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Pathogenic autoantibodies in systemic sclerosis

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Systemic sclerosis, scleroderma, is a disease characterized by widespread vascular injury and fibrosis of the skin and visceral organs. Circulating autoantibodies against several intracellular antigens are common in scleroderma patients. The specificities of such autoantibodies correlate with distinct clinical manifestations. However, till date there is no evidence that these autoantibodies, though helpful in diagnosis and prognosis, are linked to the pathogenesis of scleroderma nor that they may cause any feature of the disease. Recently, the discovery of novel agonistic autoantibodies targeting the PDGF receptor has provided important insight into the molecular pathogenesis of scleroderma and the intracellular mechanisms leading to fibrosis. Although their pathogenic role awaits validation in *in vivo* models, these antibodies represent the molecular link between the immune system and fibrosis.

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Introduction

Scleroderma is an autoimmune connective tissue disease of unknown cause characterized by substantial morbidity and mortality. The interest in scleroderma stems from the fact that an elucidation of its pathogenesis will lead to a better understanding of other chronic diseases characterized by fibrosis such as cirrhosis of the liver, idiopathic pulmonary fibrosis, atherosclerosis, and glomerulosclerosis.

A challenge in the development of novel therapies for scleroderma is the identification of the essential components of the pathologic network that targets fibroblasts, endothelial, and immune cells. The functional alterations in these cells lead to severe and progressive fibrosis, obliteration of the lumen of small arteries, and humoral and cellular immunologic dys-regulation. However, it is not clear whether these abnormalities are consequence or cause of the relevant manifestations. Available evidence indicates that activation of the immune cells has a central role in the pathophysiology of scleroderma. In this context, nonspecific signs of activation of the humoral 'arm' of the immune system have been known in scleroderma for a long time and include polyclonal hypergammaglobulinemia and antibodies to nuclear antigens that occur in 95% of patients [1]. Associations have been found between the presence of antibodies to specific nuclear antigens and clinical manifestations of scleroderma, making serologic tests helpful in diagnosis and prognosis. Yet, there is no evidence that such autoantibodies are responsible for the clinical manifestations of the disease.

Recent studies have suggested, however, that B cells may play a crucial role in the pathogenesis of scleroderma. Whitfield *et al.* have shown upregulation of B lymphocyte genes in clinically affected skin from scleroderma patients [2•]. Sato and colleagues have emphasized the role of B cells *in vivo* by showing that downregulation of B cell function results into improved skin fibrosis in the tight-skin-mouse model of scleroderma [3•].

In scleroderma patients, peripheral B lymphocytes are increased compared to controls and they are characterized by an expanded naïve B cell population and by reduced, but highly activated, memory B cell subset and plasmoblast component [4••]. Moreover, CD19 has been found overexpressed on naïve B cells and, to a greater extent, in memory B cells from SSc patients [5], suggesting that CD19 overexpression in SSc memory B cells induces the activated B cell phenotype and increases IgG production. The mechanism underlying CD19 overexpression in SSc is not clear. Increased serum levels of BAFF or B-cell-activating factor, a potent B cell survival factor, have been detected in an unselected scleroderma population and found to positively correlate with the severity of skin fibrosis [6]. Moreover, BAFF receptor expression on B cells as well as the production of IgGs and IL-6 by SSc B cells following BAFF stimulation have been found significantly stimulated [6]. B cell activation is mirrored by a predominant Th2 cytokine profile in scleroderma [7,8].

All these reports implicate the contribution of B cell activation to the clinical features of scleroderma.

B cell activation may contribute to the clinical features of scleroderma through two main pathways: first, increased production of IL-6 by B cells can stimulate extracellular

matrix (ECM) production [9] and second, IL-4, resulting from Th2 predominant immune response, induces the expression of TGF-β, a potent profibrotic growth factor [10], also in B cells [11•].

Autoantibodies may also be involved in the pathogenesis of scleroderma either by amplifying the immune response or targeting those cell types that are relevant in the pathophysiology of the disease.

This article reviews the spectrum of autoantibodies found in scleroderma patients, with emphasis on those characterized by stimulatory agonistic biological activity.

Anti-endothelial cell antibodies

Anti-endothelial cell antibodies (AECA) have been observed in a significant proportion of scleroderma patients, apparently defining patients with more prominent vascular involvement [12,13]. A potential pathogenic role of AECA has been suggested by their ability to induce apoptosis in human dermal microvascular endothelial cells, but not in human umbilical vein endothelial cells, in the presence of activated NK cells via the *Fas* pathway [14]. In partial contrast, a previous work has not implicated the Fas pathway in AECA-induced apoptosis of endothelial cells [15]. The proapoptotic effect of AECA has been demonstrated also *in vivo* in a subsequent study by transferring serum of the UCD 200 chicken model of systemic sclerosis into normal chicken embryos [16]. However, till date the antigen targeted by AECA is still unknown. Also, it is not clear whether these antibodies represent a primary mechanism inducing endothelial cell damage or if they are consequence of cell death. Using a different experimental approach, Lunardi *et al.* [17] have identified in scleroderma patients circulating antibodies recognizing the human cytomegalovirus (HCMV) late protein UL94. These antibodies are capable to induce endothelial cell apoptosis *in vitro* by crossreaction with the cell surface integrin-Nag-2 protein complex. Recently, the same group has shown that Nag-2 is expressed also on dermal fibroblasts and that anti-Nag-2 antibodies induce, upon binding to fibroblasts, approximately 1000 (989) transcripts. These sequences include genes involved in ECM deposition and genes encoding growth factors, chemochines and cytokines [18]. These findings are of interest for several reasons: first, they point to a possible target of AECA; second, they suggest a link between HCMV and scleroderma and a novel pathogenic mechanism by which HCMV may cause vascular injury; third, they support the molecular mimicry model of autoimmunity. These data await confirmation by *in vivo* studies.

Servettaz *et al.* have shown by use of a quantitative immunoblotting method that the main target of AECA in patients with limited cutaneous scleroderma is the nuclear centromeric protein B (CENP-B) [19]. The pathogenic relevance of this finding remains, however, elusive, given the nuclear localization of the antigen.

A possible link between endothelial cell apoptosis and fibrosis, probably active in scleroderma, has been proposed by Laplante *et al.* [20•]. Apoptotic endothelial cells would release soluble mediators that suppress apoptosis in fibroblasts by altering the ratio between Bim-EL/Bcl-xl protein levels. Resistance to apoptosis can be reproduced in fibroblasts by expressing a synthetic peptide containing an epidermal growth factor motif present on the C-terminal fragment of perlecan, a large multidomain proteoglycan that binds and crosslinks ECM [20•].

Anti-DNA topoisomerase I and anti-centromere antibodies (ACA)

The autoantibodies classically associated with scleroderma are markers of clinical subsets with no known relationship to disease pathogenesis. Anti-centromere antibodies (ACA) and anti-DNA topoisomerase I (also known as anti-Scl-70) antibodies are the ones most commonly found.

A role of these autoantibodies in some of the clinical manifestations that characterize scleroderma has been recently proposed on the basis of clinical association, without any experimental evidence. ACA isolated from patients with ischemic digits recognize fragments of nuclear centromeric protein C (CENP-C) and have been implicated in the pathogenesis of ischemic lesions [21]. Anti-Scl-70 antibodies have been reported to directly bind to the cell surface of unpermeabilized fibroblasts by flow cytometry, immunofluorescence, and confocal microscopy [22]. A significant association has been found between the presence of these antibodies and pulmonary hypertension and increased mortality [22]. Purified topoisomerase I can bind in a dose-dependent fashion to normal human dermal fibroblasts, where it can be recognized by anti-Scl-70 antibodies isolated from scleroderma patients. The binding of the complex constituted by topoisomerase I and anti-topoisomerase I antibodies to fibroblasts can stimulate adhesion and activation of cocultured monocytes. In the authors' opinion, this chain of events would lead to increased release of cytokines by activated monocytes and to local secretion of profibrotic cytokines by activated fibroblasts [23].

At the clinical level, the titer of anti-Scl-70 IgGs seems to positively correlate with the patients' skin score. In a small group of patients followed longitudinally, changes in the anti-Scl-70 antibody titer have been found to parallel changes in the total skin score. Furthermore, higher levels have been detected in patients with active disease, when compared to patients with inactive disease. Although these findings do not necessarily implicate anti-topoisomerase I antibodies in the pathogenesis of scleroderma, yet they seem to link these antibodies to

the process leading to fibrosis [24]. Most probably, anti-topoisomerase I antibodies are the result of immunization to nuclear antigens exposed upon cell death.

Anti-fibroblast and other circulating antibodies

Fibroblasts have been extensively investigated in scleroderma. Autoantibodies to fibroblasts have been shown to induce an activated fibroblast phenotype [25•] upon interaction with constitutively expressed cell surface molecules and subsequent internalization of the antibodies via an Fc-independent mechanism [26].

Autoantibodies against fibroblasts have also been found in patients with primary pulmonary hypertension (PAH) and in a subgroup of patients with scleroderma-associated PAH [27••].

Following the demonstration of circulating ant-ifibrillin-1 autoantibodies in the Tsk 1 mouse model of scleroderma, circulating antibodies targeting the prolin-rich C region of the ECM protein fibrillin-1 have been detected in a significant proportion of scleroderma patients and have been shown to activate normal human fibroblasts *in vitro* [28,29]. In fact, normal human fibroblasts exposed *in vitro* to affinity-purified ant-ifibrillin-1 autoantibodies displayed increased expression of several ECM components and nuclear translocation of phosphorylated Smad3. The authors have hypothesized that immunity against fibrillin-1 may cause the release of sequestered TGF-β1 from fibrillin-1-containing microfibrils in the ECM, with subsequent fibroblast activation. However, Brinckmann *et al.* using an ELISA based on correctly folded recombinant fibrillin-1 have not been able to detect anti-fibrillin-1 autoantibodies in scleroderma patients [30].

Antibodies against matrix metalloproteinases (MMP)-1 and MMP-3 have also been reported to be specific markers of scleroderma. It has been speculated that they may hamper ECM degradation, thus promoting fibrosis [31,32].

Anti-PDGF receptor (PDGFR) autoantibodies

No autoantibody found in systemic sclerosis appears to be an agonistic or stimulatory antibody. The above-mentioned antibodies most probably derive from immunization against intracellular components released during inflammation and upon cell death.

Recently, a novel class of autoantibodies has been discovered in systemic sclerosis patients. These antibodies recognize and activate the human PDGF receptor, thus stimulating production of reactive oxygen species (ROS) and collagen. The relevance of these antibodies is due to the agonistic nature of their biological action targeted to an important receptor linked to fibrosis. These antibodies represent the first identified link between the immune system and tissue fibrosis [33••].

PDGFR autoantibodies found in scleroderma patients can bind to αα, αβ, and ββ PDGF receptors. The epitope bound by the stimulatory autoantibodies is a conformational motif recognized only in its native configuration and awaits molecular identification.

The stimulatory nature of the antibody–receptor interaction has been demonstrated by induction of tyrosine phosphorylation of the receptor [33••]. Furthermore, IgGs purified from serum of scleroderma patients induce overexpression of two genes that characterize scleroderma fibroblasts: α-smooth muscle actin and type I collagen genes. The expression of these genes is not stimulated by control IgGs [33••].

The key events triggered by the autoantibodies to PDGFR can be summarized in the following way. Upon binding to the receptor, SSc-IgGs induce receptor dimerization and autophosphorylation of tyrosine residues *in trans* in the two receptor molecules composing the dimer. Tyrosine phosphorylation of the engaged receptor stimulates production of ROS by NADPH oxidase [34•]. ROS inhibits tyrosine phosphatases and set off ERK1/2 that stabilizes the GTPase Ha Ras. Ras is able to stimulate ERK1/2, which can further activate NADPH oxidase [34•]. Inhibition of any component of this loop (ERK1/2, Ras, and ROS) downregulates the system and abolishes the biological effects of SSc-IgG-induced Ras–ROS activation, for example, transcription of α-smooth muscle actin and collagen genes. The loop initially triggered by PDGFR autoantibodies may then become relatively autonomous, because it is maintained by ROS produced through activation of NADPH oxidase by Ras–ERK1/2. However, PDGFR signaling is required for long-term ROS production, because inhibition of the PDGFR kinase for 4–12 hours reduces Ras–ROS–ERK1/2–collagen levels [34•].

We wish to point out that the autoantibodies to PDGFR are not comparable to PDGF in terms of biological activity. Whereas PDGF biological effects on ROS and ERK1/2 are short-lived (15 min), these anti-PDGFR autoantibodies are long-lasting stimulators of ROS generation, because they elicit a prolonged activation of PDGFR. We have data indicating that the physiological turnover of PDGFR is substantially modified by these antibodies. Inhibition of PDGFR downregulation seems to be a direct consequence of ROS increase induced by SSc-IgG activation of the PDGFR. Stabilization of the receptor on the cell surface prevents signaling termination and sustains persistent activation of ROS–Ras–ERK1/2 cascade (unpublished data).

We suggest that fibrosis in scleroderma is generated by persistent stimulation of PDGFR-ROS signaling. These PDGFR autoantibodies trigger in fibroblasts the ROS–Ras–ERK1/2 cascade, which results in the acquisition of a

myofibroblast phenotype that accounts for a persistent profibrotic response, that is, collagen overproduction. Long-lasting action of these antibodies sustains ROS production and explains its resistance to immunosuppressive treatments. Fibroblasts remain activated as long as the antioxidant defense of the cells can buffer the oxidative stress. Once ROS overwhelm the antioxidants defense, fibroblasts undergo senescence.

The culprits are gone and what is left is the increased ECM deposition with organ derangement. Hydrogen peroxide produced during the oxidative burst can freely diffuse to and activate adjacent fibroblasts and vessels concurring to the formation of inflammation foci and spreading of the disease. It remains, however, to be ascertained whether there are antibody-dependent and antibody-independent stages of disease and whether PDGFR autoantibodies may set up a signaling cascade capable of generating hydrogen peroxide or hypoxia, that is, ischemia reperfusion events, in endothelial cells.

Do these antibodies explain all the SSc manifestations? We do not believe they do.

To our knowledge, these agonistic PDGFR autoantibodies represent an important pathogenic link between the immune system and SSc. We still need to identify the primary cause of the disease and suitable *in vivo* models to test the pathogenetic relevance of these molecules and other involved components. For example, endothelial cells do not express PDGFR as fibroblasts or monocytes do, though they are altered in the disease.

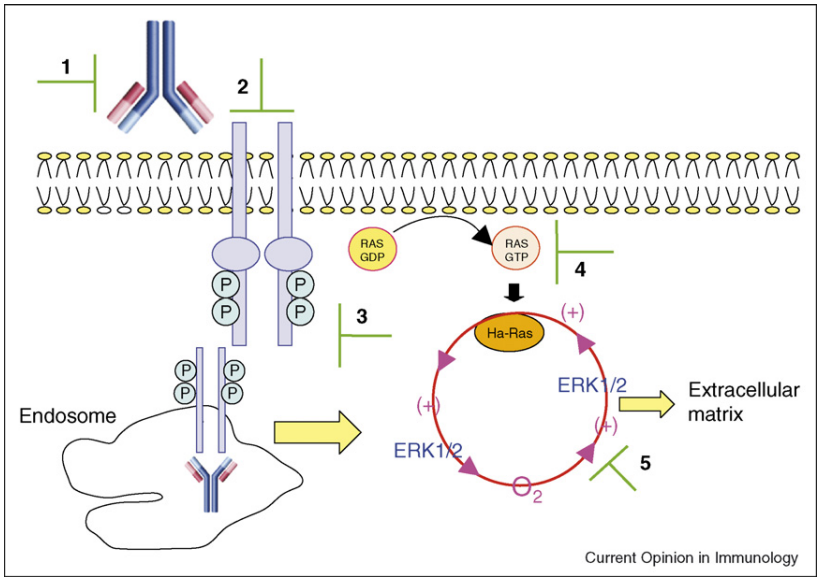
We have preliminary data indicating that in fibroblasts PDGF stimulates TGFβ signaling by increasing the TGFβ receptor levels. Also, hydrogen peroxide produced by fibroblasts and monocytes under the direct action of these antibodies can freely diffuse across the membranes and reach endothelial cells. It remains to be seen if inhibition of PDGFR impairs TGFβ signaling in endothelial cells.

Novel therapeutic venues

The unraveling of the signaling cascade triggered by autoantibodies against PDGFR paves the way to novel therapeutic approaches (Figure 1). Targeting the components of the cascade (PDGFR, Ha Ras, and ROS) may be therapeutically rewarding: first, anti-CD20 treatment can downregulate B cells and autoantibody production; second, Ha Ras is very sensitive to farnesyl transferase inhibitors or statins; third, antioxidants may reduce ROS load; fourth, tyrosine kinase inhibitors, like imatinib mesylate, may block PDGFR activation. However, because the activity of the distinct elements of the loop can vary from patient to patient and in the single patient at different stages of the disease, we believe that only integrated and combined treatments will be likely to succeed.

In conclusion, these agonistic autoantibodies represent the first pathogenic link between some features of

Figure 1



The signaling cascade triggered by autoantibodies against PDGF receptor can be downregulated at different levels. Therapies to block antibody production (target 1) and phosphorylation of PDGF receptor (target 3) are available. Ha Ras is sensitive to farnesyl transferase inhibitors or statins (target 4). High doses of free radical scavenger *N*-acetyl cysteine counteracts free radicals (target 5). Interaction between agonistic autoantibodies and PDGF receptor (target 2) can be blocked by aptly devised drugs.

systemic sclerosis (fibrosis and oxidative stress) and the immune system. By using them as bait, we hope to unravel the entire chain of events, including the primary cause leading to their selection and synthesis.

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