aAbs are present in HCs and disease controls as well. Moreover, in the second study, anti-AT1R aAbs were also present in patients with idiopathic pulmonary arterial hypertension (iPAH, frequency 21%), chronic thromboembolic pulmonary hypertension (CTEPH, 8%), and congenital heart disease pulmonary hypertension (CHDPH, 21.4%).[105] Anti-ETAR aAbs were also present in 11.3% of iPAH, 0% of CTEPH and 14.3% of CHDPH patients. Moreover, anti-ETAR aAbs have been reported in patients with SLE and PAH (frequency of 42.1% vs 16.3% in SLE without PAH),[106] vascular dementia (91%),[107] benign prostatic hypertrophy (60%),[108] and iPAH (87.5%).[109] Anti-AT1R aAbs of unknown isotype have been detected in malignant hypertension (14%–33%), pre-eclampsia (90%), and renal allograft rejection (100%).[110] Elevated levels of both aAbs have also been reported in patients with cystic fibrosis.[111]

The widespread distribution of these aAbs, including in non-inflammatory and non-autoimmune disease controls, suggests that major subsets of anti-AT1R and anti-ETAR are natural aAbs. Anti-AT1R and anti-ETAR positivity is clearly not specific for SSc.

Nevertheless, in the study of Riemekasten et al.[104], higher levels of anti-AT1R and anti-ETAR aAbs were

associated with severe SSc vascular manifestations, including digital ulcers, PAH, and renal crisis. Using the same assay, Avouac[112] reported that anti-ETAR aAbs were independent predictive markers of new digital ulcers in patients with active or past history of digital ulcers. Taken altogether, data from Riemekasten and Avouac may fulfill in part clinical pathogenicity criterion no. 3. Although significantly higher serum levels of these aAbs were observed in those patients compared to other control patients, whether non-specific polyclonal hypergammaglobulinemia could account for this result was not determined.

Although Becker et al.[105] reported that anti-AT1R and anti-ETAR aAbs are more frequent in PAH associated with SSc or other SARDs compared with other causes of pulmonary hypertension (PH), and could serve as predictive markers, this result should be interpreted with caution: aAb levels were overall equally high or even slightly higher in patients with SSc who did *not* develop PAH and, moreover, these results have yet to be reproduced. The Abs did not show significant correlations with hemodynamic parameters or N-terminal pro b-type natriuretic peptide (NT-proBNP) levels.[105]

Finally, given that endothelin receptor blockers such as bosentan and angiotensin receptor antagonists such as captopril are therapeutic agents in SSc, this raises the question whether the beneficial effect of these drugs could be mediated by blocking the agonistic effect of anti-AT1R and anti-ETAR aAbs. However, this has not been demonstrated thus far. Interestingly, immunoadsorption of functional aAbs (anti-α1 adrenergic receptor and anti-ETAR) on cardiac proteins has been successfully used in dilated cardiomyopathy, a common cause of heart failure.[109] However, no study has been reported on immunoadsorption of anti-AT1R and anti-ETAR in SSc and it remains to be established whether removal of these aAbs would fulfill clinical pathogenicity criterion no 4.

Experimental pathogenicity criteria

The capacity of anti-AT1R and anti-ETAR aAbs to cause lesions relevant to SSc pathophysiology was assessed both in vitro and in vivo using purified whole IgG from SSc serum samples testing positive for anti-AT1R and anti-ETAR aAbs (SSc-IgG) or IgG from HCs.[104,113] A major pitfall is that results were obtained using pooled total SSc-IgG testing positive for both aAbs so that the specific mechanistic effect of each aAb could not be ascertained. In addition, no data were provided with respect to the presence in the serum samples studied of other SSc aAbs, such as anti-topo or anti-CENP-B, so that it cannot be ruled out that some of the biological effects observed were due not to anti-AT1R or anti-ETAR but to other aAb specificities present in the total IgG samples.

Nevertheless, in vitro tests were performed using fibroblasts, human microvascular endothelial cells (HMECs) and immune cells, the complex interplay of which is relevant to SSc pathophysiology. Both anti-AT1R and anti-ETAR containing whole IgG fractions were biologically active, as they increased profibrotic transforming growth factor beta (TGF-β) gene expression in HMECs[104] and increased type-1 collagen expression in treated healthy fibroblasts.[113] In addition, HMEC activated by SSc-IgG showed dose-dependent increased secretion of IL-8, although with high interindividual variability.[113] Furthermore, supernatants of SSc-IgG-treated HMECs increased HC polymorphonuclear neutrophils migration and generation of reactive oxygen species (ROS). SSc-IgG also reduced cell-layer repair in a scratch assay performed with HMECs.[114] Interestingly, the effects on migration, wound repair, and collagen expression were dependent on anti-AT1R and anti-ETAR levels and were attenuated by receptor antagonists, thus arguing for direct receptor activation by binding of each aAb.

Both AT1R and ETAR have been detected on the surface of peripheral blood mononuclear cells (PBMCs) from HCs and SSc patients.[114] Interestingly, expression of both receptors was decreased in SSc patients compared with HCs but with some differences: AT1R density was reduced on SSc CD3T cells and SSc CD14 monocytes, whereas ETAR density was reduced only on SSc CD3T-cells. Moreover, expression of both receptors correlated negatively with disease duration, suggesting that anti-AT1R and anti-ETAR aAbs may diminish the expression of their cognate receptors in PBMCs of SSc patients in the long term. In addition, both aAbs induced T-cell migration in an aAb level-dependent manner and production of IL-8 and CCL18 by PBMCs at higher levels than did the IgG of HCs.

Interestingly, to explore whether anti-AT1R and anti-ETAR could be pathogenic in vivo, passive transfer into mice was performed with repeated intravenous infusions of pooled total SSc-IgG testing positive for anti-AT1R and anti-ETAR antibodies in healthy C57BL-6 mice.[105,113] Results are of potential interest with respect to pathogenicity criteria nos. 5 and 7, since human IgG was detected in frozen murine lung sections 7 days after a single injection of SSc-IgG but not with HC-IgG. However, the antigenic specificity of bound IgG was not determined.[105] Moreover, histological analysis demonstrated structural lung alterations, with increased cellular density, increased cellular interstitial infiltrations,[113] and inflammatory pulmonary vasculopathy.[105] No staining specific for collagen was reported. Increased neutrophil count was found in bronchoalveolar lavage fluid of SSc-IgG-treated mice as compared with HC IgG-treated mice, whereas no differences were observed in the counts for macrophages, lymphocytes, or eosinophils.[113] Of note, in the study by Kill et al., 71% of patients whose sera were used for animal experiments had anti-topo aAbs, but the titer of anti-topo in the pooled IgG fractions was not reported. Therefore, it cannot be ruled out that the results described are due to other aAbs.

Conclusion—some evidence in favor of pathogenicity criteria 5 and 7 for anti-AT1R and anti-ETAR

Although these data are of potential interest, their interpretation is currently limited by several issues, including lack of disease specificity of anti-AT1R and anti-ETAR for SSc, a potential confounding effect of SSc polyclonal B-cell activation on serum aAb levels, the use of pooled total serum IgG rather than affinity-purified anti-AT1R and anti-ETAR in the various in vitro and in vivo experiments, and cross-reactivity between AT1R and ETAR. No clinical or experimental pathogenicity criterion is definitely fulfilled by anti-AT1R and anti-ETAR at present. However, some evidence supports criteria nos. 5 and 7 (Table 3). Therefore, although anti-AT1R and anti-ETAR may be *pathogenetic* in SSc, additional research is needed before a definitive conclusion can be drawn with respect to any pathogenic role in this disease.

In future studies, using affinity-purified anti-AT1R and anti-ETAR aAbs in passive-transfer experiments would be of interest. This would eliminate the confounding functional effects of other aAbs, including anti-topo 1. Given the apparent association with SSc vascular complications, determining whether passively transferred purified anti-AT1R or anti-ETAR are deposited in murine blood vessels would also be of interest. Future studies could also take stock from studies of anti-ETAR aAbs in patients with dementia or benign prostatic hypertrophy showing that these aAbs may have different and specific epitopes.[107,108] Given the presence of anti-AT1R and anti-ETAR aAbs in HCs as well as in a variety of disease controls and in SSc patients without apparent vascular lesions, epitope mapping studies and better characterization of the specific effect of anti-AT1R and anti-ETAR may reveal if pathogenic effects, if any, are related to recognition of SSc-specific epitope(s).

Functional aAbs to muscarinic type-3 receptor

The potential role of muscarinic type-3 receptor in SSc

The gastrointestinal (GI) tract is the most commonly affected internal organ in SSc, with the esophagus often being the first affected internal organ in early disease.[173] While dysmotility accounts for the vast majority of SSc GI manifestations and may affect up to 90% of patients during disease course, its pathogenesis is poorly understood. SSc-associated GI dysmotility may evolve in four steps, beginning by early vascular involvement followed by neural dysfunction, then smooth muscle atrophy and finally muscle fibrosis.[174]

Muscarinic receptors are GPCRs and comprise five distinct subtypes named muscarinic M1, M2, M3, M4, and M5 receptors (for a review, see the study by Eglen[175]). Acetylcholine secreted after stimulation of the muscarinic type 3 receptor (M3R) is the principal excitatory mediator of GI tract motility acting on intrinsic neurons in the myenteric plexus. Transgenic mice studies support a major role for these receptors as, in mutated mice lacking functional M3R, isolated GI smooth muscle motility to muscarinic agonists is impaired by 77%, the residual contraction being mediated by M2 receptors.[175] Interestingly, M3R are also found in the vascular system where they have been implicated in vasodilatation, vasoconstriction, and endothelial barrier function. In diseases with damaged endothelium layer, such as SSc microangiopathy, M3R activation could cause vasoconstriction and ischemia, similar to the first stage previously evoked.[174] These data led to the hypothesis that circulating antagonistic aAbs inhibiting M3R signaling might inhibit excitatory enteric neurotransmission, therefore causing dysmotility in SSc patients.

Clinical pathogenicity criteria

Using indirect immunofluorescence with rat intestine as substrate, Howe et al.[115] were the first to report the presence of anti-myenteric neuronal IgG aAbs in 19 of 41 (46%) patients with SSc. No aAbs were found in sera from 22 HCs and in disease controls (idiopathic GI dysmotility, n = 5; RA, n = 20; SLE, n = 20). However, the frequency of GI dysfunction in patients with anti-myenteric neuronal aAbs was not significantly different from patients without these aAbs (69% vs 76%, respectively). Goldblatt et al.[116] reported that M3R-mediated colonic contractions were inhibited by IgG fractions from 7 of 9 patients with SSc, 4 of 4 patients with primary SjS and 3 of 3 patients with secondary SjS while IgG fractions from HCs were not inhibitory, providing indirect evidence that anti-M3R may exist in patients with these SARDs.

The largest SSc cohort investigated for anti-M3R antibodies was reported by Kawaguchi et al.[117] Using an enzyme immunoassay–detecting aAbs against the second loop domain of M3R and a cut-off optical density (OD) determined in comparison with 70 HCs, the mean OD was significantly higher in SSc patients.[117] Anti-M3R aAbs were found in 12 of 76 (15.7%) SSc patients but their frequency in HCs was not mentioned. When patients were classified as with (group 1, n = 14) or without (group 2, n = 62) severe GI dysmotility in the first 2 years of their disease, anti-M3R aAbs were significantly more prevalent in group 1 than in group 2 (64% vs 5%, $p < 0.0001$). Moreover, the mean OD of anti-MR3 was significantly higher in group 1 versus group 2. Interestingly, as reported by Howe with anti-myenteric neuronal aAbs,[115] anti-CENP

aAbs were significantly less prevalent in patients with anti-M3R than in patients without anti-M3R aAbs (0% vs 37.5%, p=0.01).[117] Patients with anti-M3R also had a higher frequency of GI involvement and lcSSc and a lower frequency of interstitial lung disease (ILD) compared with patients without anti-M3R, although overall no distinctive phenotype was demonstrated.

In summary, although the frequency of GI involvement appears higher in the presence of anti-M3R aAbs, the high frequency of GI involvement in patients *without* such aAbs (n=12/12, 100% vs n=43/64, 67%, respectively) suggests that the pathophysiology of GI dysmotility in SSc may be multifactorial. Moreover, anti-M3R aAbs are not specific for SSc patients, as they have been reported in various conditions, including primary or secondary SjS,[118–120] orthostatic hypotension[121] and primary biliary cirrhosis.[122,123] Longitudinal studies of anti-M3R aAbs are not available. Thus, none of clinical pathogenicity criteria nos. 1 to 4 are definitely fulfilled at present, although some evidence favors criterion no 3 (Table 3).

Experimental pathogenicity criteria

Several in vitro studies assessed the effect of purified total serum IgG from SSc patients on M3R-mediated neurotransmission and SMC contraction using rodent colon tissues, and human or rat internal anal sphincter.[124,125] In vitro, IgGs from SSc patients but not from HCs caused significant, concentration-dependent, inhibition of M3R-mediated contractions. Western-blot studies also demonstrated the presence of SSc-IgG-M3R complexes in rat and human tissue lysates, suggesting that GI dysmotility in SSc may be caused by aAbs that inhibit muscarinic neurotransmission.[124,125]

Interestingly, one study reported differential binding of SSc-IgG to myenteric neurons (MNs) or SMCs according to disease duration.[126] SSc-IgG demonstrated higher binding to MNs than to SMCs but, with progression of disease duration, binding at MNs and SMCs increased in a linear fashion. This led to the conclusion that GI dysmotility in SSc occurs sequentially, beginning with SSc-IgG-induced blockage of cholinergic neurotransmission (i.e. neuropathy), which then progresses to inhibition of acetylcholine action at SMCs (i.e. myopathy).[126]

In an in vivo study, passive transfer into a rat model of purified whole IgG from a single SSc patient with anti-myenteric neuronal aAbs led to intestinal myoelectric activity alterations.[127] No in vivo experimental study has been reported using purified IgG anti-M3R SSc aAbs. Finally, Preuss et al.[119] developed a highly reproducible in vitro luminescence-based method for the detection of functional antagonist aAbs that inhibit M3R. However, although inhibitory aAbs to M3R were present in 50% of 40 patients with primary SjS, they were present in none of 47 patients with SSc.

Conclusion—some evidence in favor of pathogenicity criteria nos. 3, 5, and 7 for anti-M3R aAbs

Anti-M3R aAbs are clearly not specific for SSc, but nevertheless they seem associated with GI involvement in this disease. Moreover, in vitro assays suggest that anti-M3R aAbs may impair M3R-mediated neurotransmission and contraction of SMCs. However, the pathogenic effect of these aAbs remains to be established. As in the case of anti-AT1R and anti-ETAR aAbs, the major pitfall is that results were obtained using total SSc-IgG from sera that tested positive for anti-M3R so that the specific pathogenic effect of the aAbs could not be assessed. Overall, anti-MR3 are *pathogenetic* in SSc but available evidence only weakly supports pathogenicity criteria nos. 3 and 5, whereas some more robust evidence is in favor of criterion 7 (Table 3).

Future studies should focus on the development of standardized assays for the detection of anti-M3R because the current absence of such assays likely affects the reported frequencies and clinical associations of these aAbs. For example, using the same assay as Kawaguchi et al.,[117] Naito et al.[118] reported that the frequencies of anti-M3R aAbs in primary versus secondary SjS were 9% and 14%, respectively. However, another team using also an enzyme immunoassay but with a different target epitope reported a much higher frequency (90%) of anti-M3R in primary SjS.[120] These discrepancies suggest that epitopes targeted by anti-M3R aAbs may vary from one disease to another. Using standardized functional assays for one specific disease would also help to better characterize the potential pathogenic effects of anti-M3R aAbs. Finally, longitudinal studies of anti-MR3 would be of interest.

Functional aAbs to platelet-derived growth factor receptor

The platelet-derived growth factor and its receptor in SSc

The platelet-derived growth factor (PDGF) stimulates proliferation of fibroblasts and SMCs (for a review, see study by Andrae et al.[176]). PDGF is secreted by platelets, monocytes, macrophages and ECs, and exerts its biological functions by activating two tyrosine kinase receptors, PDGFRα and PDGFRβ.[176] PDGFR activation leads to the induction of several signaling pathways resulting in cellular proliferation, chemotaxis, and actin reorganization. PDGF signaling has been associated with several human

disorders including atherosclerosis, cancer, PH as well as fibrosis of various organs.[176]

Studies conducted in SSc have shown:[177,178]

- Increased presence of PDGF and PDGFR in SSc skin biopsies;
- Expression of PDGF in ECs of small capillaries and in mononuclear perivascular infiltrates;
- Elevated levels of PDGF-A and PDGF-B in the bronchoalveolar fluid or skin blister fluid obtained from SSc patients;
- Response of SSc fibroblasts (unlike normal fibroblasts), to TGF-β with upregulation of PDGFRα; and
- Existence of an autocrine PDGF-A/PDGFRα loop operating in SSc fibroblasts.

Given these data showing that PDGF signaling could be involved in the fibrosis, immune dysfunction and vasculopathy of SSc,[177] whether agonistic aAbs to PDGFR may contribute to the persistent PDGF signaling in SSc was therefore investigated.

Clinical pathogenicity criteria

In 2006, using a functional assay as detection method, Baroni et al.[128] reported the presence of agonistic aAbs to PDGFR in whole IgG purified from the sera of all 46 (100%) investigated SSc patients. The agonistic activity of these aAbs was higher in patients with early lcSSc than in patients with late disease. Moreover, in patients with dcSSc, there was a trend toward higher values among patients with early disease (duration <3 years) than among patients with late disease (>6 years). No correlation was found with other clinical and serologic features, including for severity of skin fibrosis.

As no agonistic anti-PDGFR aAbs were detected in serum of patients with SLE, RA, primary RP, or ILD without SSc, this was taken as evidence that anti-PDGFR aAbs were highly sensitive and specific for SSc.[128] However, using the same assay, anti-PDGFR aAbs were also detected in patients with extensive cutaneous graft versus host disease (GVHD) after allogeneic bone marrow transplantation.[129] Although GVHD and SSc are commonly cited as having a similar pathophysiology, the latter study primarily demonstrated that agonistic anti-PDGFR aAbs are not specific for SSc.

Subsequently, using different but well-characterized functional assays, two other groups failed to detect agonistic anti-PDGFR aAbs in SSc sera.[130,131] Moreover, using an electrochemiluminescence PDGFR binding assay, Loizos et al.[131] demonstrated that 34% of sera from HCs and only 33% of sera from SSc patients contained detectable anti-PDGFR aAbs. In-keeping with the latter findings, Balada et al.[132] reported that frequencies of anti-PDGFR as measured by ELISA and immunoblotting are closely similar in women with SSc (20.7%) as compared to healthy women (19.4%). These data suggest that a major subset of anti-PDGFR are natural aAbs. With respect to pathogenicity criteria nos. 1 and 3, although patients with dcSSc seemed to have higher levels of anti-PDGFR than those with lcSSc, this difference was not statistically significant and no correlation was found with any clinical or immunological feature.[132]

In order to investigate these discrepant results, the immune repertoire of one SSc patient was directly investigated by Moroncini et al.[133] and led to the identification of two distinct conformational epitopes recognized by agonistic aAbs to PDGFR and one linear epitope recognized by non-agonistic aAbs. Two different epitope-based assays were further developed allowing either detection of only agonistic anti-PDGFRα aAbs or of all anti-PDGFRα aAbs, regardless of the epitopes recognized.[133]

Using this assay, total anti-PDGFRα aAbs were detected in 66 of 70 SSc patients (94.3%), 63 of 130 HCs (48.5%), 11 of 26 primary RP patients (42.3%), and 11 of 29 SLE patients (37.9%; $p < 0.0001$ between SSc patients versus the other groups). In contrast, agonistic aAbs were found in 24 of 34 (70.6%) SSc patients, but neither in HCs nor in primary RP or SLE. It was concluded that both agonistic and non-agonistic aAbs to PDGFRα may coexist in the same SSc patient and that agonistic aAbs to PDGFRα are enriched in SSc sera.[133]

It would be important to confirm independently these results. Furthermore, as clinical features were not reported in the study, it cannot be concluded whether the presence of agonistic anti-PDGFR aAbs is specifically associated with a more severe phenotype in SSc patients compared to patients with non-agonistic aAbs.

Experimental pathogenicity criteria

Using mouse embryo fibroblasts expressing human PDGFRα, total serum IgG containing agonistic anti-PDGFR aAbs from SSc patients were able to recognize native PDGFR, induce tyrosine phosphorylation and ROS accumulation.[128] Purified total IgG induced the Ha-Ras-ERK1/2 and ROS cascades and stimulated type-I collagen gene expression and myofibroblast phenotype conversion in normal human primary fibroblasts, similar to histological findings observed in SSc patients.

Moreover, total IgG with anti-PDGFR aAbs stimulated ROS production in mouse embryo fibroblast in a dose-dependent manner, and ROS-inducing activity was higher in patients with early lcSSc (duration <5 years) than with late disease (duration >10 years; $p < 0.01$).[128] It is noteworthy that non-fibroblastic cell lines (32D myeloid cells and porcine aortic ECs) were used in the two studies[130,131] that did not confirm the results obtained by Baroni et al.[128] with the mouse embryo fibroblast model. Silica, an environmental risk factor for SSc,

induces AFAs in asbestos-exposed mice and anti-topo and anti-PDGFR aAbs are components of these AFAs, both of which could potentially contribute to the profibrotic fibroblast phenotype encountered in SSc.[134] Whether agonistic anti-PDGFR aAbs detected in SSc patients by Baroni et al.[128] are specific AFAs as well remains to be determined.

Finally, a single in vivo study investigated the role of agonistic anti-PDGFR in a skin-humanized severe combined immunodeficiency (SCID) mice model engrafted with skin derived from SSc patients or HCs.[135] SSc skin grafts originally exhibited an SSc-like cutaneous phenotype which was not sustained, that is, by 24 weeks, it was indistinguishable from HC skin graft. This was in accordance with previous demonstration that SSc fibroblasts need extracellular factors to maintain their SSc phenotype when explanted in vitro. In contrast, in mice carrying HC skin grafts and injected with pooled IgG containing anti-PGDFR aAbs from several SSc patients, this resulted in the appearance of a cutaneous SSc-type phenotype confirmed by increased collagen deposition and fibroblast activation markers. Similar results were observed when HC skin grafts were injected with human agonistic anti-PDGFR monoclonal antibodies (generated from SSc B-cells). Interestingly, oral administration of nilotinib, a tyrosine kinase receptor inhibitor (although not PDGFR-specific), inhibited the increase in type-I collagen dermal deposition induced by SSc-IgG.[135]

Conclusion—inconclusive clinical pathogenicity criteria but some intriguing experimental evidence in favor of a pathogenic role for anti-PDGFR aAbs

At least, a subset of anti-PDGFR aAbs are non-pathogenic natural aAbs. The disease specificity of anti-PDGFR aAbs for SSc is difficult to establish and depends on the assays used for detection. Assays that are immunodetection-based have clearly demonstrated that anti-PDGFR are not specific for SSc, whereas the functional assays of Baroni et al.[128] argue for the specific and sensitive presence of agonistic anti-PDGFR aAbs in SSc patients. However, in the latter assays, sensitivity largely depends on the type of functional assay used and has yet to be confirmed independently. Moreover, no clear correlation has been established with clinical features or severity of SSc, such as the extent of skin fibrosis or the presence of lung fibrosis. Therefore, data appear inconclusive at present and multicenter comparative studies will be needed to assess the exact frequency and specificity of agonistic anti-PDGFR aAbs in SSc, other fibrotic diseases and other SARDs. This knowledge would be of interest, given experimental evidence in favor of pathogenicity criterion no. 5 (Table 3). As in the case of anti-ETAR and anti-AT1R aAbs, interpretation of pathogenicity data (including blockage by the tyrosine kinase inhibitor nilotinib) is limited by the use of total serum IgG rather than purified anti-PDGFR aAbs.

Last, it is unlikely that aAbs directed to this unique PDGFR target could explain completely the pathogenesis of the various SSc phenotypes.[5]

Functional aAbs to chemokine receptors CXCR3 and CXCR4

Chemokine receptors CXCR3 and CXCR4 are GPCRs that allow the migration of several cell types and are involved in the pathogenesis of fibrosis. The first report on anti-CXCR3 and/or anti-CXCR4 aAbs in SSc was by Weigold et al.[136] Anti-CXCR3 and anti-CXCR4 aAb levels were measured by commercial ELISA in 449 serum samples obtained from 327 SSc patients and in 234 sera from HCs and analyzed both cross-sectionally and longitudinally for associations with clinical features.

The frequency of anti-CXCR3 and anti-CXCR4 in the two groups was not clearly specified and no other disease control group was reported. The levels of the two aAbs strongly correlated with each other ($r = 0.85$). Compared with HCs, SSc patients had higher median values of anti-CXCR4 aAbs but not of anti-CXCR3 aAbs. However, when patients were classified according to SSc subsets, dcSSc patients had higher levels of both aAbs than lcSSc patients and HCs. Moreover, anti-topo positive patients also exhibited higher levels of anti-CXCR3 aAbs than anti-topo negative patients.[136]

Studies of aAb levels according to the presence of lung involvement yielded conflicting and counterintuitive results. In the cross-sectional study, patients with SSc-ILD and forced vital capacity (FVC) $<70\%$ of predicted normal value had significantly lower median anti-CXCR3 and anti-CXCR4 levels than patients with FVC $\geqslant 70\%$ ($p < 0.01$ and $p < 0.05$, respectively). Paradoxically, significant negative correlations were found between aAb levels and lung function parameters, suggesting that higher aAbs levels were associated with more severely impaired lung function. Moreover, median aAb levels were higher in SSc patients with ILD versus those without ILD. No correlation was found with the modified Rodnan skin score (mRSS) and cardiac parameters.[136]

However, in the longitudinal study, high aAb levels corresponded to and predicted stable lung function. For example, 44% of patients with low levels of anti-CXCR3, as defined by a value <6.2 U, showed a reduction in FVC $>10\%$ over 3 years of follow-up compared to only 13% of the patients with higher levels.[136] Similar results were obtained with anti-CXCR4 aAbs (55% vs 11%, respectively). Thus, anti-CXCR3 and anti-CXCR4 levels have a conflicting prognostic value, being both markers of more severe but more stable lung disease (Table 3).

Experimentally, data suggested that SSc sera preferentially bound intracellular epitopes of CXCR3, while aAbs from HCs targeted an extracellular epitope in the N-terminal domain.[137] Hopefully, these results will assist in developing a novel assay defining the aAb frequency in SSc and in disease controls, including other SARDs associated or not with ILD, and help in clarifying their prognostic value in SSc.

From a pathogenic point of view, the fact that aAbs present in SSc patients preferentially target the intracellular domain of CXCR3 is intriguing, as these epitopes are not directly accessible to aAbs. It could be hypothetized that aAbs directed against the extracellular epitope, preferentially recognized by aAbs in HCs, may have a protective effect rather than a pathogenic effect during fibrogenesis.

Functional aAbs to estrogen receptor α

Estrogens (E2) regulate immune responses via transcriptional activities of intracellular estrogen receptors (ER) α and β or by membrane-associated ER. All immune cells express intracellular ER while membrane ER have been detected on the plasma membrane of lymphocytes (for a review, see study by Ortona et al.[179]). The regulation of immune responses via E2 depends not only on its concentration but also on the type of target immune cell and occurs at multiple levels, including cell proliferation, cytokine production, stimulation of antibody production, apoptosis of immune cells, and finally, enhancing the number and function of CD4+ CD25+ regulatory T-cells (T^{reg}).

Anti-ERα aAbs have been detected in sera of up to 45% of SLE patients.[138,139] An association between anti-ERα titer and SLE activity was also observed, and in vitro assays demonstrated that these aAbs induced proliferation of anti-CD3-stimulated T-cell as well as T-cell activation and consequent apoptotic cell death of resting lymphocytes.[139] As SLE and SSc share some epidemiological and biological features, namely female predominance and a partially common interferon signature, the presence of aAbs to ERα in SSc was investigated. Thus, Giovannetti et al.[140] reported the presence of IgG anti-ERα aAbs in sera from 30 of 71 (42%) SSc patients while no aAbs were detected in HCs. When patients were subclassified according to anti-ERα status, the presence of anti-ERα was significantly associated with dcSSc, positive anti-topo aAbs and a late capillaroscopic pattern, and they also correlated with SSc activity. Anti-ERα levels were also significantly associated with alterations of immunological features, including increased T-cell apoptotic susceptibility and changes in T^{reg} homeostasis (Table 3).[140]

Thus, anti-ERα are not specific for SSc, as their frequency in this disease is similar to SLE. However, they seem to correlate with SSc activity, although this may be due to their association with anti-topo aAbs. Further in vitro and in vivo studies will be needed to clearly assess whether these intriguing aAbs may exert a pathogenic role by modulating the immune system of some SSc patients.

Functional anti-CD22 aAbs

CD22 is a negative regulator of B-cell receptor signaling. As B-cells are hyperactivated in SSc, the presence and functional properties of anti-CD22 IgG aAbs in patients with SSc and Tight Skin (TSK/+) mice were investigated.[141] Anti-CD22 were detected by ELISA in 80% (n = 8/10) of sera from TSK/+ mice and in 22% (n = 11/50) of SSc patients, but not in HCs. Reactivity of positive SSc sera by ELISA was confirmed by immunoblotting using recombinant human CD22. Anti-CD22 aAbs were not specific to SSc, as their frequency in SLE patients was similar (20% vs 22%, respectively).

The frequency of anti-CD22 aAbs was higher in patients with dcSSc versus lcSSc (35% vs 13%, respectively), but this was not statistically significant. Anti-CD22-positive SSc patients had higher mRSS than those negative for these aAbs (median 13 vs 5, $p < 0.05$) and tended to have more frequent esophageal involvement (89% vs 47%, $p = 0.06$). Patients with anti-CD22 had higher median levels of pulmonary surfactant protein D, a biological marker of ILD (137 ng/L vs 73.2 ng/L, $p < 0.05$). Among patients with dcSSc, the presence of anti-CD22 also tended to be associated with lower values of vital capacity (78.2% vs 99.2%, $p = 0.06$). Of note, the frequency of anti-topo aAbs and median values of serum IgG levels were higher, although not significantly so, in patients with versus those without anti-CD22. It would be of interest to explore further these potential associations in larger cohorts of SSc patients.

In vitro experiments showed that anti-CD22 in sera of SSc patients was not only able to modulate B-cell response by reducing tyrosine phosphorylation of CD22 but also by increasing tyrosine phosphorylation of CD19.[141] Interestingly, sera from SLE patients positive for anti-CD22 were also able to reduce phosphorylation of CD22 at the same levels observed with SSc-positive sera. By reacting with CD22, a major inhibitory B-cell response regulator, functional anti-CD22 aAbs, although not disease specific, may stimulate B-cell activation in SSc and SLE.[141]

Finally, as acknowledged by the authors, anti-CD22 aAbs may not have per se the ability to induce fibrosis, but they may serve in a subset of SSc patients as an aggravating pathogenic factor in conjunction with other profibrotic mechanisms specific for SSc that are not dysregulated in SLE.[141]

Anti-matrix metalloproteinases 1 and 3

SSc is characterized by multi-organ fibrosis due to excessive accumulation of extracellular matrix (ECM) components

resulting from imbalance between its production and degradation. As ECM degradation is regulated mainly by matrix metalloproteinases (MMPs), aAbs to MMPs (anti-MMPs) in SSc were investigated.

The presence of anti-MMP-1 aAbs in SSc patient sera was first reported by Sato et al.[142] who also reported subsequently on anti-MMP-3 aAbs.[143] In the former study, frequency and levels of IgG and IgM anti-MMP-1 were investigated by ELISA in 57 patients with SSc (33 lcSSc, 24 dcSSc), 19 with SLE, 16 with diabetes mellitus (DM) and 30 HCs.[142] IgG anti-MMP1 were significantly more common in SSc patients (40%, $n = 23$) than in SLE (5%, $n = 1$, $p < 0.05$) and DM (5%, $n = 1$, $p < 0.01$) patients or in HCs (3%, $n = 1$, $p < 0.001$). IgM anti-MMP-1 were detected in 23% of SSc ($n = 13$), 11% of SLE ($n = 2$), 6% of DM ($n = 1$) patients and in no HC. Within SSc subsets, IgG anti-MMP-1 aAbs were more frequent in dcSSc (75%, $n = 18/24$) than in lcSSc patients (15%, $n = 5/33$) ($p < 0.001$), whereas IgM anti-MMP-1 were detected in 29% ($n = 7$) of dcSSc and 18% ($n = 6$) of lcSSc.

In addition, IgG anti-MMP-1 levels were higher in dcSSc than SLE and DM ($p < 0.0005$) or HCs ($p < 0.0001$).[142] In contrast, IgM anti-MMP-1 levels were significantly higher only in patients with dcSSc versus HCs ($p < 0.05$). IgG and IgM anti-MMP-1 correlated neither with total IgG and IgM serum levels nor with levels of anti-topo or ACAs. Lung fibrosis was more prevalent in patients with versus those without IgG anti-MMP-1 (61% vs 29%, respectively, $p < 0.05$). IgG anti-MMP1 levels correlated positively with mRSS ($p < 0.0002$) and negatively with the diffusing capacity of the lungs for carbon monoxide (DLCO; $p < 0.005$) and vital capacity ($p < 0.01$). Thus, IgG anti-MMP-1 appeared specific for SSc and correlated with severity of skin and lung fibrosis. Results obtained by the same team on the frequency, levels, and clinical correlations of IgG anti-MMP-3 aAbs in SSc sera were very similar to anti-MMP-1 Abs.[143]

Potential pathogenicity of anti-MMP-1 aAbs was assessed in vitro by inhibition of MMP-1 activity. Thus, total serum IgG from SSc patients with anti-MMP-1 inhibited collagenase activity of MMP-1 by 77% compared to HCs ($p < 0.001$).[142] Comparison of dcSSc versus lcSSc patients was not reported. Similarly, MMP-3 activity was inhibited by total serum IgG from patients with anti-MMP-3 aAbs.[143] Finally, Sato et al. showed that anti-MMP-1 and anti-MMP-3 aAb levels correlated both for IgM and IgG isotypes but without cross-reactivity.

While these results will need to be expanded, they suggest that anti-MMP-1 and anti-MMP-3 aAbs represent independent but cooperating aAb systems that may contribute to the development of fibrosis in SSc by inhibiting MMP-1 and MMP-3 activity, respectively, thereby reducing ECM turnover (Table 3).

Anti-fibrillin 1 aAbs

Fibrillin 1 gene mutations have been implicated in the pathogenesis of the TSK-1 murine model of SSc as well as in human diseases like SSc in the Choctaw American Indian population and in Marfan syndrome. As circulating anti-fibrillin 1 aAbs (anti-FBN1) have been detected in TSK-1 mice, the presence of these aAbs in SSc patients has been investigated in carefully controlled studies.[144,145]

The first study used recombinant FBN1 containing the proline-rich C region of FBN1 targeted by anti-FBN1 in TSK-1 mice, allowing detection of IgG and IgM anti-FBN1 aAbs[144] in SSc patients. In comparison to control patients originating from the same ethnic groups, IgG anti-FBN1 were detected with the highest frequencies in Choctaw American Indian and Japanese SSc patients (81% and 78%, respectively) and less commonly in Caucasians (31%). A majority of Choctaw and Japanese patients expressed high titers of IgM anti-FBN1 as well. In contrast, the frequency of IgG anti-FBN1 in African American SSc patients was similar to normal controls (4% vs 5%, respectively).[144] When patients were classified into disease subsets, the frequency of IgG anti-FBN1 was significantly higher in dcSSc compared to lcSSc (37% vs 9%) patients, but curiously, the frequency was higher in patients with the full CREST syndrome (51%). A low frequency of IgG anti-FBN1 was observed in SLE (1%) and SjS (6%), whereas they were not detected in RA or morphea. Interestingly, a high frequency of anti-FBN1 was also observed in patients with mixed connective tissue disease (MCTD, 34%), MCTD plus SSc (30%), and in polymyositis dermatomyositis (PMDM, 40%), indicating that IgG anti-FBN1 aAbs are not specific for SSc.

These ethnic differences were later reexamined by the same team,[145] using three recombinant peptides representing different domains of FBN1. Ethnic differences in epitope specificity of anti-FBN1 were reported.[145] However, this time, the highest frequencies of IgG and IgM were observed not only in Choctaw and Japanese SSc patients, but also in African American SSc patients (100%, 80%, and 80% of sera with IgG anti-FBN1 recognizing at least one epitope, respectively), while Caucasian SSc patients still had the lowest frequency (42%) and PMDM patients showed no increased frequency compared to ethnically matched controls. The presence of anti-FBN1 did not correlate with any major clinical manifestations or SSc classic aAbs. In the same study, the overall frequency of anti-FBN1 was 47% in another multiethnic SSc cohort.[145]

In vitro, normal human fibroblasts were treated with affinity purified IgG anti-FBN1 isolated from SSc patients and then examined for alterations in gene and protein expression of collagen and other ECM components.[146] As neutralization of TGF-β1 signaling significantly diminished the activation of fibroblasts by anti-FBN1, a

pathogenic mechanism was proposed where interaction of anti-FBN1 aAbs with the ECM leads to TGF-β1 release and activation of fibroblasts into a profibrotic phenotype resembling that of SSc.[146]

Although these experimental results may support some pathogenic role for anti-FBN1 aAbs, the absence of correlation with clinical features such as cutaneous involvement appears contradictory. Moreover, using two recombinant, overlapping, and correctly folded peptides of FBN1 and another method of detection, Brinckmann et al.[147] failed to detect anti-FBN1 aAbs in a cohort of Caucasian SSc patients and ethnically matched controls. It was concluded that the presence of anti-correctly folded FBN1 aAbs is not a primary event in the pathogenesis of SSc in Caucasian patients.[147]

Miscellaneous recently described aAbs

In this last section, recently described aAbs of potential pathogenic interest are briefly reviewed. In most instances, few reports are available and little experimental data have been published thus far.

Anti-IFI16 aAbs

The presence of aAbs against interferon-inducible gene IFI16 in SSc was investigated in two cohorts.[148,149] In the first study, patients with SLE, primary SjS, RA, chronic urticaria, hepatitis C virus (HCV) infection, and HCs were investigated.[148] Anti-IFI16 titers above the 95th percentile for control subjects was observed in primary SjS (50%), while SSc and SLE exhibited similar frequencies of 21% and 26%, respectively. The prevalence of anti-IFI16 was slightly elevated in HCV patients (13%), and low in RA (4%) and chronic urticaria (5%). Overall, this study highlights the importance of studying several control diseases to determine the specificity of novel aAbs.

Interestingly, in patients with SSc, the prevalence of anti-IFI16 was higher in patients with lcSSc (28%) than with dcSSc (4%). Apart from the association with lcSSc, no other association with clinical and epidemiological parameters was observed.[148]

In the second study, the prevalence of anti-IFI16 aAbs in SSc was established at 29%.[149] The frequency of anti-IFI16 was not statistically different between lcSSc and dcSSc (32%, n=68/207, vs 23%, n=32/137, p=0.063). An association of anti-IFI16 with cardiac involvement was noted (p=0.026).

In conclusion, the absence of disease specificity for SSc, the possible confounding effect of hypergammaglobulinemia, the lack of clearly established association with clinical or epidemiological features and the absence thus far of experimental evidence argue against a pathogenic role for anti-IFI16 aAbs in SSc.

Anti-eIF2B aAbs

Eukaryotic initiation factor 2B (eIF2B) is a multi-subunit protein implicated in protein synthesis initiation. Inherited mutations of eIF2B cause leukoencephalopathy with vanishing white matter.[180] Anti-eIF2B IgG aAbs were recently identified by Betteridge by immunoprecipitation of SSc sera negative for known SSc aAbs.[47] In a first cohort of 548 patients, anti-eIF2B aAbs were identified in 7 (1.3%) patients and were absent in a large number of HCs and disease controls, including SLE, myositis, and ILD patients. A strong clinical association was found with dcSSc (n=6/7) and ILD (n=6/6). Two patients had myositis and two other had an RA-SSc overlap. These strong clinical associations were confirmed in a second study by the same group that focused on a large cohort of 128 SSc patients with a negative ANA.[150] Anti-eIF2B aAbs were present in 9 (7%) patients, the majority of whom had dcSSc (89%, n=8/9). ILD was present in all anti-eIF2B patients for whom chest imaging was available (n=7/9).[150]

In conclusion, anti-eIF2B aAbs appear highly specific for dcSSc and ILD and were not identified in other SARDs, although aAbs against other proteins of the eukaryotic protein initiation pathway have been detected in SLE (eIF2C)[181] and polymyositis (eIF2C).[182] No experimental data are available yet to determine the potential pathogenic role of eIF2B-AB in SSc.

Anti-ICAM-1 aAbs

ICAM-1 is an adhesion molecule expressed on several cell types, including SSc fibroblasts and injured ECs, that may result in further endothelium damage via recruitment of inflammatory cells. ICAM-1 is proposed as a biomarker for SSc, given that increased serum levels have been observed especially in patients with dcSSc, digital ulcers and renal crisis, and correlated with disease activity (for a review, see the study by Hasegawa[167]).

As anti-ICAM-1 aAbs (anti-ICAM1) were identified in sera from cardiac transplant recipients with chronic transplant vasculopathy, the presence of these aAbs in SSc patients was investigated.[151] Elevated serum levels of anti-ICAM1 of IgG isotype were detected in 32% of dcSSc and 39% of lcSSc patients. The frequency of these aAbs in HCs was not provided but analysis of the report shows that nearly 30% of HCs also had anti-ICAM1.[151] Anti-ICAM1 of IgM isotype were detected in nearly 60% of sera from both dcSSc and lcSSc patients and in almost 40% of sera from HCs. Taken altogether, these isotypic data suggest that a subset of anti-ICAM1 are natural aAbs.

The correlation between positive anti-ICAM1 and disease manifestations was not investigated but the elevated aAb levels in some lcSSc led the authors to postulate that these aAbs may be protective rather than pathogenic.[151]

However, this contradicts experimental results as purified IgG anti-ICAM1 bound to human umbilical vein ECs, leading to generation of ROS and expression of another adhesion molecule by these cells, vascular cell adhesion protein 1 (VCAM-1).[151] Thus, these results suggest that anti-ICAM1 may be pathogenic by causing proinflammatory activation of human ECs as seen in SSc, although no other EC types were investigated. Additional studies will be needed to assess the correlation with specific clinical features and characterize the potential pathogenic role of anti-ICAM1 in SSc.

Anti-RuvBL1/RuvBL2 aAbs

Autoantibodies to a complex consisting of RuvBL1 and RuvBL2 (anti-RuvBL1/2) have recently been identified in SSc patients. A monocentric Japanese cohort of 316 consecutive patients with SSc, 290 patients with other SARDs, ILD alone or autoimmune hepatitis, and 50 HCs was investigated by RNA and protein immunoprecipitation.[46] Anti-RuvBL1/2 IgG aAbs were identified in six SSc sera (1.9%) but not in other conditions. In a second Japanese cohort of 272 SSc patients, 4 additional patients were identified (1.5%). Thus, the frequency of anti-RuvBL1/2 in Japanese SSc patients is 1.7%, whereas it is 5.5% in American SSc patients.[46] SSc patients with anti-RuvBL1/2 in both the Japanese and American cohorts had higher frequencies of SSc in overlap with myositis (60% and 59%, respectively) than those without anti-RuvBL1/2. Moreover, among these patients with SSc in overlap with myositis, dcSSc was the most prevalent form, being present in 63% to 83% of patients.[46]

A subsequent study confirmed the low frequency of anti-RuvBL1/2, detected in only 2 of 128 patients (1.5%) with SSc-overlap syndromes.[150] One of these two patients had dcSSc and myositis, whereas the second patient had lcSSc and SjS. In conclusion, anti-RuvBL1/2 aAbs appear specific for dcSSc in overlap with myositis. No experimental data are available yet to clarify the potential pathogenic role of RuvBL1/2-AB in fibrogenesis and muscular inflammation.

Summary

Anti-topo is the single aAb specificity with the most evidence in favor of a pathogenic role in SSc, followed by anti-CENP-B. However, these aAbs have not been demontrated yet to fulfill completely the proposed criteria for pathogenicity. With respect to functional aAbs, their presence in normal controls and various disease controls suggests that major subsets of these immunoglobulins are natural aAbs. Current data are difficult to interpret with respect to pathogenicity because of several experimental limitations. While some of these novel aAbs may be *pathogenetic* in SSc, establishing that they are *pathogenic* is a work in progress.

Declaration of conflicting interests

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