
Non organ based laboratory markers in systemic sclerosis

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ABSTRACT

Candidate markers for endothelial cell, lymphocyte and fibroblast function in systemic sclerosis have been reviewed. Autoantibody measurement and general inflammatory markers were considered the minimum requirements, with other markers needing further evaluation.

Introduction

The subcommittee members initially reviewed the literature in order to identify those markers that have the best credentials for reflecting the activity of cell types thought to be most intimately involved in the pathogenesis of systemic sclerosis (SSc). It is likely that different cell types may play important roles in certain stages of the disease cycle. For instance, endothelial injury may be important early in disease evolution whereas fibroblast activity may be of greater significance at a later stage. Treatment strategies may be directed to specific cellular targets (e.g. anti-T cell therapy). Consequently, it would be ideal to have a panel of markers that can be chosen dependent on the purpose of the particular research being conducted.

Our search has focused on markers of endothelial activation and damage, chemokines and lymphokines, autoantibodies, fibroblasts and extracellular matrix deposition. The choice of marker has been made by taking into account its suitability and specificity for the underlying cellular event, its importance for disease diagnosis, activity or outcome based on interpretation of the published literature, and the reliability and feasibility of its measurement (Table I). Less well-established markers that may have a potential role in future studies are also mentioned.

Candidate variables

Products of endothelial cells (ECs)
Vascular and EC injury including EC

apoptosis, EC activation, intimal proliferation of arterioles and capillary necrosis with platelet aggregation are prominent features in the pathogenesis of SSc (1). The occurrence of EC dysfunction, together with or even before skin and visceral fibrosis, make markers of EC activation and damage good candidate molecules to assess activity and severity of the disease.

Von Willebrand factor (vWF) is the best-studied marker of EC injury in SSc. vWF protein is synthesized constitutively by vascular ECs and circulates in human plasma at concentrations of 10 µg/ml. vWF is not a specific marker for endothelial cells, since it is also synthesized by megakaryocytes and is contained in platelets, which accounts physiologically for about 15% of the circulating vWF protein in blood. Mediators of inflammation (and thrombosis) increase the secretion of vWF from intact ECs, and vWF leaks from damaged ECs resulting in elevated plasma levels of vWF protein in a wide variety of clinical conditions.

In patients with SSc and Raynaud's disease, plasma levels of vWF protein have been found to be elevated in several studies (2, 3). Increased levels of vWF have been related to the extent of visceral organ involvement and an overall poor prognosis in SSc (4, 5). Some investigators detected higher levels of vWF in patients with diffuse cutaneous (dc) SSc compared to patients with limited cutaneous (lc) SSc, while in other studies a correlation with the skin score, pulmonary function and kidney function were noted.

Levels of vWF are also increased in related rheumatic conditions such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and Sjögren's syndrome. Notably exercise, ABO blood groups, hypoxic reperfusion and exposure to cold influence plasma levels of vWF, which results in high intra-individual variability. The vWF propeptide is released from ECs

Table I. Candidate markers based on cell type.

Cell type	Endothelial cells	T cells	B cells	Fibroblasts
Best candidate	vWF	sIL-2R	Autoantibody	PIIINPP
Disease diagnosis (and/or subgroups)	No	No	Yes	No but may identify subgroups
Disease activity	Probable	Possible	Possible (anti-topo I)	Probable
Disease outcome (severity)	Probable	Possible	Probable	Probable
Reliability	Yes	Yes (central lab)	Yes (depending on assay)	Yes
Feasibility	Yes	Yes	Yes (with central facility)	Yes
Core set	If vascular marker essential	No	Yes	If fibroblast marker essential
Alternative markers	sVCAM, E-selectin, vWF propeptide, VEGF, Endothelin-1	CD30, IL-17 MCP1 (chemokine)	None	TIMP-1, CTGF, KL6

together with mature vWF in equimolar amounts, but is less influenced by external factors. Since vWF propeptide also has been shown to be increased in SSc, use of this marker could overcome some of the limitations in the determination of the mature vWF protein (6). Endothelin-1 (ET-1) first was described as an EC-derived factor with strong vasoconstricting properties. Later studies showed that ET-1 is synthesized by a wide variety of cell types and is able to induce proliferation and gene expression of extracellular matrix proteins in dermal fibroblasts (7).

As analyzed by radioimmunoassay, plasma levels of ET-1 were found to be increased in patients with SSc and other inflammatory and vascular diseases including patients primary Raynaud's phenomenon (8, 9). One group could not confirm these findings using ELISA for detection of ET-1 (10). Levels of ET-1 correlated with the skin score, disease duration, dc SSc and with the presence of pulmonary hypertension in patients with lc SSc (9, 11). Adhesion molecules are synthesized by ECs in response to inflammatory mediators and after interaction with anti-endothelial cell antibodies (12). In skin biopsies from patients with SSc, adhesion molecules are additionally expressed by fibroblasts and infiltrating mononuclear cells and thus also reflect the activation of these cell types. Adhesion molecules are upregulated in par-

ticular in the early edematous phase of SSc (13). They are cleaved enzymatically from the cell surface membrane and can be detected as soluble forms in the circulation.

Serum levels of soluble intercellular adhesion molecule-1 (sICAM-1) were found to be increased in patients with SSc compared to healthy controls. Patients with dc disease of recent onset and those with fingertip ulcers showed the highest levels of sICAM-1, but no association was detected with the extent of internal organ involvement (14).

Elevated levels of circulating vascular cell adhesion molecule-1 (sVCAM-1) have also been reported in SSc (13, 15), but not confirmed (16). Associations with disease severity are conflicting, but patients with dc SSc, scleroderma renal crisis, pulmonary fibrosis, joint involvement and impaired left ventricular diastolic function showed increased levels of sVCAM-1 compared with their counterparts (15, 17). Interestingly, in a study by Denton *et al.*, with serial measurements of adhesion molecules, changes in circulating VCAM-1 or E-selectin, but not ICAM-1 were associated with changes in disease severity (18).

In conclusion, vWF is currently the best-characterized marker for EC injury in SSc and might also correlate with disease severity and/or activity. Serial measurements of VCAM-1 and

E-selectin also could be of use to monitor EC activation and disease severity.

Products of T cells

Chemokines

Chemokines are a family of small molecules (molecular weight 8-12 kDa), which currently consists of approximately 50 members. According to the position of cysteine amino acids at the NH₂-terminus, chemokines are grouped into CXC-, CC-, CX₃C- and C-chemokines. While leukocyte chemotaxis and lymphocyte development are by far the best-characterized biologic functions of chemokines, recent data suggest that they might also be involved in the regulation of collagen turnover.

Only a limited number of chemokines has been examined in SSc. SSc patients showed higher levels of the CXC-chemokine interleukin-8 (IL-8, CXCL8) in bronchoalveolar lavage (BAL) fluids compared to controls, and these levels were correlated with the percentages of neutrophils in BAL fluids (19, 20). However, serum levels of interleukin-8 were detectable only in a minority of patients (21).

The CC chemokines macrophage inflammatory protein-1a (MIP-1, CCL3) and MIP-1 (CCL4) have been found to be elevated in the serum of SSc patients. Elevated serum MIP-1 correlated with the presence of pulmonary fibrosis. Similarly, levels of

monocyte chemoattractant protein-1 (MCP-1, CCL2) were elevated in SSc and were associated with the presence of pulmonary fibrosis (22). In SSc skin, MCP-1 is abundantly expressed by keratinocytes, fibroblasts, inflammatory cells and ECs, whereas no expression is detectable in healthy skin (23). Moreover, data from animal models suggest that interaction of MCP-1 with its receptor CCR2 might be directly involved in the development of fibrosis (24). In summary, chemokines, and in particular MCP-1, appear to play a significant role in the pathogenesis of SSc. However, more detailed analyses will be necessary before they can be considered reliable markers for disease activity and/or severity.

Lymphokines

Immune system activation is considered to be one of the three key steps in the pathogenesis of the SSc. For example, hyperreactivity of SSc fibroblasts may be mediated by an alteration in T cell products and/or cytokines. The available literature suggests a large number of potentially helpful immune markers of SSc activity. However, apart from measurement of the skin score, studies have been confounded by the lack of validated clinical measures of disease activity or outcome, the small numbers of patients studied, and lack of stratification for disease subtype. Finally, several bioassays used to evaluate the levels of cellular products are cumbersome and may be performed only in specialized research laboratories.

Soluble IL2 receptor (sIL-2r) has been extensively studied in recent years. Serum levels of IL-2r and are significantly elevated in SSc patients (25-35) Furthermore, some reports have shown that sIL2r levels correlate with the disease activity and display sensitivity to change over time, suggesting a potential role in assessing disease progression.

Soluble CD30 (36-39) and IL-17 (40) may be useful in assessing disease activity and the efficacy of new therapeutic agents in clinical trials. In fact, patients with active SSc display high levels of the soluble form of the CD30 molecule, a member of the tumor ne-

crisis/nerve growth factor receptor superfamily (41, 42). Similar findings have been described in other rheumatic diseases such as SLE and RA. Since CD30 is expressed and released by functionally primed Th0 and Th2 cells, it is attractive to speculate that the increased release of sCD30 in SSc results from activation of the Th2 subset, which seems to be strongly correlated with the pathogenetic mechanisms of the disease. Furthermore, a significant correlation of sCD30 with skin score and erythrocyte sedimentation rate (ESR) has been reported in SSc patients. These data suggest that the level of sCD30 in the peripheral blood may be helpful in following disease progression and outcome. Similarly, IL-17 levels were increased in the serum of SSc patients and high levels of IL-17 messenger RNA was expressed in unstimulated lymphocytes from the skin and lungs of the same patients, but not in control samples from patients with different autoimmune diseases or healthy donors. IL-17 overproduction was significantly related to the early stage of SSc.

In conclusion, although several cytokines have been reported as potential laboratory markers of disease activity, presently their reliability and feasibility are still low and both cross-sectional and longitudinal studies are needed to clarify their utility. The best validated marker at present for specifically monitoring T cell activity is sIL-2R.

Products of B cells

Autoantibodies are found in over 95% of patients with SSc using sensitive assays for their detection. Furthermore, certain autoantibodies are highly specific for the condition, are associated with clinical subtypes of disease and hence are very useful diagnostic markers. Most SSc patients have one of three almost mutually exclusive, disease-specific serum autoantibodies that recognize centromere proteins (CENPs) (43), topoisomerase I (topo I) (44) or RNA polymerase III (RNAP III) (45, 46). Anti-centromere antibodies (ACAs) are usually found in patients with the less severe lc SSc, anti-topo I antibodies are associated with pulmonary

interstitial fibrosis, and patients with anti-RNAP III antibodies tend to have dc-SSc and are at increased risk of severe renal involvement. Since autoantibodies are present at an early stage of disease, the availability of an accurate autoantibody profile is an invaluable aid to diagnosis and prognosis in individual patients.

Other less common autoantibodies also occur in SSc, and these include: anti-nucleolar antibodies (ANoAs) such as anti-PM-Scl antibodies, which are usually found in patients with lc SSc/poly-myositis (PM) in overlap (47); anti-U3 RNP antibodies, which are associated with dc SSc, primary pulmonary arterial hypertension, skeletal muscle involvement and early disease onset (48, 49); and anti-Th RNP antibodies, which are indicative of lcSSc with either pulmonary fibrosis or pulmonary arterial hypertension (50). Several other ANAs are also detected: anti-U1 RNP antibodies are associated with SSc/SLE/PM overlap syndrome (51, 52), while anti-Ro antibodies have been linked with a very severe and rapidly progressive disease course, including renal failure and pulmonary involvement (46). In addition, anti-Ku, anti-Jo-1 (anti-histidyl-tRNA synthetase), and anti-PL-7 (anti-threonyl-tRNA synthetase) antibodies occur in a small proportion of SSc patients (51,53), particularly those with the SSc/PM overlap syndrome.

There is absence of SSc-associated autoantibodies in first degree relatives of probands with SSc (54). In monozygotic twins discordant for SSc, autoantibodies segregate with presence of disease (55). Anti-topo I antibodies can be found in the tight skin mouse (TSK1) model of SSc, illustrating the cross-species association of autoantibody specificity with disease phenotype (56). Also of interest have been recent reports of autoantibodies to the extracellular matrix microfibrillar protein fibrillin-1 in patients with SSc (57), which is especially important since duplication in the fibrillin-1 gene has been implicated as the cause of the TSK1 phenotype.

There is evidence that SSc-associated autoantibodies also may identify envi-

ronmental and genetic patient subgroups. For example, anti-topo I antibodies are over-represented in SSc associated with silica dust and solvent exposure (58, 59). MHC class II associations with SSc are relatively weak overall, but notably stronger with serologically defined subgroups (60). Therefore, any study of environmental or genetic causes of SSc would be well advised to take account of autoantibody-defined subgroups.

Although an autoantibody profile is likely to be important in determining prognosis, there are few well-controlled studies reported. In a five-year survival analysis, anti-topo I antibodies were associated with increased mortality (61). The presence of anti-centromere and/or anti-topo I antibodies in patients with Raynaud's phenomenon is associated with the later development of features of SSc (62). Anti-RNA polymerase III antibodies identify patients with a relatively poor prognosis, extrapolating from studies that have been mostly retrospective in design (51). However, the detection of anti-RNA polymerase autoantibodies requires techniques that are not commonly available, hence the application of this autoantibody specificity in clinical studies to date has been limited.

Autoantibodies are normally present from the earliest sample measured and show the characteristics of an antigen-driven immune response (i.e. high titre, polyclonal, IgG isotype, and recognize multiple epitopes on cognate antigens). Levels of anti-topo I antibodies may provide useful clinical information. In *TSK 1* mice serum anti-topo I antibodies correlate with histopathological and biochemical alterations (63). A distinct subset of anti-topo I positive SSc patients who lose anti-topo I autoantibody during the course of the disease and have a favourable outcome has been reported (64). In another longitudinal study, decreasing levels of anti-topo I antibody were accompanied by atrophic skin change, whereas increasing levels were associated with new onset or worsening of organ involvement (65). The development of validated reliable high-throughput assays for autoantibodies may offer useful indices

of disease activity in the future, but need further validation in prospective studies.

A variety of serological techniques are used for measuring and confirming individual autoantibody specificities, some of which are more reliable than others. Indirect immunofluorescence using HEp-2 tissue culture cells is a sensitive and reliable screen for most anti-nuclear autoantibodies and the only technique necessary for identifying anti-centromere antibodies. Gel diffusion techniques (e.g. Ouchterlony double immunodiffusion, counter-immune electrophoresis) allow detection of autoantibodies to saline-extractable antigens (e.g. Ro, La, UIRNP, Sm, Jo-1, PM-Scl and topo I), and are reliable techniques best suited to the identification of high titre autoantibodies. Solid phase ELISA assays are commercially available for a number of autoantibodies but are prone to false positive results. ELISAs have the advantage of semi-quantitation and larger-scale screening for single autoantibody specificities but the disadvantage of limitation to measurement of a single autoantibody per test. Certain autoantibody specificities such as those to nucleolar constituents may only be reliably detected by more specialized techniques. The 'gold standard' for the detection of anti-RNA polymerase specificities is immunoprecipitation.

One major advantage of autoantibody serology is the stability of immunoglobulin in stored serum, despite multiple freeze/thaw cycles. Very small amounts of serum are required (5 – 50 ul) for the assays concerned. Also, serum can be kept unfrozen for lengthy periods of time (e.g. several days while being transported) without compromising subsequent autoantibody detection. Some autoantibodies (e.g. ACA) may be reliably detected in most local laboratories. However, for reasons of reliability and standardization and in order to obtain potentially important information on autoantibodies for which detection techniques are not widely available, we recommend that autoantibody measurement in multi-centre clinical studies be undertaken by a central laboratory with the necessary expertise. A

minimum requirement would be one baseline measurement, but ideally serum should be stored on serial visits in case of the future availability of more validated markers.

Products of fibroblasts

Markers of fibroblast activity should be good candidates for assessing disease activity in a condition characterized by the excess accumulation of extracellular matrix deposition. Products of collagen metabolism such as type III procollagen N-terminal peptide propeptide (PIIINPP) are increased in SSc (66-70) and some studies suggest an increase in association with the skin score (70), anti-Scl-70 positive patients (70), restrictive lung disease (70), active interstitial lung disease (71) and kidney disease and Raynaud's phenomenon (67, 72). PIIINPP has also been detected in increased amounts in BAL fluid in SSc patients compared to those having cryptogenic fibrosing alveolitis (71).

Connective tissue growth factor (CTGF) is a marker for fibrosis in SSc and the serum level of CTGF may serve as a useful marker for disease activity, dcSSc and pulmonary fibrosis (73-75). Serum activity of xylosyltransferase, an initial enzyme in the biosynthesis of chondroitin sulfate and proteoglycans in fibroblasts, was significantly increased in SSc patients compared with blood donors of corresponding age. Female patients with dcSSc showed higher serum xylosyltransferase activity than those with lcSSc (76). Serum levels of tissue inhibitors of metalloproteinase (TIMPs) were measured in patients with dcSSc, lcSSc, RP and controls. TIMP-1 levels were significantly raised in dcSSc and lcSSc and highest in early versus late stage disease (77). In another study, the majority of SSc patients investigated showed elevated TIMP-1 levels (dcSSc > lcSSc), and patients with dcSSc and elevated TIMP-1 levels had a significantly greater frequency of lung fibrosis and anti-Scl 70 antibodies than those with normal serum TIMP-1 levels (77). Another group has shown that TIMP-2 levels are significantly more elevated in highly active compared to less active SSc (78).

Table II. Core set variables recommended by the subcommittee.

	Core variables	Potential future variables
Endothelial	vWF (VCAM, E-selectin) for vascular-based studies	vWF propeptide
T cell	SIL-2R for T cell-based studies	CD30, IL-17
B cell	Autoantibodies (ACA, anti-topo I, anti-RNA pol I-III as minimum)	
Fibroblast	PIIINPP for fibroblast-based studies	TIMP-1, CTGF
Other	ESR, haemoglobin, CPK, serum, plasma	CRP, genetic material

Recently KL-6, a glycoprotein derived from pneumocytes, was reported to be increased in the serum of patients with SSc and SSc-related interstitial lung disease (79). There was a trend for levels to be higher in dcSSc.

In summary, PIIINP is probably the best studied fibroblast activity marker in SSc. Its detection is reasonably straightforward by RIA. CTGF and TIMP-1 levels are promising alternatives. KL-6 is an interesting candidate, but not yet well studied.

Acute phase proteins

There is some evidence that the acute phase response may be blunted in SSc (80, 81) as it is in SLE. ESR should be considered as a valuable acute phase reactant, with C-reactive protein as a less-well-studied alternative (82). Measurement of haemoglobin concentration or haematocrit is part of a 9-organ disease severity scale for SSc developed and published by an international study group (83). Creatine phospho-kinase (CPK) is a widely accepted marker for muscle damage in SSc and other conditions, especially in patients with overlap syndromes that include PM.

Discussion

Identification of core set variables

The most satisfactory approach to the assessment of patients with SSc should include the evaluation of parameters reflecting the activation of each cell type believed to be involved in the pathogenesis of the disease, as well as the evaluation of inflammatory parameters (Table I). For this reason the subcommittee considered recommending the set of laboratory markers listed in Table II. In addition, we strongly feel that it is imperative to store serum and plasma whenever possible, to furnish material

for future prospective multicentre studies in SSc.

Since after extensive discussion in the final plenary session most of the participants in this consensus conference argued that a number of the parameters in Table II are not yet routinely measured in most centres, the subcommittee members agreed to restrict the core set variables to the assessment of antinuclear antibodies, including anticentromere antibodies and anti-Scl70 antibodies, and to the evaluation of inflammatory parameters.

Rationale for selecting the core set variables

The choice of core set of parameters was based on the need for comparability among different studies and therefore the necessity to select the most feasible items.

Rationale for excluding other variables

Some parameters are still not widely used despite their recognized value in the assessment of SSc patients and for this reason were not included in the core set. This is the case, for example, of the antipolymerase I-III antibodies, which can definitely help the clinician to predict the disease course but are not yet investigated in most tertiary centres.

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