Pathophysiology of systemic sclerosis: State of the art in 2014

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Summary

Major work has been done in order to improve the understanding of systemic sclerosis (SSc) pathogenesis. A number of new experimental models have been set up, that should help to understand the disease pathogenesis and test new therapeutic targets. Reactive oxygen species represent a hallmark of the pathogenesis of SSc, both at the fibroblast and at the endothelial cell levels. Although a large number of genetic studies have been conducted, it is still difficult to identify a genetic background specific to SSc, and the major progress in this setting is probably the identification of an interferon signature. Besides endothelial cells and fibroblasts, major development has been made in the understanding of the role of B cells and autoantibodies in the pathogenesis of SSc. Plasmacytoid dendritic cells seem to play a major role in the pathogenesis of SSc through the secretion of CXCL4, although these data will need to be confirmed in the near future.

Systemic sclerosis (SSc) is a rare connective tissue disease characterized by vascular involvement responsible for vascular hyperactivity and remodeling, together with fibroblasts activation and extra-cellular matrix synthesis [1]. SSc mostly occurs in females (3 to 8 females for 1 male). Its prevalence varies between 30 and 240 per million inhabitants, being higher in North America and Australia than in Japan [2]. In Europe, the prevalence varies between 50 and more than 150, with 158 per million in France [3]. Reports of sporadic clusters of higher prevalence suggest the existence of environmental risk factors, but only silica and solvents exposure have been consistently associated with SSc [2]. Patients with SSc are usually classified into two main groups, according to the extent of skin involvement: limited SSc (ISSc), with skin involvement essentially limited to the hands and face; and diffuse SSc (dSSc), with skin involvement proximal to the elbows and knees. In patients with...
ISSc, visceral involvement is rare, whereas patients with dSSc more frequently experience visceral involvement [4]. It is essential to distinguish between limited and diffuse SSc in order to elucidate the pathophysiology of the disease, since distinct mechanisms probably contribute to the occurrence of the two forms of the disease.

In recent years, significant progress has been made in the understanding of mechanisms contributing to the occurrence of vasculopathy and fibrosis, both in human samples and animal models. However, experimental models, although very useful, do not allow to characterize all the mechanisms at play in SSc, contributing to explain why we are still lacking a universal treatment of SSc. In this review article, we will provide the reader with an overview of the pathogenesis of SSc, with emphasis of recent acquisitions in the field (figure 1).

Environmental factors

A number of environmental factors that may contribute to the occurrence of SSc have been identified. Various activities, including manufacturing and rural activities have been associated with the occurrence of SSc, notably the exposure to silica, dust and hydrocarbons [2]. The first reports of the association between silica and SSc were made in limited numbers of patients [5] followed by retrospective comparative studies, which confirmed that exposure to silica conferred a high risk to develop SSc [6]. Odds Ratio of 3.93 (1.84–8.54) [7] and 5.57 (1.69–18.37) [8] were calculated in studies conducted in Australia and France, respectively, leading a number of countries including France, Germany, Canada and South Africa to consider SSc as an occupational disease. In addition to silica, case control studies pointed out that past exposure to solvents was associated with SSc [5], with discrepancies among studies regarding the types of solvents involved and/or patient gender. Thus, paint thinner or removers, mineral spirits, trichloroethylene, trichloroethane, perchloroethylene, gasoline, aliphatic hydrocarbons, halogenated hydrocarbons, benzene, toluene or xylene-solvents have been proposed as the most at risk solvents, although discrepancies were identified among studies. Nietert et al. found that SSc males were more frequently exposed to organic solvents (in particular trichloroethylene) than controls (OR 2.9 [1.1–7.6]), which was not the case for females [9]. In a recent meta-analysis of the literature, the occurrence of SSc was associated with increased ORs for silica, chlorinated solvents, trichloroethylene and welding fumes for male patients, aromatic solvents and ketones for female patients and white spirit for both [10]. Several others toxic
products including epoxy resins and pesticides, as well as paints, adhesive, hair dye, contact lenses and fabric dyes have been investigated in SSc case controls studies but were never found significantly at risk for SSc. Finally, although silicone breast implants were suspected as potentially responsible for SSc induction [11], two meta-analyses [6,10] concluded to the absence of association between SSc and breast implants.

In addition, outbreaks of SSc-like disorders have clearly been linked to chemical exposure, such as polyvinyl chloride intoxication [12] or toxic oil syndrome [5]. Some viruses or certain chemicals, such as solvents, may also play a triggering role in the initiation and maintenance of the disease. Notably, Lunardi et al. have reported the presence of a "SSc peptide" recognized by serum immunoglobulin from 93% of patients with SSc. Interestingly, this peptide shows an important homology with UL94, a cytomegalovirus (CMV) late protein that can be recognized by purified IgG from patients [13]. In vitro, patient antibodies targeting UL94 also seem to induce apoptosis of endothelial cells (EC) similarly to SSc specific antibodies, suggesting a possible role of CMV in the triggering and the maintenance of SSc. Thus, it turns out that a cascade of extrinsic and intrinsic events seems necessary for the induction of the disease [14].

Genetic factors
General approaches

Many studies have been conducted in recent years in an attempt to identify genetic risk factors in SSc. From three U.S. cohorts, involving 703 families, including 11 multiplex SSc, first degree relatives were identified to get increased susceptibility to develop SSc, with a relative risk close to 13 (10–16 across cohorts), with a recurrence rate of 1.6% versus 0.026% in the general population [15]. For siblings, the relative risk in siblings (λs) is increased to 15 (10–27 across cohorts). For comparison, the risk (λs) varies between 3 and 15 in rheumatoid arthritis (RA) and between 20 and 29 in systemic lupus erythematosus (SLE). Only one study has been performed in twins. Based on the analysis of 42 twin pairs (24 monozygotic), this study demonstrates a poor agreement with the clinical expression of the disease (4.7%) [16]. However, there is a higher concordance for the presence of anti-nuclear antibodies (ANA): 40% for dizygotic and 90% for monozygotic. These results suggest that genetic predisposition alone is not sufficient to develop SSc but might influence the autoantibody profile.

Following the development of molecular biology, many data have been published on association studies. Unfortunately, a number of them were not repeated in large cohorts and/or could not be independently replicated by another group, which is essential for the validation of a genetic association signal.

This was particularly the case for genes suspected to be involved in the regulation of fibrosis such as SPARC (encoding the secreted protein acidic rich in cysteine), TGFβ (encoding connective tissue growth factor), TGB (encoding the tissue growth factor β) or FBN1 (encoding fibrillin 1) [17] or vascular factors including KCNA5 (encoding the potassium voltage-gated channel 5) or uPAR (encoding the urokinase-type plasminogen activator receptor) [18,19]. Interestingly, uPAR extinction in a mouse model led to dermal and pulmonary fibrosis together with microvasculopathy [20]. In parallel, a decrease of full-length uPAR protein expression in dermal biopsies of patients with SSc as compared to normal skin has been reported, suggesting a protective role of this protein in SSc. In addition, the potential influence of KCNA5 SNP on SSc prevalence remains controversial [21].

Immune system and molecules of intracellular signaling

The tumor necrosis factor ligand superfamily 4 gene (TNFSF4) encodes OX40L (CD252), the ligand of OX40 (CD134). OX40L, involved in antigen presentation in addition of T and B lymphocyte activation, is known as a susceptibility factor for SLE [22]. Interestingly, a study performed in 1,059 patients with SSc and 698 controls showed an association between OX40L polymorphism and SSc and the major disease subgroups [23]. This result has been confirmed in replication and meta-analysis studies [24,25].

Type I interferons (IFN) are key mediators of innate immunity and antimicrobial defense. IRF5 (interferon-regulatory factor 5 encoded by IRF5) is a transcription factor involved in the signaling of toll-like receptors (TLRs) and activation of target genes of interferon. Candidate genes studies have shown its association with the disease and its contribution to pulmonary fibrosis associated with SSc, independently of other risk factors identified [26,27]. These studies permanently identified IRF5 as a susceptibility factor to SSc. Moreover, recent updates highlighted the major role of IRF5 in the progression and in the occurrence of interstitial lung disease (ILD) in SSc [28,29].

Signal transducer and activator of transcription 4 (STAT4) is a transcription factor of the STAT family that induces the expression of type I IFN, interleukin (IL)-12 and IL-23, and stimulates the synthesis of IFN-γ and IL-17. Thus, a variant of the gene located in the third intron (rs7574865), without specific biological function, has independently been found to be associated with several autoimmune diseases in two European, one U.S. and a Japanese cohorts [30]. However, STAT4 is probably not directly responsible for the autoimmune state, since the combination of IRF5 and STAT4 [27] variants was associated with an increased risk of developing pulmonary fibrosis in patients with SSc (OR 2.72 [1.86–3.97], P = 3.07 × 10^-7 in presence of 3 or 4 at-risk alleles). In that study, STAT4 was found to be the second outside association major histocompatibility complex (MHC)
Interestingly, called reduced cytothes (PAH) ter scaffold. The association with several inflammatory cytokines production including TNF-α, IL-2 and IL-6. Thus, STAT4, which plays a major role in an inflammatory model of fibrosis, might represent a potential therapeutic target in SSc. Two genes, PTPN22, encoding the protein tyrosine phosphatase called Lyn, and CSK encoding the c-src tyrosine kinase also called CSK, which modulates signaling through the T cell receptor (TCR), have been found to increase susceptibility to SSc. PTPN22 was one of the first susceptibility genes associated with several autoimmune diseases. Its main variant (rs2476601, 1858C > T, R620 W) has been reported to be modestly associated with SSc [32,33], particularly in patients with anti-centromere autoantibodies; however this association is attenuated by the fact that a significant proportion of patients with SSc also develop autoimmune thyroiditis, which is associated with polymorphisms of PTPN22. Furthermore, the genome wide association studies (GWAS) identified an association of a CSK variant (rs1378942) with SSc frequency which was unrelated to auto-antibody specificities \( (P = 5.04 \times 10^{-12}; \text{OR} = 1.20) \) [34]. Interestingly, in the 2 GWAS performed, CD247 (rs2056626; OR 0.86 [0.81 to 0.90] \( P = 2.09 \times 10^{-7} \)), which encodes a subunit of the TCR\(_\gamma\) (component of TCR/CD3 complex), was significantly associated with a risk of developing SSc [35,36].

Several studies confirmed the major role of B cells in SSc. Thus, two variants (rs10516487 and rs3733197) of B cell-specific scaffold protein with ankyrin repeats (BANKT) gene, a promoter of the tyrosine kinase LYN phosphorylation, were found to be associated with dSSc [37,38], whereas another B lymphocytes specific gene, BLK (B lymphocyte kinase), which transduces the signal downstream of the BCR and is also involved in SLE (C8orf13-BLK region, rs13277113 and rs2736340) was associated with subgroups of ISSc and anti-centromere positive patients [39,40].

The variant of TNFAIP3 (6q23) was also identified through the GWAS as a susceptibility factor for autoimmune diseases [41]. TNFAIP3 is an intracellular protein that regulates negatively the NF-κB signaling pathway (by deubiquitination) downstream to the TNF-R super-family, TLRs, IL-1R and nucleotide-binding oligomerization domain protein 2 (NOD2) receptor. The rare G allele of rs5029939 was associated with SSc with an OR of 2.08 [1.59 to 2.72], \( P = 1.16 \times 10^{-7} \), with dSSc with an OR of 2.71 [1.94 to 3.79], \( P = 5.2 \times 10^{-9} \) and with pulmonary arterial hypertension (PAH) with an OR of 3.11 [1.86 to 5.17], \( P = 1.3 \times 10^{-5} \).

Taken together, these data reinforce the autoimmune component in SSc, with evidence for involvement of different pathways contributing to autoimmunity in other diseases, although fine mechanisms contributing to the occurrence of SSc remain to be identified.

Genetics might help to identify new biomarkers that could allow better stratification of patients at risk of developing visceral manifestations and offering them targeted therapies before the occurrence of severe organ involvement. However, identified OR are often weak even if they combine several gene polymorphisms. Thus, new genetic approaches might probably be helpful in the near future to go further in this direction.

**Major histocompatibility complex**

As for the vast majority of autoimmune diseases, the association of human leukocyte antigen (HLA) locus with SSc has been identified many years ago. It has been clarified by working specifically on HLA class II from 1300 SSc patients from USA and 1000 controls [42]. The strongest associations with SSc in Caucasians and Hispanics were found for DRB1*1104, DQA1*0501, DQB1*0301, DQB1 alleles encoding a protein without leucine at position 26 (DQB126*epi). On the other hand, DRB1*0701, DQA1*0201, DQB1*0202 and DRB1*1501 alleles were protective. In a recent GWAS, several SNPs located in 6p.21 in the MHC region were strongly associated with SSc, whereas the rs6457617 variant SNP located in the HLA DQB1 gene was strongly associated with SSc (\( P = 2.31 \times 10^{-18} \)) [43]. These results are associated with a strong weight of ethnicity and heterogeneity of the disease.

In addition, many other alleles or haplotypes appear very specific to certain subgroups, particularly characterized by auto-antibody status [44]. Notably, DRB1*1101 and DPB1*1301 alleles have been associated with anti-topoisomerase I positivity when DRB1*0401-22 and DRB1*0801-11 alleles are associated with anti-centromere positivity [45].

**Experimental models**

A relatively large number of animal models of SSc have been set up over the years, including at least 5 genetic and 3 inducible models that reproduce, at least partly, human SSc [46–65]. Most of them are listed in (figure 2). For a purpose of clarity we will not detail them all.

Tsk-1 mouse model is very well described and results from a partial duplication of the fibrillin 1 (Fbn1) leading to a characteristic phenotype of dermal fibrosis associated with increased extracellular matrix (ECM) production by fibroblasts and detection of SSc specific and other autoantibodies (anti-topoisomerase, anti-deoxyribonucleic acid [DNA]… [46–48]. Fibroblasts cultured from the skin of Tsk-1 mice also display a pro-fibrotic phenotype and can thus be used for the assessment of drugs in vitro [49]. Interestingly, in this mouse model, B cell deficiency can attenuate fibrosis progression by modulating IL-6 production [50]. Finally, Tsk-1 mouse is associated with pulmonary emphysema and not with pulmonary fibrosis, which has to be considered with the recent description of...
### Figure 2

**Genetic and inducible systemic sclerosis (SSc) experimental models** [46,52,54,56–58,61,62,65]

Dark grey: symptom similar to human SSc; Light grey: symptom partially mimicking human SSc; White: no evidences of symptom similar to SSc; CFA: complete Freund’s adjuvant, GVHD: graft versus host disease; Fra: fos related antigen, Fli: friend leukemia integration 1, KO: knock out, ROS: reactive oxygen species; SSc: systemic sclerosis, TGF-β: transforming growth factor–β, Topo-1: topoisomerase 1, Tsk: tight skin, UCD: University of California at Davis.

<table>
<thead>
<tr>
<th>Experimental model</th>
<th>Vasculopathy</th>
<th>Fibrosis</th>
<th>Inflammation</th>
<th>Autoantibodies</th>
<th>Limits/specificities</th>
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<td>Tsk-1 [46]</td>
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<td>Tsk-2 [52]</td>
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<td>UCD-200 UCD-206 [54]</td>
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<td>Chicken model with poor background</td>
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<td>TgRIIΔk and TBRICΔ [56, 57]</td>
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<td>Absence of autoimmunity</td>
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<td>Caveolin 1Δ [61]</td>
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<td>Fli1CAT::ΔTA [58]</td>
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<td>Fli1 endothelial cell KD [58]</td>
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<td>Overestimation of drug effects</td>
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<td>TGF-β independent model</td>
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<td>Unclear function of anti-topoisomerase I antibody</td>
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<td>Angiotensin II induced SSc [81]</td>
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<td>Poor links with human SSc</td>
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<tr>
<td>Sclerodermatous GVHD [81]</td>
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*Combined pulmonary fibrosis and emphysema (CPF E) in SSc [51]. Another animal model, the Tsk-2 mouse model occurs after administration of ethyl-nitrosourea, a mutagenic agent [52]. This model is associated with systemic inflammation. However, these two mouse models lack typical vascular phenotype and combine diverse organ involvements unclearly related to SSc [53].

UCD-200 and UCD-206 chicken models closely reproduce the human disease with vasculopathy characterized by Raynaud’s phenomenon or ischemic lesions on toes, skin fibrosis with activated fibroblasts, inflammation and autoantibodies. Nevertheless, the absence of molecular background in non-mammalian, particularly in chicken, drastically limits the development of researches on this model [54,55].

As TGF-β has been identified as a profibrotic mediator in human SSc and an activator of fibroblasts, two mouse models are focused on TGF-β pathways: TBRICΔ (Cre-ER) mice and TgRIIΔk mice. In the first case, under the control of tamoxifen, the active
form of TGF-β is overexpressed after birth [56]. Upon this increased secretion of active TGF-β, mice develop progressive and generalized skin fibrosis with activation of fibroblasts and vasculopathy in small arteries in lungs, kidneys and several organs also affected in human SSc. Unexpectedly, histological analysis of TβRI−/− mice differ from human SSc lesion, which limits the understanding of TGF-β pathways dysregulation in SSc patients. TβRI−/− construct encodes for the cellular and transmembrane domains of human TGF-βRII, specifically in fibroblasts. Increased expression of this receptor leads to fibroblasts activation and stimulates ECM protein secretion. Interestingly, this construct affects mice only 6 weeks after birth and reproduces human dermal and pulmonary fibrosis [57].

Fli1ACATA and Fli1 endothelial cell knock out (ECKO) are 2 recently described genetic models implicating the same transcription factor identified in Friend virus-induced erythroleukemia model in mice [58]. This protein is known to be epigenetically down-modulated in skin lesion of patients with SSc [59,60]. In C57BL/6 mice, an ECKO or a specific deletion of a potent transcriptional repressor through its carboxy-terminal activation (CTA) domain leads to significant up regulation of collagen synthesis in mouse skin. However, if mouse skin presents ultrastructure with collagen fibrils similar to human dermal lesion in SSc and vasculopathy, skin thickness is comparable with that of wt mice. These results suggest a potential role of Fli1 in the genetic predisposition to SSc. However, the absence of autoimmunity and systemic inflammation represent true limits for these models [61].

An interesting new model of SSc has also been developed using reactive oxygen species (ROS) in BALB/c strain [62]. Subcutaneous injections of a ROS solution, containing HOCI, O_2•, ONOO⁻ and/or OH⁻, everyday for 6 weeks leads to the development of skin fibrosis but also systemic reaction with lung fibrosis and renal injury. Interestingly, exposure to these oxidative molecules triggers a systemic autoimmune response characterized by the detection of anti-topoisomerase I and anti-centromere antibodies in ROS-treated mice. This autoimmune compound is not obtained in severe combined immunodeficiency (SCID) mice, which present only skin fibrosis after ROS treatment. Moreover, sera of wt mice treated with ROS solution could induce H_2O_2 production by mouse EC, similarly to what we observe when human EC exposed to SSc patient’s serum [63]. Recent study in ROS induced SSc have also shown an increase of splenic T cells with higher production of IL-4 and IL-13, two cytokines implicated in human SSc [64]. Thus, TGF-β independent model suggests a possible new role of ROS in the oxidation of self-antigens that could lead to auto-immune response against modified self-antigens and particularly, topoisomerase 1 and centromere antigens.

Finally, bleomycin-induced dermal fibrosis model is the widest used model in of SSc. Pro-fibrotic effects of bleomycin have been known for a long time since high doses of bleomycin instillation during cancer treatments frequently promote lung injury and pulmonary fibrosis [65]. Subcutaneous high doses of bleomycin typically induced localized dermal fibrosis and also induced systemic manifestation like lung injury and pulmonary fibrosis and inflammatory changes in skin. Few studies report activation of fibroblasts and SSc-like phenotypes characterized by the presence of autoantibodies, increased ECM secretion and systemic inflammation [66,67]. Easy handling model, bleomycin-induced dermal fibrosis remains nevertheless an artificial model in which anti-inflammatory drug effects are frequently overestimated as compared to results obtained in SSc patients [68].

Fra-2, caveolin 1/2, sclerodermatous GVHD mice models are also available and are characterized by SSc-like symptoms. However, in these models skin fibrosis is dominant, whereas the autoimmune reaction is limited [61].

**Oxidative stress**

As suggested in the experimental model chapter, oxidative stress plays a major role in the pathogenesis of SSc, and data have accumulated over the past 15 years to document it. Thus, ischemic phenomena leading to superoxide anions production do occur in SSc patients [69]. Interestingly, silica, an environmental agent responsible for the occurrence of SSc, is responsible for the induction of oxidative stress and NF-kB pathways in lungs of a luciferase reporter mouse model of respiratory insufficiency [70]. Indirect markers of ROS involvement have also been reported in sera from patients with SSc, such as oxidative proteins and lipid peroxidation [71,72]. Finally, monocytes and fibroblasts isolated from patients with SSc show an increased synthesis of superoxide anions [73] that could directly increase fibroblasts proliferation and ECM production [63].

More recently, exposure of EC and fibroblasts to sera from patients with SSc has been shown to induce a higher production of H_2O_2 by both HUVEC, an EC line, and the two fibroblast cell lines HEP-2 and NIH 3T3, than the exposure to sera from healthy controls [74]. Interestingly, clinical status of patients correlated with impact of the serum on ROS production by EC and fibroblast. This production of ROS is combined with an increased proliferation of fibroblasts. The deleterious effects of SSc patients’ sera could be reversed by N-acetyl cysteine and reduced glutathion for H_2O_2 production by EC, by bosentan for NO production by EC and D-penicillamine for fibroblasts proliferation.

This amplification of ROS production has also been linked to Ras protein and ERK1/2 activation pathways in patients with SSc, in synergy with platelet derived growth factor (PDGF) excess in the serum of patients [75]. A direct action of ROS on DNA has also been suggested since urine levels of 8-hydroxy-2′-deoxyguanosine, a marker of endogenous oxidative damage of DNA,
have been found higher in patients with SSc than in controls [76].
Thus, modifications of ROS targets, and particularly topoisomerase 1 and centromere proteins, two targets of SSc antibodies, have been proposed to contribute to the dysregulations observed in SSc [62]. Moreover, autoantibody binding seems to be dependent on specific metal deposits and ROS production in patients with SSc [77].

**Endothelial cell activation**

In patients with SSc, arterial and arteriolar hyperreactivity and myointimal proliferation can lead to vascular occlusion occurring in digital arteries, as well as in heart, lung and kidney arteries. The equilibrium of vascular endothelium in physiological conditions results from the balance between vasodilator and vasoconstrictor molecules, as well as the expression of adhesion molecules at the surface of EC. In patients with SSc, the vascular tone is dysregulated, leading to perturbed interactions between EC, vascular smooth muscle cells (VSMC) and ECM components participating in vascular remodeling and occlusion [78].

In patients with SSc, EC lesions are associated with increased plasma levels of factor VIII, decreased angiotensin converting enzyme production, increased activity of von Willebrand factor (vWF) and detection of circulating platelet aggregates [79]. At an early stage of the disease, EC involvement in SSc is characterized by increased apoptosis, loss of physiological barrier with permeabilisation of blood vessels and abnormal vascular tone regulation [80].

It has been proposed that EC activation and proliferation might occur as a consequence of the binding of anti-endothelial cell antibodies (AECA). Thus, in patients with SSc, AECA have been reported to induce EC apoptosis through antibody dependent cell cytotoxicity, involving NK cells and Fas/FasL pathway [81]. However, although of interest, these *in vitro* data do not preclude the pathogenic role of these antibodies *in vivo*. In addition, AECA are not specific for SSc and can be found in various auto-immune diseases, binding to a relatively large number of EC antigens. Finally, AECA failed to be identified in a number of experimental models of SSc including the Tsk-1 mouse model. In the same line, the prevalence of antiphospholipid syndrome is very low in SSc, although anti-cardiolipin antibodies and increased vWF activity have been associated with the detection of PAH, suggesting that anti-cardiolipin antibodies could represent a marker of EC damage in PAH associated with SSc [83].

Other mechanisms are involved in the perturbation of the vascular tone in patients with SSc. Thus, increased endothelin-1 (ET-1) levels, which are known to enhance vasoconstriction, inflammation, and mediate fibrosis and vascular remodeling, have been detected in the serum of SSc patients [82]. ET-1 is known to also activate fibroblasts and induce *in vitro* ECM production [83]. In patients with SSc, ET-1 levels have been shown to correlate with the severity of PAH. Moreover, ET-1 has been found to be increased in the lung at the mRNA and protein levels in patients with idiopathic PAH as compared to healthy controls. In kidney biopsies from SSc patients who experienced scleroderma renal crisis, immunohistochemistry staining demonstrated the presence of ET-1 in glomeruli, arterioles and arcuate arteries, which was not the case in other vascular diseases involving the kidney [84]. Interestingly, ET-1 receptors (ETR) inhibitors, including bosentan, ambrisentan and more recently macitentan, have demonstrated efficacy in the treatment of idiopathic PAH and PAH associated with SSc [85]. In addition, defective prostacyclin synthesis and perturbed NO synthesis have also been reported to a possible key elements to explain EC dysfunction in patients with SSc [86].

Finally, vascular epidermal growth factor (VEGF) levels have been reported to be decreased in patients with SSc as compared to healthy control [87]. Increased synthesis of MCP-1 and VCAM-1 by EC could also increase the recruitment of lymphocytes and promote the local and systemic inflammation in patients with SSc [88].

**Fibroblasts involvement and extra-cellular matrix synthesis**

Collagen accumulation in the dermis is one of the hallmarks of SSc. Extra-cellular matrix (ECM) accumulation is mostly the consequence of fibroblasts activation. *In vitro*, fibroblasts isolated from involved skin of patient with SSc produce more collagen IV, proteoglycan and fibronectin than those obtained from the skin of healthy controls. Phenotypical changes of these fibroblasts have also been described, with the expression of alpha-smooth muscle actin (α-SMA), a marker of differentiation into myofibroblast [89]. These myofibroblasts are known to secrete more collagen and display VSMC properties including migration phenotype. Distinctly from EC, which experience increased apoptosis, fibroblasts isolated from SSc patients have a defect in Fas/FasL induced apoptosis pathway which probably participates to the maintenance of the disease process [90]. Interestingly, a defect in metalloproteinase synthesis, which regulates formation of ECM, has also been reported [91].

In addition to intrinsic phenotype modifications of fibroblasts, extrinsic factors also contribute to explain excessive fibroblast activation in patients with SSc. Transforming growth factor β (TGF-β) has been proposed as an utmost important factor in fibroblast activation. Thus, fibroblasts phenotype modification in patients with SSc could be easily reproduced with incubation of normal fibroblasts with TGF-β [92]. As in TGF-β-dependent experimental models, in patients with SSc, the expression of TGF-β receptors at the fibroblast surface has been found increased as compared to healthy control fibroblasts [93]. This result, together with the
observation of increased levels of endogenous TGF-β in lung and dermal SSc lesions suggests an over stimulation of this pathway, leading to fibroblast phenotypical modifications including over proliferation, ECM production and differentiation into myofibroblasts. In addition, the expression of endogenous inhibitors of TGF-β pathway, including Fli-1 and SMAD7, is also defective in SSc patient’s fibroblasts [94,95]. However, the persistence of phenotypical modifications of fibroblasts from patients with SSc despite multiple passages, in vitro, and recent updates on epigenetic and DNA methylation mechanisms, suggest the existence of other mechanisms independent of TGF-β stimulation [96].

In addition to TGF-β, IL-4, a cytokine secreted by T helper 2 (Th2) cells has been identified as a key cytokine in SSc. IL-4 express pro-fibrotic properties on fibroblasts and increased IL-4 production has been documented in the spleen of ROS-induced SSc mouse model as well as in the serum of patients with SSc [97]. Interestingly, in bronchoalveolar fluid of SSc patients with interstitial lung disease, Luzina et al. have observed activated CD8+ T lymphocytes that present an activated phenotype and expression of mRNA encoding IL-4 and latent TGF-β mRNA [98]. Another important local source of IL-4 could also be a subset of double positive CD4/CD8 T lymphocytes, present in dermal lesions of patients with SSc [99]. In addition to IL-4, other growth factors have been identified in patients with SSc, including connective tissue growth factor (CTGF) or PDGF [100]. Alternatively to these growth factors, autoantibodies may activate fibroblasts. Anti-fibroblast antibodies (AFA) are not specific to patients with SSc and could lead to fibroblasts activation by direct targeting surface adhesion molecules. Two studies notably reported that exposure of normal fibroblasts to AFA leads to the internalization of the antibody that could interact with caveolin pathway and induce the secretion of pro-fibrotic chemokines including CCL2, CXCL1, CXCL8, CLKL and ECGF1 [101,102].

AFA directed at the PDGF receptor have been detected in patients with SSc. These autoantibodies have been reported to enhance in vitro the activation and pro-fibrotic phenotype of isolated fibroblasts [75,101]. Unfortunately, these results remain controversial [103].

PDGF can also be targeted by an inhibitor of the PDGF receptor, imatinib, a serine-protein kinase receptor inhibitor, that has been developed in the treatment of chronic myeloid leukemia [104,105]. Imatinib binds to c-Abl, a signaling protein downstream to TGF-β receptor, and blocks both TGF-β receptor and PDGF receptor. Because of promising results in the bleomycin induced model of SSc [106], clinical trials investigated the efficacy of imatinib in patient with SSc [107,108]. However, the results were disappointing in terms of tolerance, with a large proportion of dropouts and poor efficacy [109]. Novel therapeutic strategies have been proposed in experimental models of SSc and fibroblasts from patients with SSc in culture including inhibitors of SRC kinase family, Rho associated kinases, Fos related antigen-2 (Fra2) or histone acetylases and DNA methyl transferases [110,111]. Several of these strategies will be investigated in the treatment of patients with SSc in the near future.

**Immune system activation**

In addition to EC and fibroblasts, the immune system plays an important role in the pathogenesis of SSC. Thus, autoantibodies, imbalance in B and T lymphocytes subpopulations and perturbations of dendritic cells have been reported in SSc. Although the disease cannot be qualified as autoimmune, since autoantibodies have not been shown to induce/transfer the disease, the identification of specific autoantibodies is of major help in the diagnosis and/or the evaluation of the progression of SSc [112]. The distribution of autoantibodies in SSc patients correlates with phenotypes. Thus, anti-topoisomerase I, anti-RNA polymerase III and anti-U3 RNP correlate with dSSc, while anti-centromere, anti-Prm/Scl, anti-Th/To and anti-U1 RNP are associated with iSSc. Various other antibodies have also been found in SSc patients, directed against several targets such as fibrillin-1, metalloproteinases or PDGF receptor. These autoantibodies argue for a pathogenic role of B cells [113]. Thus, circulating B cells from SSc patients differ in their phenotype as compared to healthy controls, with increased proportions of naive B cells and decreased numbers of memory B cells and plasma cells [114]. CD19 and CD21, two activation co-receptors of the B-cell receptor (BCR), are overexpressed in these naive and memory B cells. The activation receptors CD80, CD86 and CD95 are upregulated on memory B cells, suggesting their participation to the pathogenetic process. High levels of B cell activating factor (BAFF) have been measured in the serum of patients with SSc, together with an overexpression of BAFF-R at the surface of peripheral B cells from SSc patients [115]. BAFF activates the NF-κB pathway, promotes B cells survival and participates to the differentiation of autoreactive B cells. In addition, it has been documented that B cells infiltrate the dermis of SSc patients [116,117], that circulating levels of IL-6 are increased in SSc patients as compared to healthy controls and correlated with the extent of skin fibrosis [118]. In Tsk-1 mouse, the depletion of B cells leads to a decrease of total IL-6 mRNA and improvement of fibrotic lesions [119]. Finally, IL-6 is known to stimulate collagen secretion by fibroblasts and represent a potential “B cell link” to fibroblast activation. T cells are also involved in the pathogenesis of SSc. Thus, a Th-2 bias has been documented in SSc patients [120]. Increased levels of IL-4 and IL-13 have been measured in the serum of SSc patient. These two cytokines have been classically described as synergic Th-2 polarization cytokines via STAT6. Although the trigger contributing to the initiation of secretion of these
cytokines is not identified, it has been documented that mature and activated Th-2 secrete IL-4, IL-6 and IL-13, enhancing a positive loop of Th-2 polarization and B cells stimulation [121]. Interestingly enough, the treatment of Tsk-1 mice with an anti-IL-4 monoclonal antibody prevented the induction of skin lesions [122], underlining the major role this cytokine in SSc physiopathology. Taken together, targeting B cells (anti-CD20), IL-6, IL-4 and also T cells and mature lymphocytes (anti-CD52) may lead to substantial clinical improvement in mouse models and in open series of patient with SSc [119,122–124]. These findings argue for the important role of B and T cells in the pathogenesis of SSc. In addition to B and T lymphocytes, dendritic cells appear of utmost importance in SSc. Thus, a recent proteome-wide analysis of the culture supernatant of plasmacytoid dendritic cells (pDC) isolated from patients with SSc allowed identification of increased levels of CXCL4, with higher levels in diffuse than in limited forms, and increased levels in early diffuse vs. late diffuse SSC patients [125]. Interestingly, CXCL4 was also detected in the skin of SSC patients and not in healthy control skin. Serum levels of CXCL4 correlated with clinical status of SSC patients. Notably, in patients with lung fibrosis and/or PAH, high levels of CXCL4 correlated with a poor prognosis. Finally, infusion of CXCL4 leads to increased leukocytes infiltration, skin thickening and CCL2 mRNA expression in the bleomycin-induced model of SSc. Taken together, these observations argue for a possible role of CXCL4 in the pathogenesis of SSc. Further studies will aim at the interest of targeting CXCL4 in SSC patients.

**Conclusion**

Major work has been done in order to improve the understanding of SSC pathogenesis. A number of new experimental models have been set up, that should help to test new therapeutic targets. Although a large number of genetic studies have been conducted, it is still difficult to identify a scleroderma specific genetic background, and the major progress in this setting is probably the identification of an interferon signature, as reported in SLE and Sjögren’s syndrome. Besides EC and fibroblasts, B cells and autoantibodies actually probably represent major actors in the pathogenesis of SSc. Finally, the role of dendritic cells and CXCL4 seem to be promising.

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