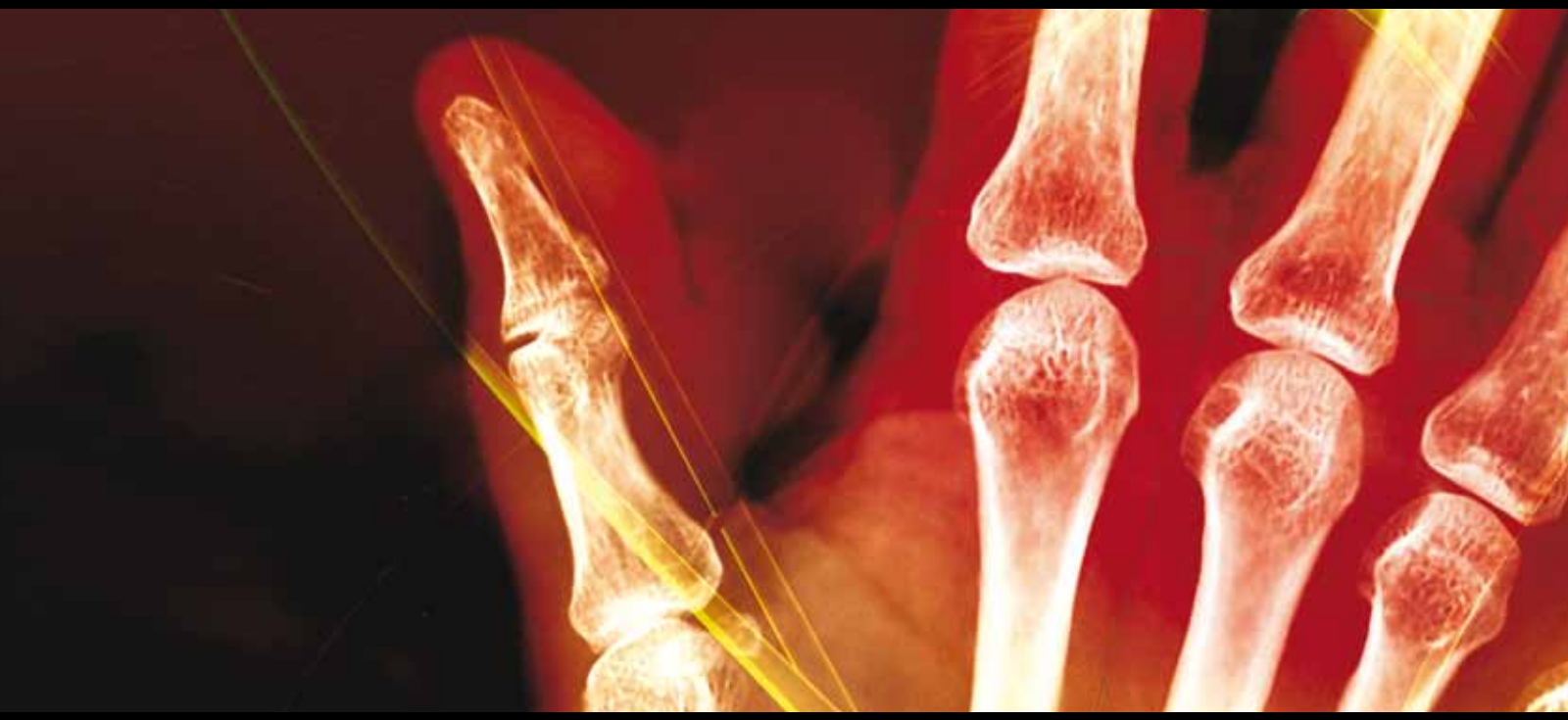
International Journal of Rheumatology

Systemic Sclerosis 2011

Guest Editors: Lorinda Chung, Oliver Distler, Laura Hummers, Eswar Krishnan, and Virginia Steen



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Editorial

Systemic Sclerosis 2011

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Systemic sclerosis (SSc) is a systemic autoimmune disease characterized by widespread fibrosis affecting the skin, internal organs, and vasculature. However, there are currently no systemic, disease-modifying therapies available for the treatment of the overall condition, and the outcomes remain poor. Studies into disease pathogenesis have identified several pathways that are dysregulated in SSc, and novel targeted therapies are currently being developed. In this special issue, we invited authors to submit original research articles, review articles, or case reports/case series describing preclinical, translational, or clinical studies related to new therapies for SSc.

A set of papers in this special issue focuses on identification of new therapeutic targets in preclinical and translational studies. V. J. Moulin's paper is a review article describing the role of apoptosis in the initiation and maintenance of disease in SSc, through effects on the immune system, vascular damage, and fibroblast proliferation. This report discusses potential therapies targeting apoptosis and the Fas/FasL pathway that could be investigated in patients with SSc. Other paper describes vascular changes in the bleomycin-induced mouse model of SSc. The authors discuss the use of this mouse model to investigate targeting fibrosis, apoptosis, and cellular adhesion molecules for the treatment of vascular disease in SSc. The paper by T. Radstake et al. reviews the evidence that hypoxia contributes to the pathogenesis of SSc, with a focus on the role of hypoxia inducible factor (HIF)-1 alpha in the vasculopathy, immune dysregulation, and fibrosis in SSc. This paper summarizes potential therapeutic interventions to bypass the dysfunctional hypoxic pathway in SSc. The paper by R. De Vries et al. describes an original research study evaluating the accumulation of advanced glycation end products (AGE) in the skin of patients with SSc compared with controls using a technique called skin autofluorescence. Although this study did not find a significant difference in AGE accumulation in SSc skin compared with control samples, use of angiotensin-converting enzyme inhibitors and angiotensin II receptor blockers in the SSc patients may have confounded the results. The paper by E. G. Kroon et al. is an original research article evaluating the expression of Types I and III interferons (IFNs) and interferon-stimulated genes (ISG) in peripheral blood mononuclear cells from SSc patients compared with controls. This study confirmed the increased basal expression of Type I IFNs and the ISG 2′5′OAS in SSc, but found no induction of Type III IFNs. This paper provides further evidence that targeting the IFN pathway may be useful in the treatment of SSc. The paper by S. M. Violette et al. reviews the preclinical data supporting the role of the integrin $\alpha v\beta 6$ in the activation of fibrosis via the transforming growth factor (TGF)-β pathway. This paper summarizes in vivo evidence of the utility of blocking $\alpha v\beta 6$ for the treatment of lung fibrosis and provides rationale for pursuing this therapeutic approach in patients with SSc-associated interstitial lung disease.

Another set of articles includes reviews of novel therapies that are currently being evaluated for the treatment of SSc. The paper by M. Anderson et al. reviews the role of

interleukin-6 (IL-6) in SSc, summarizing evidence of effects on B cells, inflammation, fibrogenesis, and endothelial cell activation. The paper reviews the rationale for ongoing clinical trials of agents blocking IL-6 transsignaling for the treatment of SSc. The paper by S.-N. Liossis et al. reviews the published literature supporting the role of B cells in SSc, summarizing data from animal models and human studies. The authors then review the results of four clinical trials assessing the effects of B cell depletion with rituximab therapy on skin disease and lung function in patients with SSc. The paper by R. F. Spiera and J. Gordon reviews the preclinical and clinical studies of tyrosine kinase inhibitors, with a focus on experience with imatinib, in the treatment of SSc and related fibrotic conditions. The authors conclude that interpretation of the results from the completed proof-of-concept studies is difficult due to the small size and heterogeneity of the populations studied and the open-label designs. The review article by K. Phillips et al. describes published studies investigating the utility of phosphodiesterase-5 inhibitors (PDE-5-I) in the treatment of Raynaud's phenomenon (RP) and/or digital ulcers (DU), detailing results of studies using sildenafil, vardenafil, and tadalafil. The authors also list the ongoing clinical studies of PDE-5-I for RP and DU. The paper by D. F. Fiorentino et al. reviews the role of endothelin-1 in the pathogenesis of SSc-associated vascular disease and summarizes the published reports evaluating the use of endothelin receptor antagonists in the treatment of RP and DU.

One of the sets in this special issue includes two articles describing rare clinical manifestations of SSc, gastric antral vascular ectasias and primary biliary cirrhosis, and management strategies for these entities. The paper B. Markewitz et al. is a case series describing the tolerability and efficacy of naltrexone for the treatment of pruritus and gastrointestinal symptoms in three patients with SSc. The paper by K. Nikolov and M. Baleva reviews the rationale for using intravenous immunoglobulins (IVIG) in the treatment of SSc. The authors also summarize the published case reports and series supporting the potential efficacy of IVIG in the treatment of skin sclerosis in SSc.

In summary, this special issue provides an interesting compilation of articles addressing potential emerging therapies for the treatment of SSc, including information gleaned from preclinical, translational, and clinical studies. We, the guest editors, hope readers find that the manuscripts included herein offer a comprehensive summary of the current status of drug development and promising therapies for SSc.

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Review Article

Primary Biliary Cirrhosis Associated with Systemic Sclerosis: Diagnostic and Clinical Challenges

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Patients with primary biliary cirrhosis (PBC) often have concurrent limited systemic sclerosis (SSc). Conversely, up to one-fourth of SSc patients are positive for PBC-specific antimitochondrial antibodies (AMA). The mechanisms responsible for the co-occurrence of these diseases are largely unknown. Genetic, epigenetic, environmental, and infectious factors appear to be important for the pathogenesis of the disease, but the hierarchy of events are not well defined. Patients with SSc and PBC have an increased morbidity and mortality compared with the general population, but whether the presence of both diseases in an affected individual worsens the prognosis and/or outcome of either disease is not clear. Some case reports suggested that the presence of SSc in PBC patents is associated with a more favorable prognosis of the liver disease, whereas others report an increased mortality in patients with PBC and SSc compared to patients with PBC alone. This paper discusses the features of patients with PBC-associated SSc. Our aims are to clarify some of the pathogenetic, diagnostic, and clinical challenges that are currently faced in the routine management of these patients. We also intend to provide some practical hints for practitioners that will assist in the early identification of patients with PBC-associated SSc.

1. Introduction

1.1. Primary Biliary Cirrhosis. Primary biliary cirrhosis (PBC) is a chronic cholestatic liver disease characterized by immune-mediated chronic nonsuppurative cholangitis that mainly affects interlobular and septal bile ducts [1–3]. PBC is a rare disease with prevalence ranging from 28 to 402 per million [4], which is highly variable based on geographical location. PBC primarily affects middle aged women [5]. Several reports indicate that the incidence and prevalence of PBC is increasing in the UK, USA, Finland, and Australia [4–7]. PBC often occurs in association with other autoimmune conditions [1–3]. The serological hallmark of PBC is the presence of high-titre serum antimitochondrial autoantibodies (AMA), usually existing in 90–95% of patients with PBC [1–3, 8–16]. The presence of AMA in asymptomatic patients is usually indicative of eventual PBC development [17]. These autoantibodies specifically recognize lipoylated domains within components of the 2-oxoacid dehydrogenase family of enzymes, particularly the E2 component of the pyruvate dehydrogenase complex, located within the inner mitochondrial membrane [1–3, 8–12]. Indirect immunofluorescence using rodent liver, kidney, and stomach sections as substrate is still the most widely used screening assay for AMA in the routine setting [18]. Other techniques such as immunoblotting and ELISA have a higher sensitivity, and the use of cloned mitochondrial antigens and bead assay testing systems allows for the identification of AMA in the sera of patients previously defined as AMA negative [19]. Additionally, PBC-specific antinuclear autoantibodies

(ANAs) can be observed in 30% of patients presenting with multiple nuclear dot (antibodies against Sp100) or nuclear membrane staining patterns (antibodies against gp210) [9, 10, 12, 14, 20], which are preferentially identified using HEp-2 cells as substrate [21]. The autoimmune nature of PBC is supported by a plethora of experimental and clinical data, such as the presence of autoreactive T cells and serum autoantibodies in patients with PBC [8, 15, 22–31].

The aetiology of PBC remains unknown, although evidence suggests a role for both genetic susceptibility and environmental factors that remain to be characterized. In fact, a number of chemicals and infectious agents have been proposed to induce the disease in predisposed individuals [22–27, 30, 32–38]. At presentation, patients with PBC may have nonspecific symptoms such as pruritus and fatigue while jaundice is less frequently seen [1–3]. Portal hypertension and its complications may also develop in patients with early, pre-cirrhotic PBC [1–3]. However, the majority of PBC patients are asymptomatic and diagnosed incidentally during the diagnostic workup or treatment for other conditions [39, 40]. Currently, a definite diagnosis of PBC is made on a combination of abnormal serum enzymes indicating cholestasis (i.e., elevated alkaline phosphatase for at least six months), the presence of serum AMA (titre $> 1:40$ by indirect immunofluorescence), and characteristic liver histology with florid bile duct lesions [1–3, 18]. A probable diagnosis is made when two out of these three criteria are present. Serum AMAs (or disease-specific antinuclear antibodies) may precede disease onset by several years, and many individuals found positive for these autoantibodies in the absence of other criteria eventually develop PBC [17].

PBC has a progressive course which may extend over many decades, with greatly variable progression rates among patients. The end of this progression is characterised by cirrhosis, liver failure. However, the patterns of clinical disease and natural history have changed significantly in the last two decades after the introduction of medical treatment with ursodeoxycholic acid (UDCA). When UDCA is administered in early PBC at adequate doses (13–15 mg/kg/day), the progression of the disease is altered, with many patients having a normal life expectancy without additional therapeutic measures [41].

Concomitant autoimmune diseases are often found in patients with PBC. PBC is found in patients with systemic sclerosis (SSc). Also, SSc is one of the most frequent autoimmune rheumatological conditions associated with PBC. This paper discusses the major characteristics of patients with PBC and SSc, and provides clues related to their immunopathogenic link (Table 1).

2. PBC-SSc

2.1. Epidemiology. PBC has been considered as the most common liver disorder in patients with systemic sclerosis (SSc) [42]. This association was first described to co-occur by Milbradt in 1934, and it has been noted historically in several case reports. One such case from 1964 reports two patients with SSc and possible (but unconfirmed) PBC [43]. Murray-Lyon et al. report two cases of SSc and PBC [44]. The first case was that of a 64-year-old female with Raynauds and scleroderma of the right hand and arm, who was found to have hepatosplenomegaly [44]. She was positive for AMA, and a liver biopsy confirmed the diagnosis of PBC [44]. The second case was similar, with AMA positivity and PBC confirmed on liver biopsy [44]. Despite several similar reports over the years, liver disease has not been considered a significant feature of scleroderma, and a higher prevalence of liver disease was found in the control populations of several large studies [45, 46]. The association of lcSSc and PBC was first described in 1970 with two cases of PBC and limited scleroderma [44]. A further six cases were reported by Reynolds et al. [47], and several other case reports have found an association between lcSSc and PBC. The first case reporting an association of PBC and scleroderma, without features of lcSSc, was described in 1972 [48]. The prevalence of clinically evident PBC among patients with SSc was recently reported to be 2.5% in a registry of 1700 SSc patients [49] and 2% in a series of 817 patients with SSc [50]. On the other hand, the prevalence of SSc in patients with PBC is estimated to be around 8%, as demonstrated by two studies comprising large cohorts of patients with PBC [49, 51]. However, case reports [44, 47, 48, 52–63] and some series reported a wider range of prevalence (3–50%) of SSc, mostly lcSSc, in PBC patients [42, 49, 51, 55, 61, 64, 65].

Large epidemiological studies on PBC note a minority of patients who also have SSc (scleroderma). A large French study found scleroderma in 1% of a cohort of PBC patients, although 1% of their first-degree relatives and 1% of controls were also noted to have scleroderma [66]. One of the most comprehensive epidemiological studies by Gershwin and colleagues found that 2% of PBC patients and 1% of their first-degree relatives had scleroderma, compared to none of the controls [39]. First-degree relatives with scleroderma were more often sisters, followed by daughters of PBC patients [39, 67]. Twin studies in both conditions are scarce. One twin study for SSc found a low concordance of 4.2% among monozygotic (MZ) twins, compared to 5.6% in dizygotic (DZ) twins [68]. Interestingly, there was a 90% concordance for ANA among MZ twins, compared to only 40% among DZ [68]. A higher concordance of 63% among MZ twins was found in the only comprehensive twin study in PBC [69]. Although both twin studies note co-existing autoimmune disease, which was often the same condition in the twin, none have noted SSc in twins with PBC or PBC in twins with SSc.

2.2. Immunopathogenesis. Despite the scarcity of case reports and large-scale studies, the association of PBC and scleroderma seems to be more than coincidental and suggests that these two diseases might have a common autoimmune basis. However, the autoimmune mechanisms behind the PBC-SSc association are still not fully understood. It has been reported that this patient group has clonally expanded CD8(+) T cells expressing one T-cell receptor beta-chain variable region, TCRBV3, which may be involved in the disease pathogenesis [70]. Genetic, epigenetic, environmental, and infectious

Table 1: Features demographic, immunological and genetic features of primary biliary cirrhosis (PBC) and systemic sclerosis (SSc).

	PBC	SSc
Prevalence (highly variable geographically)	28–402/million	50–200/million
Incidence (highly variable geographically)	2.3–27/million	0.6–122/million
Male to female ratio	1 : 8	1 : 1.5–12 (highly variable geographically)
Peak frequency age	53 years	45–64 years
Autoantibodies	AMA, ANA	*Limited disease*: ACA, anti-Th/To, anti-U1-RNP *Diffuse disease*: TOPO, anti-RNA polymerase III, anti-U3-RNP
Genes (positive associations)	*HLA*: DRB1, DQA1, DQB1, DQA2 *Non-HLA*: IRF5, STAT4, SPIB, IKZF3-ORMDL3, IL12A, IL12RB, MMEL1, DENND1B, CD80, IL7, CXCR5, TNFRSF1A, CLEC16A, NKFB1	*HLA*: HLA-DRB1*1104, DQA1*0501, DQB1*0301, HLA-DRB1*0804, DQA1*0501, DQB1*0301 *Non-HLA*: STAT4, IRF5, BANK1, TNSF4, TBX21, IL-23R, and C8orf13-BLK

AMA, anti-mitochondrial antibody; ANA, anti-nuclear antibody; ACA, anti-centromere antibody.

factors appear to be important for the induction of the underlying autoimmune pathology, but the hierarchy of events and the close interplay of these factors are not well defined.

The association between PBC and SSc has been largely based on reports indicating the presence of autoantibodies related to SSc in patients with PBC and *vice versa*. Autoantibodies which characterize limited cutaneous SSc (lcSSC) include anti-centromere antibodies (ACA), anti-Th/To, anti-U1-RNP, and PM/Scl. Diffuse cutaneous SSc (dcSSc) is characterized by anti-Scl 70 antibody (anti-topoisomerase I antibody, TOPO), anti-RNA polymerase III, and anti-U3-RNP [71]. Severe lung disease is the hallmark of anti-TOPO-positive dcSSC patients. DcSSc patients with anti-RNA polymerase III have the most severe skin disease and the highest frequency of renal crisis. Patients with the nucleolar antibody anti-U3-RNP have dcSSc with multiorgan involvement [71].

The autoimmune basis of association between PBC and SSc was first established by the presence of AMA in approximately 5% of patients with scleroderma and ACA in one-quarter of patients with PBC [55]. A positive ACA is reported in 9–30% of PBC patients [59, 72–75] and in 22–25% of all SSc patients, almost all of which have lcSSc. Conversely, up to 25% of SSc patients are AMA positive, but the high prevalence rates of AMA are probably secondary to referral bias and overestimate the frequency of AMA in SSc [76–79]. Another interesting point which needs attention is that of studies reporting a relatively high prevalence of AMA negative PBC in patients with SSc or other autoimmune diseases [51, 80] the autoantibody profile of SSc patients with AMA-negative PBC may require the use of highly sensitive immunoassays for the detection of AMA. It has been shown that such assays are able to detect AMA in serum samples from SSc patients characterized as AMA negative by indirect immunofluorescence, and this may be the case for other PBC-specific autoantibodies, such as ANA specific for sp100 [11, 12, 50].

ACA positivity is greater in PBC-SSc than in either disease in isolation, but there is no cross-reactivity between mitochondrial and centromere antigens [81]. Because ACA have been detected not only in SSc but also in other autoimmune diseases [82–85] including PBC [72, 86], the clinical significance of ACA in PBC has been the focus of ongoing research. Three major centromere antigens have been recognized: centromere protein A (CENP-A, 18 kD polypeptide), centromere protein B (CENP-B, 80 kD polypeptide), and centromere protein C (CENP-C, 140 kD polypeptide). One study attempted to identify the major epitope of ACA in sera obtained from patients with PBC and to classify the correlation between the presence of ACA epitopes and the clinical features in patients with PBC [87]. The serological results obtained were compared with clinical features of lcSSc in PBC. Forty-one patients with PBC were studied: 10 out of 16 (63%) patients with ACA (all anti-CENP A) had one or more lcSSc feature. The higher incidence of Raynaud's phenomenon seen in ACA-positive patients with PBC than that in ACA-negative patients with PBC suggested a close association of the presence of ACA with clinical features of lcSSc in patients with PBC [87]. From the results of this study, it was proposed that there is a subset of PBC patients with scleroderma who are ACA positive and differ from both ACA-negative PBC-SSc and ACA-negative PBC non-SSc patients, based on their clinical features and ACA epitope reactivity [87].

Over the past two years, a tremendous amount of data has come available as to the genetics underlying PBC and SSc. In regards to SSc, several HLA and non-HLA regions have been identified [88], with HLA regions showing variability among ethnic groups. Positive HLA associations in whites and Hispanics include HLA-DRB1*1104, DQA1*0501, DQB1*0301 [89]. Negative associations in those groups included DRB1*0701, DQA1*0201, DQB1*0202, and DRB1*1501 [89]. Positive HLA associations in African Americans included HLA-DRB1*0804,

DQA1*0501, and DQB1*0301 [89]. That study also noted that ACA positivity was closely associated with HLA-DQB1*0501 [89], and another study found an association between TOPO positivity and HLA-DRB1*1104 [90]. A smaller study of a Spanish cohort showed similar HLA findings to those noted above [90]. Several non-HLA regions have also been identified in SSc. These include STAT4 [88, 91–94], IRF5 [88, 95, 96], BANK1 [97, 98], TNSF4 [99], TBX21 [92], IL-23R [100], and C8orf13-BLK [101] among others [88]. As with SSc, several HLA and non-HLA regions have been identified in PBC. HLA regions include DRB1, DQA1, DQB1, and DQA2 [102, 103]. Non-HLA regions include IRF5, STAT4, SPIB, IKZF3-ORMDL3, IL12A, IL12RB, MMEL1, DENND1B, CD80, IL7, CXCR5, TNFRSF1A, CLEC16A, and NKFB1 [104–106]. Interestingly, PBC and SSc have several genes in common: HLA-DRB1, DQA1, DQB1, IRF5, and STAT4, although it should be noted that DR11, which is positively associated with SSc, is considered protective in PBC [88, 105].

Infectious agents have been implicated in the pathogenesis of both SSc and PBC. A number of organisms, such as *E. coli*, have been strongly associated with PBC [22, 107, 108], but not with SSc. *Helicobacter pylori* and *Chlamydia* have been implicated in both conditions [109–119]; however, some studies indicate the *Chlamydia* is not involved [72, 120, 121]. It is possible that certain infectious organisms contributes to the development of PBC or SSc in isolation and that other organisms induce the disease in both conditions.

2.3. Screening and Diagnosis of PBC in SSc Patients and Vice Versa. Given the overlap between PBC with SSc and *vice versa*, including ACA positivity in PBC patients and AMA positivity in SSc patients, the major challenge remains to clarify which screening method would be best for early diagnosis of the associated conditions.

Firstly, routine screening for PBC-specific antibodies in patients with SSc needs to be further refined. Recently, Norman et al. investigated the presence of antibodies against PBC disease-specific mitochondrial antigens and antibodies against the sp100 nuclear body antigen in 52 patients with SSc, by using two commercially available ELISAs [79]. In that study, 13% of cases were positive for AMA and 2% for ANA (anti-sp100), and one patient (2%) was diagnosed with symptomatic PBC [79]. These figures were reproduced by Mytilinaiou et al., who confirmed 13.5% positive results with ELISA testing for antibodies against PBC disease-specific mitochondrial antigens in 37 SSc patients [78]. However, this was not confirmed with the conventional indirect immunofluorescence based on unfixed rodent kidney, liver, stomach tissue sections, or HEp-2 cells as antigenic substrates, and none of the ELISA-positive patients showed features of PBC [78]. It remains to be clarified whether ELISA testing is less specific with false positive results or that it simply represents a more sensitive method with respect to indirect immunofluorescence, which should currently remain the technique of choice.

Nevertheless, the presence of AMA can precede clinical symptoms of PBC. Indeed, Mitchison et al. and Metcalf et al. showed that the vast majority of AMA-positive subjects have typical histological features of PBC despite being asymptomatic with normal biochemistry [17, 122]. Furthermore, the study by Prince et al. suggested that 36% of initially asymptomatic PBC patients would become symptomatic within a median time of 5 years [123]. Thus, AMA-positive SSc cases require immediate attention and close, long-term monitoring for early detection of symptoms, signs, and liver biochemistry suggestive of chronic cholestatic liver disease. Routine followup of AMA-positive SSc patients should include liver tests (alanine aminotransferase, aspartate aminotransferase, γ-glutamyl transpeptidase, alkaline phosphatase, albumin, bilirubin, international normalized ratio), thyroid function, and possibly an annual ultrasound abdominal scan. Transient elastography of the liver has been used to assess biliary fibrosis in patients with PBC [124]. This test is emerging as a useful screening tool to detect undiagnosed chronic liver disease in apparently healthy subjects [125]. Whether patients with SSc, who are tested positive for PBC-specific AMA, need regular checks with transient elastography or more common tests, such as liver ultrasound, needs to be evaluated in large prospective multicentre studies. Currently, there is no evidence that either of these would be of value. Figure 1 illustrates the diagnostic and screening algorithm for PBC in SSc patients.

Screening PBC patients for ACA is not mandatory but can be considered, especially in the presence of disease-related symptomatology. Nakamura et al. reported that, in PBC patients, ACA positivity was significantly associated with more severe ductular pathology on liver histology and was a significant risk factor for the development of portal hypertension [126]. In another study, ACA-positive PBC patients without clinical features of SSc were shown to have similar symptoms and signs at diagnosis [49]. Although ACA positivity is not pathognomic of SSc, it is associated with an increased risk of developing connective tissue disease [127]. One review [128] reported a sensitivity of 32% (17–56%) for SSc and 57% (32–96%) for lcSSc and specificity of at least 93%, while ACA positivity was present in 5% of patients with other connective tissue diseases and less than 1% of disease-free controls. Since ACA could be predictive of rheumatic disorders, it has been suggested that an assessment of PBC patients should always include screening for SSc-related symptoms, such as Raynaud's phenomenon and CREST-related symptoms (calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia) [129]. The use of nailfold videocapillaroscopy in patients suspected of having connective tissue disease may be a useful indicator. Some evidence suggests that this assessment can be useful for the diagnostic and/or clinical management of patients with PBC and suspected SSc. Experimental and clinical observation suggests that patients with PBC have endothelial dysfunction [130]. In an interesting study, nailfold videocapillaroscopy abnormalities were found in 91% of patients with PBC, and capillary alterations characteristic of SSc were found in 54% [131]. Eleven out of the 22 PBC patients (50%) had extrahepatic signs of connective tissue disease with most being related to SSc, while patients with other types of chronic liver disease did not present with rheumatic

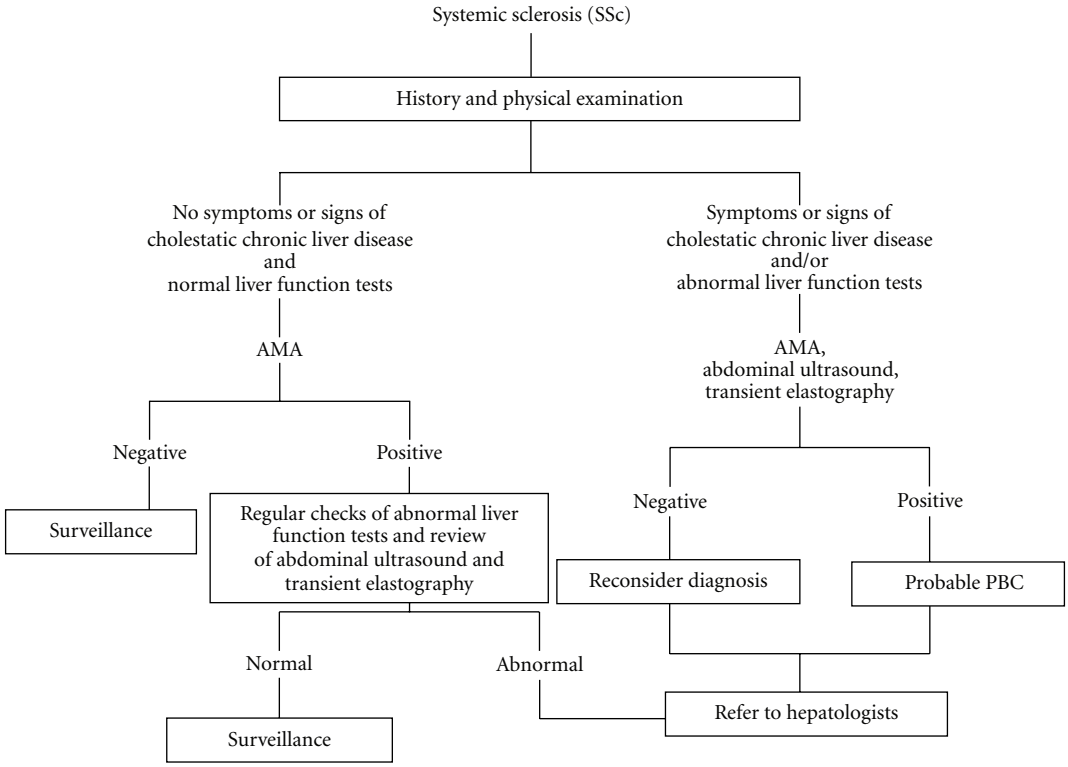

Figure 1: A proposed algorithm for the screening and diagnosis of primary biliary cirrhosis (PBC) in patients with established systemic sclerosis (SSc).

manifestations [131]. In PBC patients, there was a significant association between SSc capillary pattern and rheumatic manifestations. The high prevalence of nailfold capillary abnormalities characteristic of SSc in patients with PBC, and correlation with sclerodermal manifestations, suggests that this capillaroscopic finding could be a useful indicator to investigate rheumatic manifestations in these patients [131]. Further clinical assessment of organ involvement (especially lung by spirometry) in association with evaluation of pulmonary artery pressure on echocardiography should be considered in PBC patients with a definite diagnosis of SSc. A proposed diagnostic and screening algorithm for SSc in PBC patients is presented in Figure 2.

2.4. Clinical Presentation and Prognosis. In approximately 60% of the cases, the clinical presentation of SSc precedes that of PBC. The demographics of the disease in patients with overlapping features are not well defined. For example, it is not clear whether in the PBC-SSc group the diagnosis of PBC occurs at a lower age than that in patients with PBC alone. In a study of 43 PBC-SSc patients, the median age at diagnosis of PBC made after SSc diagnosis was lower (46.1 years) than in PBC diagnosed before SSc (51.1 years). This was lower than the diagnosis in PBC alone, with a median age of 53.2 years at diagnosis [49]. The different age at diagnosis in the PBC-SSc patients, compared to patients with PBC alone, was probably due to the effect of lead time bias (i.e., screening for PBC in SSc patients and thus early diagnosis of asymptomatic PBC, since 56% presented with SSc alone).

PBC-SSc patients were reported to have a higher incidence of a first episode of spontaneous bacterial peritonitis and septicaemia during followup with respect to patients with PBC alone. This is likely due to an increased risk of infection due to immune abnormalities and organ system manifestations associated with SSc [132].

Both SSc and PBC are associated with increased morbidity and mortality compared with the general population [123, 133–139]. Among the disease-related causes of mortality in SSc patients, pulmonary fibrosis, pulmonary arterial hypertension, and cardiac causes (mainly heart failure and arrhythmias) are reported to account for the majority of deaths. The most frequent non-SSc-related causes of death are infections, malignancies, and cardiovascular causes [140]. In PBC patients, liver-related causes account for roughly 50% of deaths, whereas cardio- and cerebrovascular causes together with malignancies are responsible for the non-liver-related deaths [139, 141]. Some case reports [62, 142] suggest that PBC in association with SSc is associated with a more favourable prognosis than PBC alone, whereas others reported increased mortality due to SSc [143]. In the study which included 43 PBC-SSc patients, liver disease had a slower progression in PBC-SSc compared to matched patients with PBC alone. A lower rate of liver transplantation and liver-related deaths was demonstrated in PBC-SSc patients compared to patients with PBC alone, and these differences were not due to earlier SSc-related deaths [49]. However, the improvement in liver-related survival in the PBC-SSc cohort was outweighed by an increase in

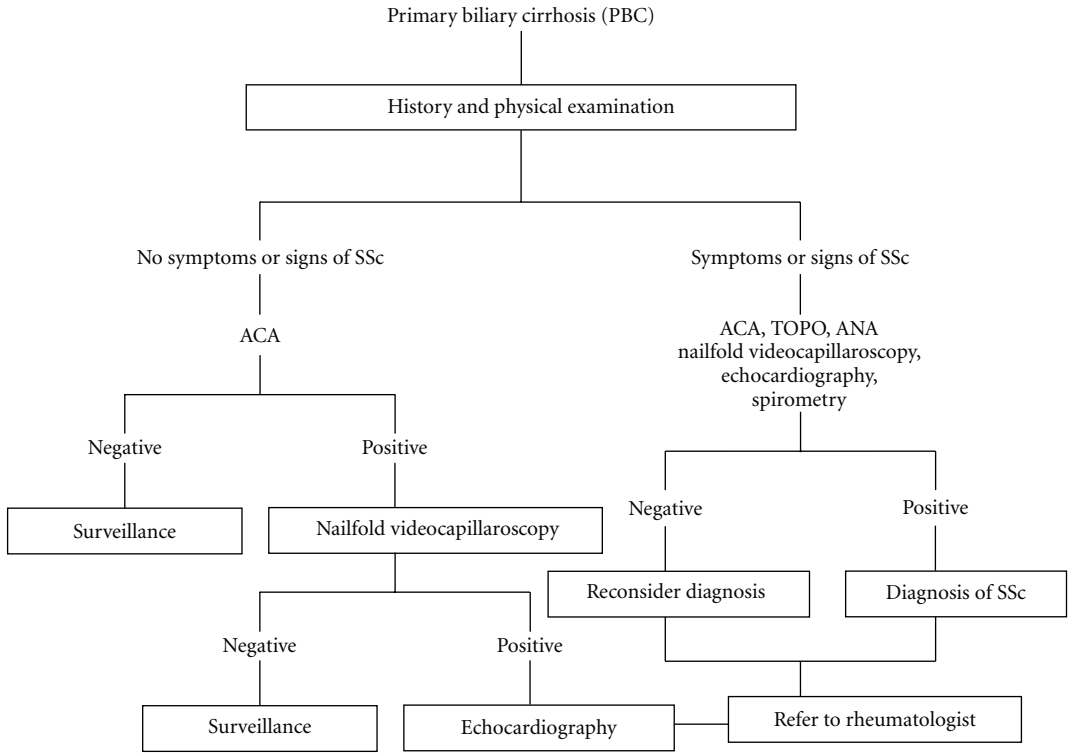

Figure 2: A proposed algorithm for the screening and diagnosis of systemic sclerosis (SSc) in patients with established primary biliary cirrhosis (PBC).

non-liver-related deaths due to SSc, and, thus, overall survival was not different in PBC-SSc patients and those with PBC alone [49]. These data emphasize the importance of comorbidity in PBC. More data on the outcome of patients with PBC and PBC with SSc are needed. If patients with PBC and SSc have a lower rate of liver transplantation and liver-related deaths compared to patients with PBC alone [49], it would be expected that patients with PBC and SSc-related ACA would also have better prognosis than their seronegative counterparts, but this does not appear to be the case [126]. It may be that the outcome of patients with ACA-positive PBC, who do not have SSc-related symptoms, differs from that of ACA-positive SSc and PBC overlap.

Prince and colleagues observed an increase in non-hepatic deaths in asymptomatic PBC, even with a reduced liver-related mortality, in comparison with symptomatic PBC [123]. Since the causes of death in PBC-SSc patients are mainly due to SSc and not to liver disease, these patients may need different prognostic models in order to better predict their liver-related survival. Prognostic models for PBC alone may not be applicable for PBC associated with SSc or for other associated autoimmune diseases to assess the risk of liver-related mortality and the need for liver transplantation.

2.5. Therapy. All PBC patients with abnormal liver biochemistry should be considered for specific therapy. UDCA at the dose of 13–15 mg/kg/day on a long-term basis is currently considered the mainstay of therapy for PBC [18]. In the early stages of PBC, UDCA protects injured cholangiocytes against the toxic effects of bile acids. In later stages of the disease, UDCA stimulates impaired hepatocellular secretion, mainly by posttranscriptional mechanisms [144]. In addition, stimulation of ductular alkaline choleresis and inhibition of bile acid-induced hepatocyte and cholangiocyte apoptosis are included among the beneficial effects of UDCA in PBC [144]. UDCA has been demonstrated to markedly decrease serum bilirubin, alkaline phosphatase, γ-glutamyl transpeptidase, cholesterol, and immunoglobulin M levels and to ameliorate histological features in patients with PBC in comparison to placebo treatment [145–149]. However, no significant effects on fatigue or pruritus were observed in these large trials nor were effects on survival [150]. Favorable long-term effects of UDCA are observed in patients with early disease and in those with a good biochemical response, which should be assessed after one year from start of treatment [18]. A good biochemical response after one year of UDCA treatment is currently defined by a serum bilirubin $\leq$1 mg/dL (17 μmol/L), alkaline phosphatase $\leq$3x ULN, and aspartate aminotransferase $\leq$3x ULN, according to the "Paris criteria" [151]. The "Barcelona criteria" indicates a good response with a 40% decrease or normalization of serum alkaline phosphatase [152].

Whether treatment with UDCA has an effect on the symptoms and the outcome of SSc remains poorly understood. Prospective studies of patients with PBC-associated SSc who are followed-up for many years under UDCA treatment are needed to address this issue.

The treatment of SSc is complex and may include drugs with hepatotoxic potential. For example, the use of endothelin-1 receptor antagonist bosentan, which is the treatment of choice for SSc-related pulmonary artery hypertension, has been associated with increased risk of elevated aminotransferases [153–155]. When PBC is present, the management of SSc patients is more challenging, as this autoimmune liver disease may pose further risk factors or unwanted complications. Whichever therapy is to be implemented, it is recommended that collaboration takes place between specialists responsible for the care of these patients.

3. Conclusions

The association of SSc and PBC is a rare but intriguing autoimmune syndrome which challenges the expertise and interests of hepatologists and rheumatologists in terms of early diagnosis and shared management. A major effort should be made for continuing collaborative research in this field aimed at achieving a better understanding of the immunopathogenesis, genetic background, and demographic features of patients at higher risk of developing the associated conditions. These findings may also contribute to the development of specific protocols for preventing development and evolution of the two associated diseases.

Abbreviations

ACA:	Anticentromere antibody
AMA:	Antimitochondrial antibody
ANA:	Antinucluear antibody
CENP-A:	Centromere protein A
CENP-B:	Centromere protein B
CENP-C:	Centromere protein C
dcSSc:	Diffuse cutaneous systemic sclerosis
ELISA:	Enzyme-linked immunosorbent assay
lcSSc:	Limited cutaneous systemic sclerosis
PBC:	Primary biliary cirrhosis
SSc:	Systemic sclerosis
Anti-TOPO:	Anti-topoisomerase (anti-Scl-70) antibody
UDCA:	Ursodeoxycholic acid
ULN:	Upper limit of normal.

Authors' Contribution

D. P. Bogdanos and A. K. Burroughs are equally contributed to the paper.

Conflict of Interest

None of the authors has a conflict of interest to declare.

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Review Article

A System Out of Breath: How Hypoxia Possibly Contributes to the Pathogenesis of Systemic Sclerosis

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Systemic sclerosis (SSc) is an autoimmune disease characterized by vascular alterations and immunological disturbances and fibrosis, the order of which remains to be fully determined. Clinically, patients show clear signs of hypoxia in skin and internal organs. The low oxygen tension is potentially caused by a yet to be indentified circuitry involving the three features that typify SSc. In addition, once present, the hypoxia creates a vicious circle of ongoing pathology. In this paper, we provide an overview of the evidence that points towards the mechanisms causing hypoxia in SSc. In addition, data that suggest how hypoxia itself may orchestrate worsening of symptoms is presented. Altogether, it is clear that hypoxia is an important hallmark in SSc patients. By providing an overview of the mechanisms at play and the possible therapeutic avenues that have emerged, we hope to stimulate researchers to provide novel clues into the conundrum in SSc patients.

1. Introduction

Systemic sclerosis (SSc) is typified by vascular alterations and immunological disturbances and fibrosis of the skin and internal organs, which culminates in severe disabilities and not seldom premature death. Although the abovementioned pathways are all clearly involved, their sequel and relative contributions are still a matter of debate. About 90% of the patients diagnosed with SSc experienced Raynaud's phenomenon long before the appearance of other clinical symptoms that drives the patient to visit a physician [1]. The diagnosis is often made when patients suffer from a full-blown SSc with rarefaction of the small capillaries as identified by capillaroscopy, digital ulcers, and progressive fibrosis of the skin [2, 3]. Both Raynaud's phenomenon and the rarefaction of capillaries suggest the presence of hypoxia during certain stages of disease. In line with these thoughts, several studies demonstrated a lowered oxygen pressure in SSc skin [4–6]. Data showing a lower pO_2 only in the lesional SSc skin and a correlation between skin thickness and pO_2 suggests a direct connection between fibrosis and tissue hypoxia. Lastly, evidence of increased oxidative stress, thought to be caused by ischemia-reperfusion events, is convincing [7, 8]. The precise contribution of the clinically clear hypoxia to the cellular derogatory mechanism is exposed bit by bit. Next to this aberrant regulation of oxygen system, a role for the dysregulated immune system is illustrated by the fact that 75–90% of patients have ANA positivity, mostly explained by the presence of anticentromere or antitopoisomerase antibodies (reviewed in [9]). Recent literature shows the potential interaction between the vascular alterations and the immunopathology found in SSc. The rarefaction of the small vessels clearly lowers oxygen pressure in the skin, and there are signs of increased oxidative stress, which both are able to alarm and activate the plethora of different cell types of the immune system Figure 1.

1.1. HIF-1α. As a result of the hypoxia in skin and other affected tissue in SSc, activation of important hypoxia pathways is plausible. The identification of hypoxia-inducible factor 1 (HIF-1) unlighted the cellular mechanism of oxygen homeostasis. As a transcription factor of genes important in

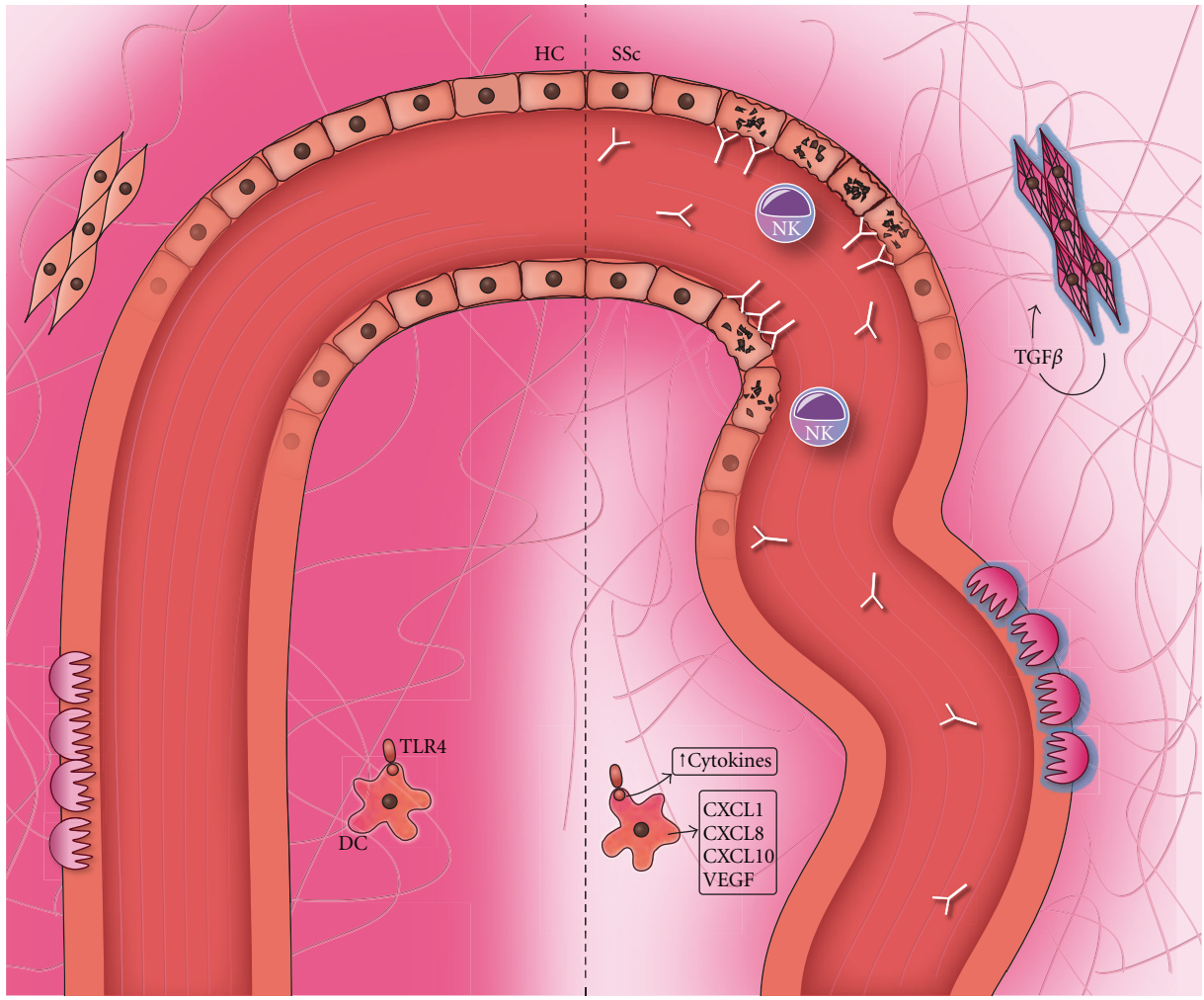

Figure 1: Hypoxia in the pathogenesis of systemic sclerosis. The left side illustrates the normal situation, with a healthy blood vessel delivering oxygen to the surrounding tissue. The right side represents the situation in SSc, where the diseased vessel and overwhelming deposition of collagen fibers prevent the oxygen from reaching the periphery. In the vessel, endothelial apoptosis as a result of antibody-dependent cytotoxicity is visible. Surrounding the ECs, activated pericytes are responsible for the deposition of collagen. The resulting hypoxia leads to a higher cyto- and chemokine production by DCs, in part triggered by TLR stimulation, and to a continuing loop of TGFβ production, collagen synthesis, and myofibroblast differentiation of fibroblasts. This in turn leads to a hindered dispersion of oxygen, keeping the vicious circle going on.

the metabolism of the cell but also angiogenesis, apoptosis, proliferation, and matrix production, this factor seems to play a more then central role in the cellular response to changing oxygen pressures (reviewed in [10]). HIF-1α is the tightly regulated form of HIF-1, which is quickly hydroxylated and degraded in normoxic conditions by prolyl hydroxylases [11]. But in case of hypoxia, the levels of HIF-1α increase dramatically [12]. Counterintuitive however, HIF-1α was found to be decreased in the epidermis of SSc patients compared to healthy controls [6]. As nicely suggested in the review by Beyer et al. [13], this could be caused by a negative feedback loop causing an increase in prolyl hydroxylases resulting in a faster degradation of HIF-1α. The persistent upregulation of VEGF (further discussed below) in SSc illustrates the activation of pathways sensitive to oxygen pressure and thus the low expression of HIF-1α must be compensated by other factors like HIF-2α and HIF-3α. The presence of these factors in SSc is currently unknown, and, moreover, the function of these factors is not mutually exclusive with HIF-1α [14]. Furthermore, a French cohort SSc patients showed an increased presence of a genetic variant of HIF-1α, implying a role in SSc pathogenesis [15]. How a defective HIF-1α regulation is precisely involved in the development of SSc will be discussed in detail later in this paper.

2. Vasculopathy

Of the three pathogenic features in SSc, vasculopathy is thought to be the first one to occur [16]. The vascular defects can be made visible with nailfold capillaroscopy, showing giant capillaries, loss of architectural arrangement, hyperpermeability, and dropout of capillaries [2]. The lower density and quality of vessels leads to a reduced blood flow and consequential tissue hypoxia.

The absence or aberrant function of dermal endothelial cells (ECs) is one mechanism often addressed to explain the onset of vasculopathy. ECs can be distinguished on immunohistochemistry by the expression of CD31, von Willebrand factor (vWF), and vascular endothelial cadherin (VE-cadherin). Dermis of early diffuse SSc patients (disease duration <2 years in 27/30 patients) shows a population of CD31+ endothelial cells with no expression of vWF and VE-cadherin [17]. Another group observed apoptotic vWF positive cells in skin section from early diffuse SSc patients, indicating apoptosis of EC. In the UCD 200/206 chicken SSc model, this apoptosis of ECs was already present before the appearance of perivascular infiltrates or fibrosis [16] and was also identified in the lungs, esophagus, and kidneys [18]. The presence of immunoglobulins (Ig) on these ECs [16] points towards a role of antiendothelial cell antibodies (AECA), which are known to be present in the serum of SSc patients [19]. The induction of EC apoptosis is thought to be the result of antibody-dependent cellular cytotoxicity [20, 21], and Sgonc et al. have suggested that natural killer (NK) cells form a likely candidate to initiate this process [22].

Next to the increased loss of ECs due to apoptosis, there might also be a problem in endothelial repair in SSc. SSc patients show a decreased amount of bone-marrow (BM) resident endothelial progenitors, while the hematopoiesis of other cell lines seems to be undisturbed [23]. The decreased amount of BM resident endothelial precursors could be a consequence of the above-mentioned AECA, which can induce apoptosis of the precursors upon exposure to AECA-positive serum [23]. Another mechanism to be considered is a higher efflux of these progenitor cells to the periphery, possibly as a result of elevated VEGF levels (as discussed in the next paragraph) [24]. Of interest in this light are the articles showing an increased amount of circulating endothelial progenitors cells (CEP) [25, 26], although there are also groups reporting a conflicting lower number of CEP [27]. These latter are more in line with a hampered recruitment of the BM progenitor cells to the periphery. There was no difference between patient and healthy control CEP when looking at VE-cadherin, CD31, and vascular endothelial growth factor receptor 2 (VEGFR-2) [27, 27]. Nevertheless, the capacity to differentiate in vitro to EC is diminished in SSc progenitors, as shown by a tempered induction of vWF after prolonged culture [27]. On the other hand, healthy control (HC) and SSc CEP show an equal ability to form tubules when cultured on a Matrigel matrix in vitro [28]. Thus, in principal, SSc CEP should be able to form coherent and functional vessels.

In normal circumstances, hypoxia induces the expression of angiogenic factors to stimulate angiogenesis. One of the key angiogenic growth factors is vascular endothelial growth factor (VEGF), which is produced by, among others, fibroblasts in response to HIF-1α [6]. Despite the low presence of HIF-1α in SSc, the expression of VEGF is increased in serum and skin of SSc patients [6, 29]. The ongoing induction of VEGF must then be caused by another mechanism, like the effect of the inflammatory cytokines interleukin 1 (IL-1) and platelet-derived growth factor (PDGF). These are highly present in SSc [30, 31] and can indeed induce the expression of VEGF in fibroblasts [6].

The effects of VEGF are regulated by its receptors, VEGF receptor 1 and 2 (VEGFR-1 and VEGFR-2). From these, VEGFR-2 is the effecting molecule and VEGFR-1s main function is to regulate the phosphorylation and activation of the other [32]. In scleroderma skin, both receptors are found to be higher present with an intenser staining for VEGFR-2 than for VEGFR-1 [6]. Exposure of EC to hypoxia *in vitro* showed a downregulation of VEGFR-2 and upregulation of VEGFR-1 in HC and SSc samples; the latter showed a lower expression in SSc EC [28]. This differential regulation can make the cells more responsive for VEGF. Correct timing of VEGF upregulation results in recruitment of EPC and formation of new vessels, which improves the oxygen supply to the tissue. In contrast, an ongoing VEGF stimulus caused by a higher VEGF and a defective regulation due to a relative decrease in VEGFR-1 elicits the formation of aberrant vessels [33], resembling the anomalous vessels seen on nailfold capillaroscopy in SSc [2].

In conclusion, in SSc, the vascular reaction to hypoxia is dominated by the growth factor VEGF. On the other hand, there is the altered quantity and phenotype of the ECs and their progenitors, which could be a causal factor in the appearance of hypoxia in SSc. The fact that this altered phenotype is not present in the EC of patients who were treated with intensive immunotherapy and subsequent stem cell transplantation is a promising thought [17]. This could imply reversibility of the vascular defects and might open novel avenues for therapeutic intervention.

3. The Dysfunctional Immune System

It is by now generally accepted that hypoxia can induce inflammation and that inflammation itself causes hypoxia in tissues. The decrease in oxygen tension during inflammation is a logic result of the increasing metabolic demand of cells, thrombosis, and compression of the vessels due to edema (interstitial hypertension). The effect of hypoxia, causing a proinflammatory environment throughout the body, is nicely illustrated by individuals that spend time at extreme height and display increased levels of IL-6 and CRP [34]. Furthermore, we know from transplantation medicine that the expression of Toll-like receptors (TLRs) and pro-inflammatory cytokines correlates with the amount of ischemia [35, 35, 36].

The main TLR-expressing cells are the cells of the innate immune system, especially the antigen-presenting cells. In response to the hypoxic environment during inflammation, these cells need to increase their expression of HIF-1α for a proper function. This was elegantly demonstrated in phagocytes from HIF knockout mice that could not efficiently remove bacteria and get persistent ulcers [37, 38]. Differentiation and maturation of dendritic cells (DCs) is inhibited under hypoxic conditions [39] but they showed an increased production of CXCL1, VEGF, CXCL8, and CXCL10 [40, 41], all of which increased in SSc [29, 42, 43]. In contrast with these latter observations, these hypoxic

DCs showed a lowered CCL2 and CCL18 production [40, 41], two pivotal chemokines that are repeatedly found to be increased in the circulation of SSc patients [43, 44]. Interestingly, other research showed an increased DC maturation and costimulatory capacity after TLR ligation during hypoxia [45, 46], suggesting that these cells are already in an activated state. Taking into account the endogenous TLR4 ligands found in SSc serum [47], the increased cytokine production in response to TLR ligands [48], and the increase in circulating cytokines mainly produced by these antigen presenting cells, it is tempting to speculate about a role for the hypoxic environment in this condition and this justifies further research to unravel this conundrum.

The effects of lowered oxygen pressure on the adaptive immune system are also quite evident. Hypoxia increases the expression of IL-10, TGF-b, galectin-1, and Foxp3, all supporting the development of regulatory T cells (Tregs) in hypoxic conditions. This is in line with several studies that reported increased numbers of Tregs in the circulation of SSc patients [49–51]. HIF-1α expression in T lymphocytes enhances the apoptotic rate and decreases T-cell function. In line with this, conditional deletion of HIF-1α in T and B cells is associated with the appearance of autoimmune responses in mice [52]. More intriguingly, the HIF-1α deficient T lymphocytes produced more pro-inflammatory cytokines in response to TCR triggering than HIF-1α expressing control cells [53, 54]. Taken into account the low HIF-1α expression in SSc patients, even in low oxygen conditions, it is tempting to suggest that the function of T lymphocytes in SSc is diverted by their impaired response to the hypoxic condition. Altogether, a hypoxic environment as present in SSc patients has clear effects on the immune system which might be augmented by an inherent defect to adept to low oxygen concentrations by SSc cells. Research focused on the effect of hypoxia on immune cells from SSc patients however is scarce and needs more attention in future investigations. Next to that, the underlying circuitry that explains this possible altered response to hypoxia by SSc cells remains elusive and warrants further investigation.

4. Fibrosis

Fibrosis is regarded to be the end stage of SSc and is often thought to cause the majority of the clinical symptoms. It is no wonder that most of the research of the last decades in this field focused on fibroblast biology and hypoxia as a causative factor for the extensive extracellular matrix (ECM) deposition.

A lower supply of oxygen to the tissue can be caused by vessel depletion or impaired diffusion. Where vasculopathy can be the cause of the former, fibrosis can lead to the latter. An important effector cell in this perivascular fibrosis is the pericyte. Pericytes can be found together with ECs around capillaries and show characteristics resembling vascular smooth muscle cells (SMCs) [55, 56]. In the perivascular area in SSc skin, pericytes with a myofibroblast phenotype have been observed, expressing alpha smooth muscle actin (α-SMA) and ED-A splice variant fibronectin (ED-A FN). These cells also show expression of the cell-surface glycoprotein Thy1 [57], which is essential in the differentiation of fibroblasts to myofibroblasts [58]. The upregulation of α-SMA in pericytes is associated with collagen production [59]. Furthermore, a reduced endothelial growth was observed when combining pericytes and EC in culture [56]. Taking together, these myofibroblast-like cells can lead to both perivascular fibrosis and endothelial dysfunction and thus cause a reduced supply of oxygen to the tissue.

The excessive production of ECM components by fibroblasts in reaction to transforming growth factor beta (TGFβ) and connective tissue growth factor (CTGF) is a paradigm in scleroderma [60]. Lately, there seems to be a paradigm shift noting that this excessive ECM production is not only due to an intrinsic defect in the fibroblasts, but also in part caused by a normal reaction of these fibroblasts to the pathological environment present in SSc patients. Indeed, in hypoxic circumstances, fibroblasts from SSc patients and HC react in the same profibrotic way. Exposure of fibroblasts to a low oxygen environment leads to HIF-1α-dependent upregulation of both TGFβ and CTGF [61–63]. Stabilization of HIF-1α, as occurs during hypoxia, also elevates the sensitivity of cells towards TGFβ, leading to a quicker TGFβ-dependent upregulation of CTGF. Herein could lay the explanation for the heightened serum CTGF in SSc [62]. Moreover, during hypoxia, the fibroblasts show an upregulation of genes involved in ECM synthesis and regulation, including fibronectin, thrombospondin, proα2(I) collagen (COL1A), and lysyl hydroxylase 2 (LH2) [64, 65]. This upregulation is in part regulated by HIF-1α, but totally dependent on TGFβ [64]. Last but not least, the hypoxic damage and oxidative stress could induce tissue damage and the subsequent release of associated molecular patterns (DAMPs), which could in turn stimulate TLRs on the fibroblasts and thus lead to activation [66].

Epithelial-to-mesenchymal transition (EMT) is a novel concept in fibrosis of lung and kidney, two severe complications of SSc. It describes a process in which epithelial cells develop mesenchymal characteristics, such as an increased expression of α-SMA and vimentin and a decrease in expression of E-cadherin. TGFβ is the prototypic stimulus of EMT [67]. EMT can also be induced by a lower oxygen pressure, as is shown by hypoxic culture of alveolar and kidney tubulus epithelial cells. The role of HIF-1α and TGFβ in this transition seems to be dependent on the origin of the epithelial cells. In alveolar cells, both molecules are required for EMT, while in kidney epithelium the process seems to be independent of these two proteins [4, 61]. Recently, EMT has been observed in the skin of a murine SSc model, suggesting that this process may be involved in both dermal and pulmonal fibrosis [68].

In contrast to the long-thought intrinsic defect in fibroblasts, data show that these cells respond in a normal way to abnormal circumstances. Taking the above mentioned together, there is evidence that in SSc hypoxia can lead to differentiation and activation of fibroblasts but might have similar effects on primary immune cells all contributing to an extensive ECM deposition.

5. Conclusion

Most human cells are able to adapt to a broad range of oxygen tension varying between 40–100 mmHg in the circulation and 4–20 mmHg in tissue. In contrast to these physiological differences, in certain condition pathological hypoxia develops. In SSc patients pathological hypoxia is obvious only by looking at their hands and seeing the digital ulcers patients suffer from. The response to this loss of oxygen has to be well coordinated and tightly regulated by factors like HIF-1α. The hypoxia caused by a combination of activated immune cells (increasing metabolic demands), rarefaction of blood vessels, and perivascular fibrosis becomes detrimental without this regulation. In SSc, there is no sign of increasing HIF-1α activity and therefore the low oxygen tension can go on activating fibroblasts (etc.), aggravating immune responses, and impairing endothelial cells and thereby further increasing the hypoxic environment. Moreover, the ongoing hypoxia will increase TGFβ levels and will therefore increase the production of extracellular matrix, further increasing fibrosis and thus the distance to the closest blood vessel [64, 69, 69]. Interestingly, another detrimental effect of hypoxia is shown in cancer research by the profibrotic and proinflammatory effect of the increased reactive oxygen species and autophagy caused by low oxygen tension [70]. Eventually, it seems inescapable that the system will choke in its own vicious circle. As shown by the effect of stem cell therapy [17], there are options to interfere in this ongoing process. For example, one would be able to interfere in the impaired response to hypoxia more specifically by the use of propyl-hydroxylase inhibitors (reviewed in [71]). In addition, the pharmacological increase of bilirubin by the use of atazanavir could directly provide more anti-inflammatory agents thereby bypassing the dysfunctional hypoxic pathway [72]. These therapeutic interventions could be given systemically but, alternatively, could also be specifically targeted to certain cells of the immune cells by exploiting recent knowledge on liposomes or even nanoparticles. In conclusion, the research field on therapeutic interventions in detrimental hypoxia is growing which will hopefully lead to the broadening of our therapeutic armamentarium to treat this disease.

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Review Article

The Role of Intravenous Immunoglobulin Preparations in the Treatment of Systemic Sclerosis

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Scleroderma is progressive autoimmune disease associated with severe disability. The major underlying pathological process in scleroderma is progressive development of fibrous tissue and obliteration of the microvasculature. Currently, there are no medical products for the treatment of scleroderma that provide both sufficient immunosuppression and low-risk side safety profile with negligible side effects. There are a large number of experimental data showing that intravenous immunoglobulin (IVIG) has multiple clinical and morphological effects. On the other hand, some authors report good effect of intravenous immune globulins in patients with scleroderma. The less frequent side effects of IVIG in doses below or equal to 2 g/kg/month divided in 5 consecutive days make IVIG a promising treatment of choice in scleroderma.

In 1981, the Swiss physician Paul Imbach prescribed IVIG to a child with immune deficiency combined with idiopathic thrombocytopenic purpura (ITP). He was surprised that, besides the increase in serum IgG levels, a dramatic improvement in the platelet count was observed. By the middle 1980s, such increase in platelet count in autoimmune thrombocytopenia after the administration of IVIG had been observed by many authors and IVIG were included in the standard treatment for ITP. Moreover, the indications for IVIG were broadened, and these preparations were administered in a wide range of diseases.

Rationale for the Use of IVIG

(1) IVIG has immunomodulatory action.

(2) IVIG contains idiotypes that neutralize different autoantibodies.

(3) IVIG blocks the Fc receptors on the surface of B-cells and macrophages.

(4) IVIG inhibits inflammatory mediators, such as cytokines, chemokines, and metalloproteinases.

(5) IVIG neutralizes toxins.

(6) IVIG reduces immune complexes.

(7) Substitutive treatment in immune deficiencies.

(8) Alternative treatment, for example, in cases where all other immunosuppressive therapeutic modalities have shown to be inefficient (*ultima ratio*).

Food and Drug Administration (FDA) has licensed the administration of IVIG for the following six therapeutic indications:

(1) treatment of primary immune deficiencies;

(2) prevention of infectious complications in patients with chronic B-cell lymphatic leukemia and hypogammaglobulinemia;

(3) prevention of coronary aneurism in Kawasaki's disease;

(4) prevention of infections and graft-versus-host disease after bone marrow transplantation;

(5) minimization of the risk for severe bacterial infections in children with HIV-infection;

(6) increasing of the platelet count and prevention of hemorrhages in patients with ITP.

Table 1: IVIG in patients with SSc.

Author/year	Number of patients	Skin lesions	Results	Remarks
Bodemer et al. 1990 [6]	1	Only in the face	Myositis improvement	SSc-dermatomyositis overlap
Levy et al. 2000 [8]	3	dcSSc	Regression of skin score, disease stabilization	No changes in PM-Scl antibodies
Levy et al. 2004 [9]	15	5 patients-lcSSc, 10 patients-dcSSc	Decreased mRSS, changes in HAQ score	Very high skin score in patients with lcSSc
Nacci et al. 2007 [10]	7	5 patients-limited SSc, 2 patients diffuse SSc	Decreased VAS, IAFD, mRSS after 6-month treatment	Effects on joints and skin
Asano et al. 2005 [11]	1	Fingers, hands, forearms, upper arms, face, chest, abdomen, lower legs, dorsum of the feet	Decreased mRSS	
Szekanecz et al. 2009 [12]	1	Disuse SSc, refractory to treatment	Good clinical effect	Combination of IVIG and plasmapheresis

In dermatology, IVIG is used mainly in patients with dermatomyositis and polymyositis [1, 2] and with autoimmune bullous dermatoses [3–5]. Recently, beneficial effect in small series of patients with different forms of scleroderma has been reported by some authors.

Scleroderma is progressive autoimmune disease associated with severe disability. It is manifested as diffuse cutaneous (dcSSc), limited cutaneous (lcSSc), or disease with multisystemic and multiorgan involvement (SSc). The major underlying pathological process in scleroderma is progressive development of fibrous tissue and obliteration of the microvasculature. Currently, there is no specific medicinal treatment of scleroderma that could provide both sufficient immunosuppression and low-risk side safety profile with negligible side effects. Several cytotoxic and immunomodulatory agents are used for the treatment of scleroderma, including cyclophosphamide, mycophenolate mofetil, cyclosporine, and, more recently, selective inhibitors of the T-(Sirolimus, Alefacept) and B-cells (Rituximab), antifibrotic agents (Imatinib), and hematopoietic stem cell transplantation.

In 1990, Bodemer et al. report the beneficial effect of IVIG 2 g/kg/month combined with prednisone for 10 months in patients with SSc-dermatomyositis overlap [6]. They point that the skin lesions are only on the face and the improvement after IVIG concerns only the myositis (Table 1). In 1998, Wollina et al. reported the beneficial effect of IVIG on the satellite infection in a patient with disabling morphea [7]. In 2000, Levy et al. report the beneficial effects of IVIG 2 g/kg/month in three patients with SSc [8]. All of them had past history for progressive and rapidly deteriorating skin symptoms not responsible to previous therapy with colchicine and one of them—colchicne and D-penicillamine. Two of the patients had been treated for 6 months without side effects, and, in the third patient, the treatment was ceased after month 3 due to renal damage and subsequent sepsis. In all three patients, regression in the skin score and disease stabilization were observed, but the levels of PM-Scl antibodies measured by indirect immunofluorescence test showed no changes during treatment (Table 1). Discussing these data, the authors point that "three patients are insufficient to evaluate a novel therapy." They assume that future studies are needed to show the effect of IVIG on scleroderma.

In 2004, a research group from the Sheba Medical Center and the University of Florence [9] reported 15 patients with SSc (5 with lcSSc and 10 with dcSSc) treated with IVIG 2 g/kg/month (Table 1). Eleven patients had been treated for 6 months, three for 4 months, and one patient for 3 months. In all patients, the skin involvement was evaluated using the modified Rodnan skin score (mRSS) at baseline and after the completion of treatment. Eight patients completed the Health Assessment Questionnaire (HAQ). The mean mRSS after therapy with IVIG was significantly decreased: shorter disease duration was associated with milder degree of improvement (21% in mRSS), longer disease duration (≥2 years)—44% in mRSS. Moreover, the HAQ score revealed that the patients report marked improvement. The authors assume that suppressing the action of profibrotic cytokines (IL-4, TGF beta) IVIG may improve the disease and quality of life of patients with SSc.

Several years later in a pilot study, the same authors reported marked improvement in articular involvement in seven Caucasian patients with severe refractive arthropathy in SSc (five with localized and 2 with diffuse systemic sclerosis) treated with IVIG 2 g/kg/month during 4 days/month for six consecutive courses [10]. The authors evaluated the effect of the treatment upon the following symptoms: joint tenderness and swelling and articular deformities. At baseline and after six-month treatment, the patients underwent the following tests: Ritchie Index (RI) evaluation of articular involvement; Dreiser Algo-Functional Index (IAFD) evaluation of hand joint function; pain visual analogue scale (VAS) to measure joint pain; HAQ to evaluate the limitations in everyday living and physical disability; mRSS for evaluation of skin involvement. After six-month therapy with IVIG in 6/7 patients VAS, IAFD and mRSS decreased significantly and HAQ score showed improvement in general

functionality (Table 1). According to the authors, the therapy with IVIG may be useful for SSc patients with severe joint involvement and those who are refractory to other treatment.

In 2005, Asano et al. reported similar results in a 60-years-old female Japanese patient with diffuse scleroderma treated with IVIG 400 mg/kg for 5 consecutive days [11]. The patient's condition improved dramatically with significant decrease in mRSS and marked improvement on the histological examination (Table 1).

In 2009, Szekanecz et al. reported a case of diffuse SSc without response to conventional therapy. After 12 months of combined repeated treatment with IVIG and plasmapheresis, they administered a good clinical effect [12].

There is a large number of experimental data showing that IVIG has multiple clinical and morphological effects. For instance, the administration of IVIG 2 g/kg in tight-skin mouse leads to significant decreases in collagen depositions and reduction of type I collagen gene expression [13].

It has been speculated that the overproduction of fibrotic tissue in SSc is due to the increased expression of TGFβ1 and IL-4 [14, 15]. The experimental studies of Blank et al. in tight-skin mice (animal model of SSc) revealed that the administration of IVIG in total dose 2 g/kg for four weeks lead to marked decrease in collagen deposition and reduction in type I collagen gene expression [13]. The authors observed parallel inhibition of TGFβ1 and IL-4 secretion by splenocytes with no changes in IFNγ levels. The studies of Asano et al. [11] revealed that the fibroblasts of scleroderma patients contain more type I procollagen, TGFβ and α-smooth muscle actin, and less matrix metalloproteinase 1. After the administration of IVIG the initial abnormalities normalize. Amemiya et al. [16] speculate that in inflammatory myopathies IVIG can bind directly to TGFβ. On the other hand, IVIG downregulate T cells with subsequent decrease in fibroblast TGFβ production. Other possible explanations of the beneficial effect of IVIG in reducing of fibrosis include inhibition of complement cascade, presence of anti-Fas antibodies in IVIG preparations that inhibit fibrogenesis by blocking the activity of Fas, and presence of antifibroblast antibodies that inhibit fibrogenesis [17]. These experimental data confirm the hypothesis that the treatment with IVIG in patients with different forms of scleroderma could change the fibroblast phenotype in this disease.

There are not so many studies about the effect of IVIG in SSc patients. We found only several publications, of which mainly case reports discussing the beneficial effect of this treatment in SSc. On the other hand, the less frequent side effects of IVIG in doses below or equal to 2 g/kg/month divided in 5 consecutive days make IVIG a promising treatment of choice in patients with scleroderma refractory to other therapy. The current use of IVIG in dermatology is at a rise, and this demands for new controlled clinical studies in different autoimmune skin diseases including SSc.

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Review Article

Gastric Antral Vascular Ectasia in Systemic Sclerosis

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Gastric antral vascular ectasia is a not so well-understood, and more rare, gastrointestinal manifestation of Systemic Sclerosis which can lead to chronic anemia. A high suspicion and better understanding of this rare manifestation is needed for early detection and treatment. Therapeutic regiments include iron supplementation with acid suppressive therapy, while endoscopic intervention has been shown to be successful in most cases, with gastrectomy or antrectomy rarely needed.

1. Introduction

Gastric antral vascular ectasia (GAVE) was first defined in 1984 by Jabbari et al. [1]. Its distinctive endoscopic appearance caused it to be also known as "watermelon stomach." On visualization it consists of parallel rugal folds with dilated blood vessels going from the gastric antrum and converging on the pylorus resembling watermelon rind and thus the nomenclature. It is often found in association with autoimmune diseases such as diffuse or limited cutaneous systemic sclerosis (dcSSc or lcSSc), Raynaud's phenomenon pernicious anemia, autoimmune hypothyroidism, primary biliary cirrhosis, polymyalgia rheumatic, and rheumatoid arthritis, but it is also found in patients suffering from other chronic medical conditions such as cirrhosis of the liver, chronic renal failure, ischemic or valvular heart disease, hypertension, acute myeloid leukemia, diabetes, and chronic obstructive pulmonary disease [2].

2. Prevalence and Incidence

GAVE is a rare manifestation of systemic sclerosis. A recent large retrospective study of 264 patients with SSc found a prevalence of 5.7% [3]. Eighty to ninety percent of patients already carry the diagnosis of SSc before GAVE manifests itself [3], but occasionally it can be the initial manifestation of the disease [4, 5]. It is commonly diagnosed early in the course of the disease, often within the first 3 years from diagnosis [3]. Prevalence though may be higher than described because patients who undergo endoscopy are only the ones who are symptomatic or have an unexplained anemia. In a recent abstract from the SCOT trial (Scleroderma cyclophosphamide or transplant study), where asymptomatic dcSSc patients had to be screened with an endoscopy before entering the study, 10.8% of them had silent GAVE [6].

3. Risk Factors

In some studies GAVE has been shown to be more prevalent in lcSSc while in other studies in dcSSc patients [3, 7, 8]. Patients with dcSSc tend to develop GAVE earlier in their disease course than patients with lcSSc, often the diagnosis is made within 2 years from the diagnosis of dcSSc, and in a recent series of twenty-eight patients with SSc and GAVE patients with dcSSc were diagnosed at a mean of 21.5 months into their disease course compared to 82.6 months in patients with lcSSc. The patients with lcSSc in this series also tended to have less severe anemia [7]. This may be a reflection of the natural history of lcSSc which has a more indolent course as compared to dcSSc.

An association between rapidly progressive skin changes and the development of early onset GAVE (i.e., development of GAVE within 18 months of the onset of SSc symptoms) may also exist. A subset of 16 patients from the series of 28

patients in the article by Ingraham et al. were diagnosed with early-onset GAVE. Nine out of these 16 patients also had rapid progression of their cutaneous disease (i.e., the presence of diffuse involvement in the upper extremities and trunk by 18 months from the onset of the first SSc symptoms) [7]. Ceribelli et al. also suggested that rapid progression of cutaneous disease is associated with the early development of GAVE [9].

The autoantibody profile may also give clues to the risk of developing GAVE, and a speckled pattern appears to carry a higher risk [7]. A recent report suggests a possible predictive role of RNA polymerase III (RNAP III) antibodies for GAVE. Ceribelli et al. reported 16 SSc patients with positive anti-RNAP III antibodies; 4 out of the 16 (25%) had GAVE [9]. This is considerably higher than the overall prevalence of GAVE in SSc patients [3]. It has been shown in several series that lack of antitopoisomerase I antibodies (anti-scl-70) is associated with an increased risk of GAVE [3, 7, 9].

4. Pathogenesis

The pathogenesis of GAVE is not fully understood though it has been noted in the past when pathology specimens from GAVE patients were examined that there is a loose attachment of the distal gastric mucosa to the underlying muscularis externa, where in the setting of dysmotility the antral mucosa may prolapse through the pylorus [1, 3, 10]. Pathology specimens show dilated capillaries, focal fibrin thrombi, spindle cell proliferation on the mucosal surface, fibromuscular hyperplasia in the lamina propria, and dilated submucosal vessels [1, 3, 11].

GAVE is considered one of the manifestations of the spectrum of vascular alterations in systemic sclerosis [8]. Studies have shown that the majority of patients with GAVE, around 60%, have telangiectasias of the skin. A smaller percentage also has telangiectasias in the gastrointestinal tract other than the stomach, in the esophagus, duodenum, ileum, colon, and rectum [3]. Supporting this theory is the fact that similar histopathologic changes can be found in the dermis on skin biopsies of scleroderma patients as are found in gastric mucosal biopsies from GAVE patients—that is, capillary dilatation, small vessel fibrin deposits, and platelet thrombosis [3, 8].

5. Clinical Evaluation and Diagnosis

Most patients with lcSSc and GAVE present with iron deficiency anemia or symptoms attributable to iron deficiency anemia, such as weakness, severe fatigue, or dyspnea on exertion. Thus a high index of suspicion is often needed in order to make the diagnosis in a timely fashion. Patients with dcSSc have a tendency to present with occult blood in the stool, and occasionally one may present with overt gastrointestinal bleeding though melena or hematemesis is unusual [3, 10]. Recurrent indolent bleeding is common, occurring in approximately one-third of cases [3].

6. Endoscopic Description

The appearance of GAVE is longitudinal red stripes or rugal folds in the antrum, radiating out in pattern resembling spokes from the pylorus. Alternatively, multiple cherry-red spots may be seen, but this mosaic pattern is far less common. Antral biopsies typically show capillary dilation with focal intravascular thrombi and fibromuscular hyperplasia in the lamina propria [1].

7. Management

Management of GAVE ranges from conservative symptomatic therapy to endoscopic interventions to surgery. The mainstays of symptomatic therapy and medical management are iron supplementation, proton pump inhibitors, and blood transfusion if anemia is severe and symptomatic [3]. There are some reports in the literature of treatment with estrogen-progesterone combination hormone therapy, but the risks and benefits must be carefully weighed in each individual case [12–14].

Early diagnosis of GAVE in SSc patients is important because endoscopic management can successfully treat this. It is important to educate primary care physicians who take care of these patients to be aware of slight drops in the hemoglobin of SSc patients and to refer such patients for endoscopic evaluation. While lcSSc patients typically have a good outcome after local therapy, dcSSc are more difficult to treat, with a need for recurrent electrocoagulation and blood transfusions and an increased risk for renal crisis.

Several unique endoscopic interventions have been reported to be successful in the management of GAVE. One popular technique is argon plasma coagulation, which has been shown to be effective in short- and medium-term followup in maintaining a rise in hemoglobin and giving transfusion independence to formerly transfusion-dependent patients within 2-3 treatment sessions [15]. Occasionally it can be associated with discomfort from gastric overdistension with argon gas [16].

Another endoscopic technique is neodymium-yttrium aluminum garnet (Nd:YAG) laser photocoagulation. A study of 45 patients treated with Nd:YAG laser reported that, at an average of two-year followup, normal hemoglobin levels were seen in 87% of all patients and transfusion independence was found in 24 of 28 initially transfusion-dependent patients [10].

There have been case reports describing many other successful therapies such as endoscopic band ligation [17], endoscopic ablation with hot biopsy forceps [16], and endoscopic therapy with monopolar electrocoagulation and injection of 5% polidocanol [18].

There have been reports of success in two small series of patients using intravenous cyclophosphamide to treat systemic sclerosis-associated GAVE or remission of GAVE when cyclophosphamide is used to treat other serious disease manifestations such as pulmonary disease [19, 20].

Gastrectomy or antrectomy is the only cure for GAVE, but it is rarely needed in the modern era with such a vast array of medical and endoscopic therapies available.

8. Conclusion

Gastric antral vascular ectasia continues to be a not well-understood manifestation of SSc patients, but an increase awareness and early diagnosis can improve outcome since local therapy can be successful. There is a need for a noninvasive therapy, though currently the best approach in the care of these patients is close followup and repeated local therapy.

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Review Article

PDE-5 Inhibitors in Scleroderma Raynaud Phenomenon and Digital Ulcers: Current Status of Clinical Trials

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Systemic sclerosis- (SSc-) related vasculopathy, as manifested by Raynaud's Phenomenon (RP) and digital ulcers (DUs), is associated with significant impairment of the quality of life and morbidity. The current vasoactive approach for SSc-RP, although employing vasodilators, is entirely off-label. PDE-5 inhibitors improve peripheral circulation, are well tolerated, and are widely used for various forms of constrictive vasculopathies. This class of medications has become one of the first lines of treatment of SSc-RP and SSc-DUs among rheumatologists that routinely treat SSc patients. Due to the lack of robust randomized clinical trials of PDE-5 inhibitors in SSc-RP/DUs, the PDE-5 inhibitors have not been FDA approved for these particular indications, which constitutes a significant barrier to prescribing this category of drugs. This paper reviews the current state of evidence-based knowledge in SSc-related vasculopathy and the use of PDE-5 inhibitors.

1. Introduction

Phosphodiesterases (PDEs) are isoenzymes that control the level of intracellular cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) by hydrolyzing them [1]. The human genome encodes 21 PDE genes which are classified in 11 families. PDE isoenzyme 5 (PDE-5) selectively breaks down the cGMP, a critical smooth muscle tone regulator. Nitric oxide (NO), produced by nitric oxide synthase, signals the conversion of GMP into cGMP which accumulates inside the cell. Inhibition of the PDE-5 enzyme increases the available intracellular cGMP which leads to vasodilatation. Aside from corpus cavernosum, PDE-5 is found on a variety of tissues, including platelets, lungs, muscle, brain, retina, thymus, heart, liver, esophagus, stomach, pancreas, small intestine [1], arterial and venous vasculature [2], and endothelial cells [3].

Sildenafil, vardenafil, and tadalafil are the three commercially available PDE-5 inhibitors (PDE-5Is). All three PDE-5Is are available in oral formulation, are rapidly absorbed from the gastrointestinal tract, and are metabolized by hepatic enzymes via cytochrome P450 [4]. Sildenafil and vardenafil have similar molecular structures, while the tadalafil molecule is different, the difference being reflected in the pharmacokinetic properties (Figure 1) [4]. Tadalafil is not affected by food ingestion and has a terminal half-life of 17.5 hours as opposed to sildenafil and vardenafil which are affected by fatty food intake and both have a half-life of approximately 4 hours [4].

The primary Food and Drug Administration- (FDA-) approved indication for the PDE-5Is is erectile dysfunction. In recent years, sildenafil (2005) [5] and tadalafil (2009) [6] have also been approved for use in pulmonary arterial hypertension. Vardenafil was recently shown to improve hemodynamic parameters in patients with pulmonary arterial hypertension in a randomized trial of 66 patients [7].

Raynaud's Phenomenon (RP) is an exaggerated vasoconstrictive response to cold and stress and is the presenting symptom in the majority of patients with systemic sclerosis (SSc) [8]. An important clinical manifestation of the scleroderma-related vasculopathy is the ischemic digital ulcer (DU) which is associated with significant morbidity [9]. Use of PDE-5Is in SSc-related RP and DU makes pathophysiologic sense and has been explored in randomized fashion.

2. Clinical Trials

As with penicillin or TNF-α blockers, the PDE-5Is history is interesting. The initial intent was to develop PDE-5Is as a new anti-ischemic therapy, but the early cardiac

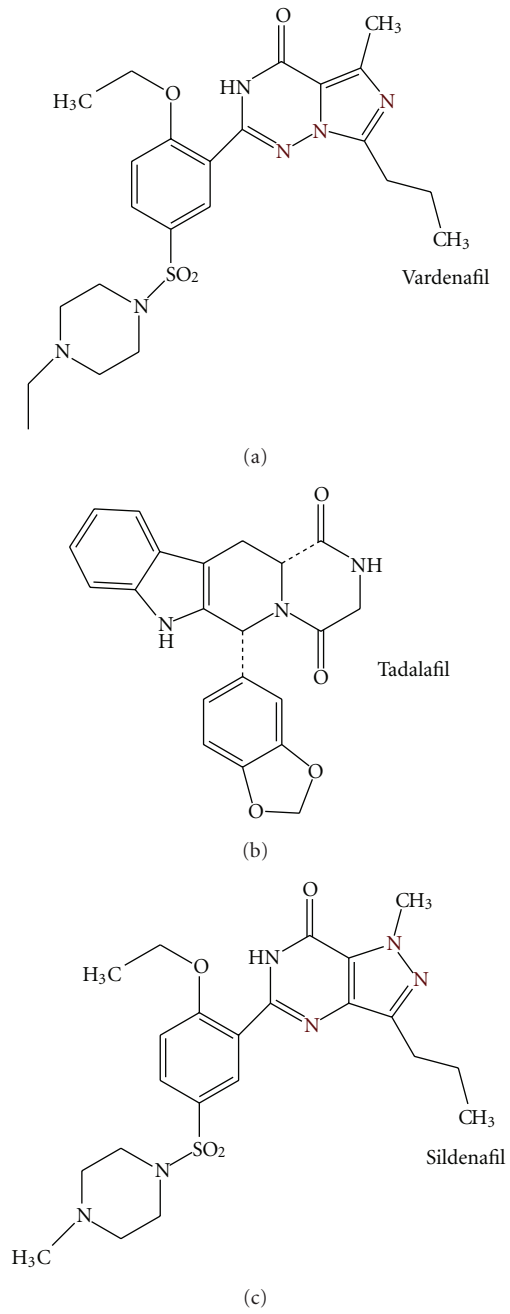

Figure 1: Chemical structures of the three available PDE-5Is [4].

trials failed to excite any interest. The "adverse" effect on penile erections led to revolutionary development of erectile dysfunction awareness and therapies [10]. Sildenafil citrate (Viagra) was approved by the FDA in 1998. Another PDE-5I, vardenafil (Levitra), came to market in September of 2003, followed shortly by the "weekend" drug, tadalafil (Cialis), in November of 2003. Each of these individual drugs' use in SSc-RP and DU will be reviewed below.

2.1. Sildenafil. The popular "blue pill" for erectile dysfunction has been used off-label by rheumatologists for symptomatic improvement of secondary RP and SSc-DUs. A retrospective chart review of 10 SSc patients at a single center briefly described the response to sildenafil dosed from 12.5 mg to 100 mg daily [11]. As the letter to the editor reports in 2005, "eight of the ten patients [· · ·] had a response within few weeks, with significant reduction in the frequency and severity of RP. Of the eight patients who had digital ulcers [· · ·] six experienced complete healing of the ulcers." No other details were provided regarding the specific measures used to quantify the RP improvement [11].

The physiological benefit of sildenafil citrate in patients with SSc-RP was assessed in a group of 5 patients and published as a letter to the editor in 2006 [12]. In this small study, the objective measure of the skin temperature response to mild cold challenge after a single dose of 50 mg of sildenafil citrate was conducted by thermography (thermal images of the hands were collected every minute for 15 minutes after the cold challenge to enable an area under the curve) and by percentage recovery to mean baseline temperature postcold challenge. Although this letter reported that 3 out of the 5 patients had clear and significant improvement in digital temperature responses to mild cold challenge, no other details were provided [12]. Based on this limited information, sildenafil citrate seems to be well tolerated in patients with SSc-RP.

A randomized double-blind cross-over trial of single dose of 50 mg sildenafil or α-tocopherol (100 mg) was reported in abstract form in 15 patients with RP [13]. The outcome measures reported were 75% improvement in forearm blood flow (as measured by near-infrared time-resolved spectroscopy) and 32% increase in serum cGMP concentration ($P < 0.01$) in the sildenafil group. Details about how many patients had SSc-RP are not available. The study reported no adverse events, aside from a mild decrease in the systolic and diastolic blood pressure ($P < 0.05$) in the patients exposed to sildenafil [13]. The only published trial that evaluated efficacy of sildenafil in RP is a double-blinded, placebo-controlled, fixed-dose, cross-over study of 50 mg sildenafil twice daily for 4 weeks versus placebo [14]. Most subjects had secondary RP (16/18), and 6 of the patients with secondary RP had DUs. Primary outcome variables included RP frequency and duration as assessed by diary cards, Raynaud's Condition Score (RCS), capillary flow velocity by laser Doppler anemometry, and healing of the DUs. At the end of the study, there was significant improvement in the frequency (35 versus 52, $P = 0.0064$) and duration (581 versus 1046 minutes, $P = 0.0038$) of RP attacks and in the RCS (2.2 versus 3.0, $P = 0.0386$). After 4 weeks of active therapy with sildenafil, 2 of the 6 subjects with DUs completely healed their ulcers, while the rest of the subjects noted visible healing. The mean capillary blood flow velocity increased by more than 400% (from 0.13 to 0.53, $P = 0.0004$) during the sildenafil treatment [14]. Despite its small size, this trial confidently shows improvement in the peripheral circulation after exposure to sildenafil and improvement of traditional measures of RP.

Aside from case reports, the effect of sildenafil on SSc-DUs was not systematically studied. In 2010, Brueckner et al. reported the results of a pilot open-label study of the effects of maximum tolerated sildenafil in SSc-DUs [15].

Sixteen patients were treated with a mean sildenafil dose of 114 mg/day for a mean duration of 5.2 months. There was significant improvement ($P < 0.001$) in the number of DUs from baseline (mean of 3.1/patient) to the end of sildenafil therapy (mean of 1.1/patient). Most patients reached a minimum number of DUs within 3 months irrespective of SSc. Nine subjects developed a total of 12 new DUs while taking sildenafil, of which one of the subjects was diagnosed with calcinosis [15]. Based on this small, open label trial, it seems that sildenafil is well tolerated, and it could be a viable option for SSc-DU therapy. An important caveat of this study is the lack of uniformity in defining SSc-DU (DUs due to calcinosis tend to respond less to vasoactive therapies) and the absence of a control group.

2.2. Vardenafil. Vardenafil is similar to sildenafil in terms of pharmacokinetic properties, with rapid onset of action, maximal benefit at 1 hour, and a half-life of about 4 hours. It is the second PDE-5I to make its debut in the erectile dysfunction arena at a dose of 10 mg as needed.

Caglayan et al. published the results of an open-label pilot study of vardenafil (10 mg twice daily for 2 weeks) in patients with RP in 2006 [16]. Of the 40 subjects recruited, 33 (82%) had secondary RP. All the vasoactive medications were discontinued at least one week prior to recruitment, and the outcome measures included RCS, frequency, and duration of RP attacks, and measures of peripheral blood flow by laser Doppler's flowmetry at room temperature and in the cold exposure test room. Laser Doppler's flowmetry revealed that 70% (28) of the subjects had improved digital flow, and, in those individuals, the digital blood flow measured at room temperature increased by a mean of 21% and 30% at 1 hour and 2 weeks compared to baseline at ($P < 0.01$). The RCS improved significantly from baseline to the second week of vardenafil therapy (5.05 versus 3.54, $P < 0.001$) [16]. As far as we could ascertain this is the only published trial of vardenifil. Based on this information vardenafil seems to be well tolerated and shows RP improvement.

2.3. Tadalafil. The longer half-life of this PDE-5I made tadalafil an attractive option as a daily dose for SSc-related peripheral vasculopathy. A small open-label study of tadalafil (between 5 and 20 mg every other day as needed) was published in abstract format in 2005 [17]. Of the 15 patients studied, 11 had SSc spectrum of diseases and the RCS was reported to be 3.8 while on tadalafil versus 6.9 without. Due to the limited data available in the abstract, no meaningful conclusions can be derived from this study.

A physiological study of tadalafil in RP (mostly primary RP, 18/20) was reported in 2007: this was a double-blind, placebo-controlled cross-over study of 20 patients with RP that received a single dose of 10 mg of tadalafil versus placebo [18]. The hypothesis that tadalafil improves cold-induced vasoconstriction was tested by measuring the digital blood flow with laser Doppler flowmetry at rest and during two graduated local heat and cold exposure cycles; skin blood flow (flux) and skin temperature were recorded at baseline and 90 minutes after receiving the drug. Tadalafil did not affect the baseline flux ($P = 0.57$) or skin temperature ($P = 0.69$). Tadalafil neither increased the maximal flux flow during heating nor decreased the vasoconstriction during cooling, which might mean that tadalafil improves RP through a different mechanism [18].

There is evidence that tadalafil improves peripheral circulation in SSc-RP. A randomized controlled trial of tadalafil (20 mg 2-3 times/week) versus pentoxifylline for 4 weeks in men with severe RP associated with autoimmune diseases was reported in abstract form in 2006 [19]. The frequency (decline of 59% with tadalafil versus 36% with pentoxifylline) and duration of RP attacks and the RCS were improved in the patients receiving tadalafil. Also, physician and patient assessments of RP improved at 4 weeks ($P < 0.05$ compared to 2 weeks, and $P < 0.05$ versus controls). Our interpretation of this study is limited as no specific information is provided about the number of SSc patients included in this study or about the statistical significance of some of the results. A more recent open-label study of 20 male patients with SSc-RP receiving 10 mg of daily tadalafil for 12 weeks was published in 2009. The primary endpoint showed improvement in the RCS, the number of RP attacks and a decrease in the plasma adrenomedullin and endothelin-1 levels compared to baseline [20].

In 2009, Schiopu et al. reported a randomized, double-blinded, placebo-controlled, crossover trial of tadalafil at a fixed dose of 20 mg daily versus placebo in 39 women with SSc-RP [21]. The trial design prohibited use of any other vasodilator therapies for RP, required a run-in period to document presence of a minimum of 6 RP attacks per week and excluded smokers. The treatment blocks were 4 weeks each with a 2-week washout in between. The outcome measures included paper RP diary and RCS. There were not sufficient DUs to permit an adequate statistical analysis of the effects on DUs. Although all measures showed overall improvement, there was no significant difference between the change from baseline RCS, duration, and frequency of attacks among the tadalafil and the placebo groups (RCS 2.43 versus 2.53, frequency 2.08 versus 2.1, duration 40.61 versus 47.0) [21]. A year later, Shenoy et al. described the results of a single-center, randomized, double-blind cross-over trial of tadalafil at 20 mg on alternate days versus placebo in subjects with SSc-RP which was conducted in India [22]. The trial design, although similar to the previous cross-over trial of tadalafil, included a longer treatment block (6 weeks) and a shorter washout (1 week), and allowed subjects to continue all the previous RP-specific therapies. When compared to the Schiopu et al. trial, the patients recruited needed to have 4 RP attacks/week, as opposed to 6, and the mean age of the 24 participants was younger (36.87 versus 52.9). The primary outcomes were similar: mean change in RCS, duration, and frequency of RP attacks from baseline. The secondary outcomes included assessment of DUs, quality of life (QoL) measures, endothelial function, and flow-mediated dilatation of brachial artery. In this study, the primary outcomes were significantly better in the tadalafil group: frequency (2.29 versus 3.37, $P < 0.001$), duration (33.81 versus 54.89, $P = 0.023$), and RCS (3.86 versus 5.20, $P < 0.0005$). All the 24 digital lesions healed

during the tadalafil treatment versus only 3 of the 13 DUs during the placebo treatment. The brachial artery reactivity was measured using B-mode ultrasound imaging and improved significantly while subjects received active drug ($P < 0.05$). The levels of E-selectin and endothelin 1 were not significantly different between placebo and the active treatment groups ($P = 0.5$ and 0.81, resp.) [22].

Following this above-mentioned single-center trial by Shenoy et al, a multicenter randomized, double-blinded, placebo-controlled study of tadalafil at 20 mg every other day versus placebo was conducted in 4 centers from North and Northeastern India and reported in an abstract form [23]. Similar to Shenoy et al., the mean age of the recruited subjects was lower than the mean age of the subjects in the US tadalafil trial (36.8 years in both Indian studies versus 52.9 years in the US study). This trial recruited 53 subjects, and, after a 2 week run-in period, they were randomized to tadalafil 20 mg every other day or placebo for the 8-week treatment period. Similar to the Shenoy et al. trial, improvement in RCS, duration, and frequency of RP attacks was demonstrated ($P < 0.05$, $P < 0.001$, $P < 0.001$, resp.), along with significant SSc-DU healing (14 out of 18 DUs healed in the tadalafil group compared to 5 out of 13 in the placebo group) [23]. It is likely that the differences in the trial design, specifically the concomitant RP therapies, along with the lower average age (less disease severity and more reversal changes, consistent with younger age) could explain the differences in the results of these controlled trials. A significant aspect on which all three studies seem to agree was the excellent tolerability of the oral tadalafil as a daily or every other day therapy for SSc-RP.

2.4. Ongoing Clinical Trials. A phase IIa, randomized double-blinded, placebo-controlled, cross-over trial to assess the efficacy of a novel, once daily PDE-5I (PF-00489791) for the treatment of primary and SSc-related secondary RP has just finished recruiting. This is a multicenter trial sponsored by Pfizer (http://www.clinicaltrials.gov/ identifier NT01090492). Another phase II/III clinical trial is comparing daily use of amlodipine (10 mg) versus udenafil daily (100 mg) in secondary RP in a double-blind, randomized, cross-over design; aside from the RP common outcome measures, the investigators are also assessing DUs and arterial flow velocity (http://www.clinicaltrials.gov/ identifier: NCT01280266).

The effects of sildenafil in SSc-RP as measured by the microcirculator blood flow, endothelial progenitor cells, and serum levels of vascular endothelium growth factor is ongoing (http://www.clinicaltrials.gov/ identifier: NCT013-47008). A multicenter double-blinded, placebo-controlled trial is also currently randomizing participants to either sildenafil (20 mg three times a day) or placebo for 90 days to assess healing of SSc-DUs (http://www.clinicaltrials.gov/ identifier: NCT01295736).

3. Conclusions

The SSc-related vasculopathy (including RP and DUs) lacks FDA-approved therapies. The class of PDE-5Is is a well-tolerated vasoactive therapy for patients with SSc-related vasculopathy. After a surge of clinical case reports, case series and open-label studies published in the mid 2000s, large, multicenter, controlled trials of PDE-5Is in SSc-related vasculopathy remain underdeveloped. Most of the published clinical trials in SSc-RP focus on sildenafil and tadalafil although the one study reported with vardenafil has shown it to be well tolerated with a beneficial impact on RP.

Currently, the available evidence for the efficacy of PDE-5I's for SSc RP/DUs is thin. Definite conclusions are hard to reach as studies have shown conflicting results. Comparisons are difficult as patient populations and outcome measures are not uniform. There are efforts to address the questions of placebo response in RP by designing trials focused on objective vascular markers, QoL, and physicians' and patients' global assessments. Robust clinical evidence that PDE-5Is, alone or in conjunction with traditional vasoactive therapy, improve SSc-RP and heal SSc-DUs is still lacking. Clinical trials involving selective PDE-5Is are ongoing.

The great clinical need for a tolerable, affordable therapeutic option for SSc-RP and SSc-DU remains. The clinical research community recognizes PDE-5Is as an excellent potential option for SSc-related vasculopathy.

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Research Article

Basal Activation of Type I Interferons (Alpha2 and Beta) and 2′5′OAS Genes: Insights into Differential Expression Profiles of Interferon System Components in Systemic Sclerosis

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Objective. Systemic sclerosis (SSc) is a complex autoimmune disease in which interferons (IFNs) may play an essential role. We hypothesized that type I and III IFNs may be found in increased levels in patients and be responsible for SSc autoimmune status. *Methods*. Type I and III IFN and ISG basal expression profiles were measured by qPCR using RNA from PBMCs of patients and controls . *Results*. Type I IFNs are increased in SSc patients, while no induction of type III IFNs was detected. This induction cannot be related to IRF7, since no upregulation of this gene was seen on patients. Of the ISGs tested, 2′5′OAS levels were increased in patients, while 6–16 and MxA levels were not. *Conclusions*. While there is no indication of type III IFN induction, increased levels of type I IFNs may lead to abnormal regulation of ISGs that can be responsible for immune system alterations described for SSc.

1. Introduction

Systemic sclerosis (SSc) is a complex autoimmune disease of unknown aetiology characterized by excessive fibrosis of the skin and internal organs, presence of autoantibodies to nuclear antigens, and vascular damage [1–4]. Several genetic and environmental agents have been proposed to be responsible for causing SSc. Among these agents are infections, toxin exposure, single nucleotide polymorphisms (SNPs), and interferon treatment [5–7]. Regardless of the origin, the immune system on SSc patients shows evidence of homeostatic alterations, including increased levels of chemokines in blood serum and different populations of lymphocytes in peripheral blood mononuclear cells (PBMCs). In addition to these alterations, there is increased evidence that the interferon (IFN) system is modified in patients with SSc [8–15].

IFNs are immunomodulatory cytokines that act as an important link between the innate and adaptive immune system in vertebrates. IFNs bind to distinct cellular receptors, and their biological activities are mediated by the regulation of interferon stimulated genes (ISGs). IFNs can be divided into three types based on receptor binding and homology. Type I and type III IFNs are important regulators of innate immunity and are produced after stimulation of pattern recognition receptors in order to initiate and regulate the immune response. One of the main pathways leading to type I and III IFNs induction depends on the induction and activation of IRF7. Almost every cell type is able to produce these IFNs after stimulation, but its main producers are cells from the immune system such as plasmacytoid dentritic cells. Type I and III IFNs have redundant biological activities, even though they bind to different cellular receptors and have distinct structures. IFN gamma, the only known type

II IFN, has distinct biological activities and is important for the regulation of adaptative immunity [16, 17]. The innate immune system and type I IFNs have been proposed as important factors in the initiation and maintenance of some autoimmune diseases, and the influence of type I IFNs in SSc has not been studied in detail [18–20]. Several activities of type I and type III IFNs are redundant, and until now there has been no description of type III IFNs participation in SSc or other autoimmune diseases. An SNP in one chain of the type III IFN receptor (IL10R2) has been associated with SSc, indicating that at least responsivity to these molecules may be important for the disease [21].

Previous studies have shown an increase of several ISGs in PBMCs of patients with autoimmune diseases such as systemic lupus erythematosus (SLE) and type I diabetes [10, 22]. Differential induction of ISGs in blood cells and fibroblasts from patients has already been described [19, 23–25]. When PBMC gene expression was compared by microarray between healthy donors and SSc patients, several ISGs were characterized as differentially induced. Some of these genes were also found in patients with SLE, which is an autoimmune disease marked by several alterations in the type I IFN system [4, 24]. Levels of 2′5′ oligoadenylate synthetase (2′5′OAS) and double-stranded RNA-activated protein kinase (PKR), two ISGs, are found at higher levels in fibroblasts from SSc patients when compared to controls [19]. To date, there is no direct evidence of abnormal induction of type I IFNs in SSc, even thought cells treated with sera from SSc patients produce more IFN alpha and other cytokines than cells treated with sera from controls [12]. These findings suggest that IFNs may also be important in SSc pathology.

In this paper, we show that PBMCs from SSc patients basally expressed more type I IFNs (alpha and beta) than PBMCs from healthy donors. In addition, there was no detectable basal induction of type III IFNs in patients or healthy donors. When ISGs were measured, we observed increased 2′5′OAS basal levels, consistent with previous studies. These findings suggest that, similar to other autoimmune diseases, type I IFNs play an important role in SSc.

2. Methods

2.1. Blood Donors. Ten patients fulfilling the American College of Rheumatology preliminary criteria for diagnosis of diffuse SSc and four healthy subjects were chosen as blood donors (Table 1). Before donating blood, each subject read and signed an informed consent previously approved by the Ethics Committee of the Universidade Federal de Minas Gerais, Brazil.

2.2. Cells and PBMC Fractionation. Vero cells (African green monkey kidney cell line) were obtained from the ATCC and grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine, 5% fetal calf serum (Cultilab, Campinas, SP, Brazil), and antibiotics. PBMCs from healthy donors or SSc patients were purified using the Ficoll-Hypaque purification technique [26].

Table 1: Information about the subjects that consented to take part in this research.

	Sex	Age (years)	Disease time (years)[1]	Treatment	ANA titer
P1	F	31	1	Prednisone	>1/320
P2	F	54	5	Methotrexate Prednisone	<1/10240
P3	M	35	5	Methotrexate Prednisone	>1/5120
P4	M	29	1	Prednisone	>1/640
P5	M	40	7	—	<1/10240
P6	F	30	2	—	>1/320
P7	F	50	5	—	>1/80
P8	M	56	6	—	<1/10240
P9	F	58	11	Methotrexate Prednisone	>1/80
P10	F	70	20	—	>1/640
C1	M	39	—	—	Not tested
C2	F	32	—	—	Not tested
C3	M	25	—	—	Not tested
C4	F	24	—	—	Not tested

C: control; P: patient; M: male; F: female; —: not treated.
[1]Dated from the onset of the first non-Raynaud's symptom.

Briefly, fresh blood collected in vacuum tubes containing heparin was diluted in an equal volume of 1X phosphate buffered saline (PBS). Twenty milliliters of the diluted blood were carefully added over 10 mL of Ficoll and centrifuged at 400 × g for 30 min. The layer containing PBMCs was collected and washed once in 1X PBS, and the cells were counted. One million cells were pelleted and used for RNA extraction.

2.3. RNA Extraction, DNase Treatment, Reverse Transcription and Quantitative PCR (qPCR). Total cellular RNA was extracted using the RNeasy mini kit (QIAGEN). After the extraction, one microgram of RNA was treated with 1 U of DNase I enzyme (BIOLABS), and after treatment the RNA was used as template in reverse transcriptions carried out using MMLV reverse transcriptase (PROMEGA). These steps were performed as described by the manufacturers. Real time PCRs were performed in a Step-One Real-Time PCR machine (Applied Biosystem) using the relative quantification methodology. The results were analyzed using StepOne Software v2.2, and all data were expressed as a ratio relative to the beta-actin level. PCR primers used for human genes are listed below: IFN alpha2 forward 5′-TTGACCTTTGCTTTACTGGT-3 and reverse 5′-CACAAGGGCTGTATTTCTTC-3′. IFN beta forward 5′-CCTGTGGCAATTGAATGGGAGGC-3′ and reverse 5′-CAGGTAGATGGTATAGCGTGG-3′. IFN lambda1 forward 5′-CTTCCAAGCCCACCCCAACT-3′ and reverse 5′-GGCCTCCAGGACCTTCAGC-3′. IFN lambda2/3 forward 5′-TTTAAGAGGGCCAAAGATGC-3′ and reverse 5′-TGGGCTGAGGCTGGATACAG-3′. IRF-7 forward 5′-CAAGTGCAAGGTGTACTGG-3′ and reverse 5′-CAGGTAGATGGTATAGCGTGG-3. 2′5′OAS forward

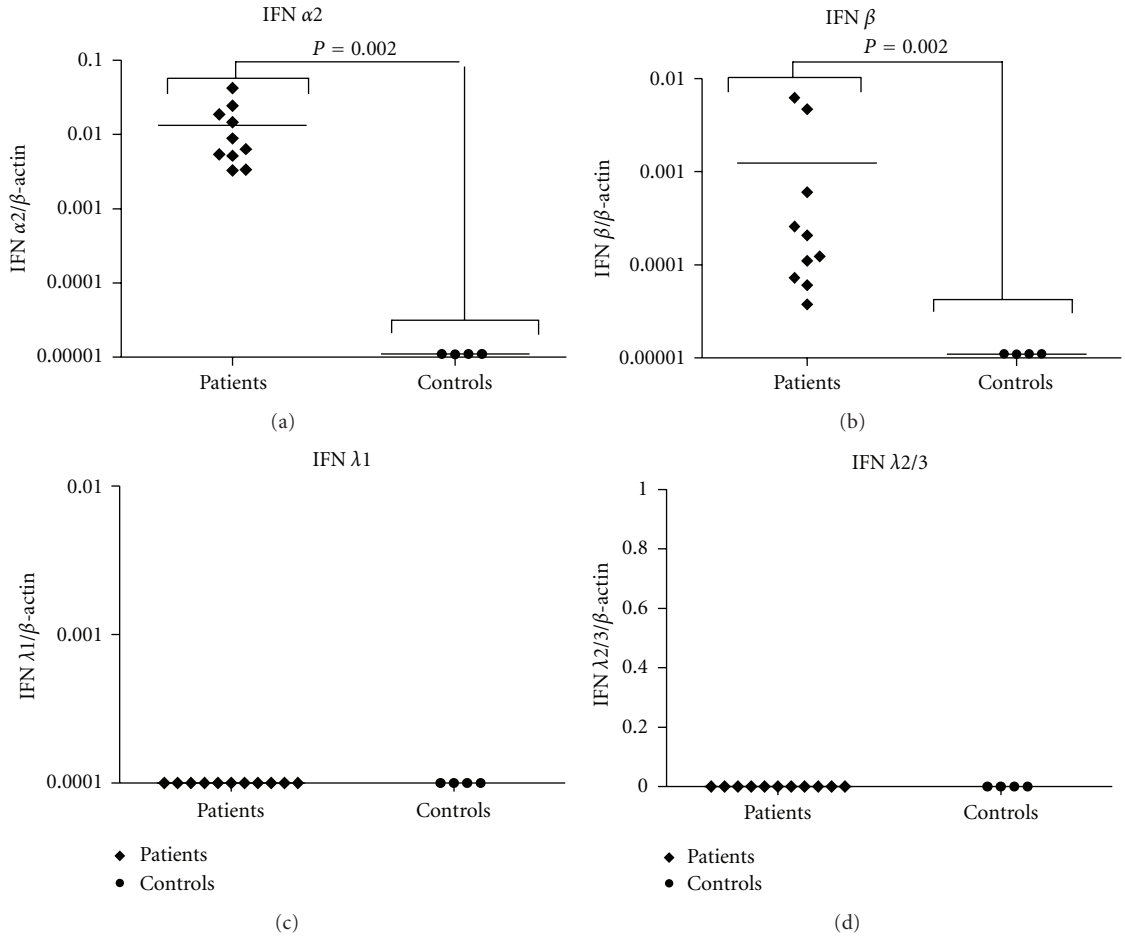

Figure 1: Type I and type III IFN mRNA basal levels in PBMCs from healthy donors and SSc patients. PBMCs from healthy donors and SSc patients were purified, and total RNA extraction was performed. The RNA obtained was used as template in reverse transcription reactions, and the resulting cDNA was used in real-time PCRs to measure IFN alpha (a), IFN beta (b), IFN lambda1 (c), and IFN lambda 2/3 (d).

5′-AACTGCTTCCGACAATCAAC-3′ and reverse 5′-CCTCCTTCTCCCTCCAAAA-3′. MxA forward 5′-ATCCTGGGATTTTGGGGCTT-3′ and reverse 5′-CCGCTTGTCGCTGGTGTCG-3′. 6–16 forward 5′-CATGCGGCAGAAGGCGGTAT-3′ and reverse 5′-CGACGGCCATGAAGGTCAGG-3′. Beta-actin forward 5′-CCAACCGCGAGAAGATGA-3′ and reverse 5′-CCAGAGGCGTACAGGGATAG-3′.

2.4. IFN Titration. After cell fractionation by the Ficoll-Hypaque technique, one milliliter of sera was collected from each donor and frozen at −70°C until use. At the time of titration, each serum sample was serially diluted from 1 : 2 to 1 : 4096 and used to treat 96-well plates of Vero cells with 90% confluency. Eighteen hours after treatment, the medium was discarded and the cells were infected with 10^4 TCID50/mL of *Encephalomyocarditis virus* (EMCV). The infection was monitored for 48 hours, at which time the plates were fixed with 3.7% formaldehyde before being stained with 1% crystal violet (adapted from [27]). Alongside the samples, the following controls were used: 600 U/mL of recombinant human IFN alpha 2a (Roche) and a negative serum sample to which 600 U/mL of recombinant human IFN alpha 2a (Roche) was added.

2.5. Statistical Analysis. Student's t-test and nonparametric Mann-Whitney test were used to analyze the results. Differences of $P < 0.05$ were considered to be statistically significant. Analyses were made using the GraphPad Software (USA)

3. Results

The expression profile of type I and type III IFNs was measured by qPCR using RNA from PBMCs freshly collected from SSc patients or controls. We observed that the basal levels of IFN alpha2 and IFN beta were increased in patients while there was little or no expression of these genes on healthy donors (Figures 1(a) and 1(b)). When type III IFNs were measured, we observed that the basal level of IFN lambda1 and lambda2/3 was the same in both groups (Figures 1(c) and 1(d)). These results were obtained from cells without any treatment or culture, indicating that these genes might be normally induced in patients. We also

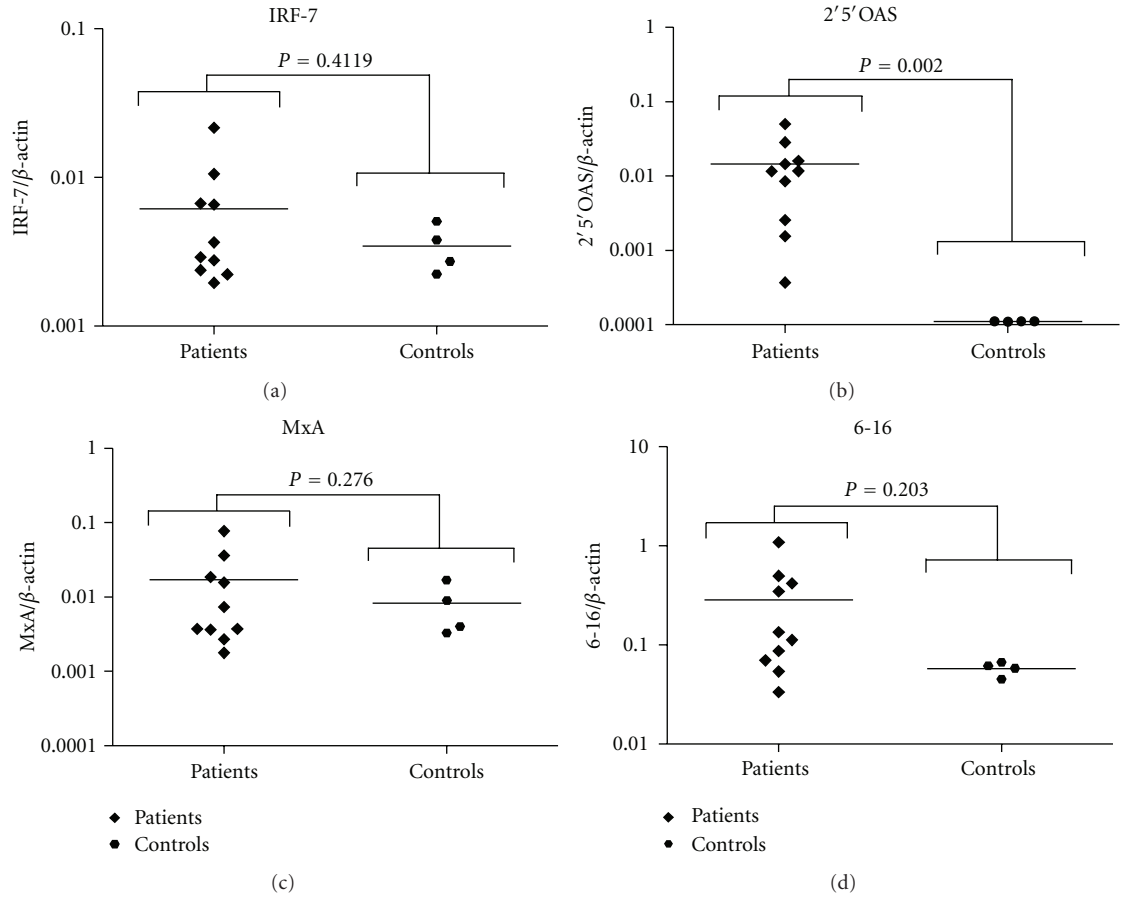

Figure 2: Basal levels of IRF-7 and ISGs in PBMCs from healthy donors and SSc patients. PBMCs from healthy donors and SSc patients were purified, and total RNA extraction was performed. The RNA obtained was used as template in reverse transcription reactions, and the resulting cDNA was used in real-time PCRs to measure IRF-7 (a), 2′5′OAS (b), 6–16 (c), and MxA (d) levels.

attempted to detect IFN proteins in sera from patients and controls through a biological assay commonly used for IFN titration. However we were unable to detect any activity higher than the detection limit of the assay (20 IU/mL). Our controls worked perfectly, indicating that the negative results obtained were not due to sera toxicity or experimental artifacts (data not shown).

In addition to IFN basal levels, we also measured IRF7, 2′5′OAS, MxA, and 6–16 basal levels in PBMCs from patients and controls. IRF7 is an important factor for type I and type III IFN induction, whereas the other genes are ISGs that are commonly induced by type I and type III IFNs. There was no statistically significant difference in the levels of IRF7 (Figure 2(a)) between patients and controls. Similarly, there was no difference between levels of MxA (Figure 2(c)) and 6–16 (Figure 2(d)). However a difference on 2′5′OAS levels was detected, and this gene basal expression level was increased in patients but not in controls (Figure 2(b)).

4. Conclusions

IFNs are cytokines with antiviral, antiproliferative, and immunomodulatory activities. Type I IFNs have been implicated in the pathogenesis of several known autoimmune diseases. However, the influence of type III IFNs in these diseases is not yet known, despite the similarities in the biological activities of these IFN types. SSc is an autoimmune disease with unknown etiology in which the immune system homeostasis is severely compromised. To date there has been little information about the influence of IFNs in SSc patients in the literature and no direct evidence of type I or type III IFN basal induction in patients.

In this paper, we show that some components of the IFN system can be found at higher levels in freshly purified PBMCs from SSc patients, compared with cells from controls. Increased basal expression of IFN alpha2 and IFN beta (type I IFNs) was detected in SSc patients, while there was no difference between the basal levels of IFN lambda1 and IFN lambda2/3 (type III IFNs) in patients and controls (Figure 1). These results show that, at least for mRNA levels, there is a higher expression of type I IFNs by PBMCs from SSc patients than from controls. This abnormal expression, especially in the case of IFN alpha2, is consistent with the status of autoimmunity in several other diseases and can have a major role in initiating and maintaining the disease. High levels of IFN beta can be even more dramatic for an autoimmune status, since this IFN has similar biological activities to IFNs alpha but is even more potent [28]. We

also tried to titrate IFN protein levels on sera obtained from patients and controls, but the levels were below our limit of detection (20 IU/mL). Considering that this titration method is able to detect any mixture of IFNs capable of inducing antiviral activity in host cells, we can hypothesize that even though IFN levels are increased on SSc, they are not found in high levels. Methodologies with greater sensitivity must be used in order to detect and measure type I IFN proteins in SSc patients sera.

Expression levels of IRF7, an inducible transcription factor responsible for type I and type III IFN gene induction [29], were not altered between patients and controls (Figure 2(a)). This finding leads us to conclude that IFN alpha2 and beta are being induced by another stimulus in the disease, which is specific to their induction and is not common to type III IFNs induction. 2′5′OAS basal expression levels were higher in patients than in controls (Figure 2(b)). This gene is also found at higher levels in SLE patients [30]. Differential induction of 2′5′OAS is consistent with a previous study that used fibroblasts from SSc patients, in which higher basal levels of 2′5′OAS were detected when compared to control fibroblasts [19]. It also indicates that there is, at least to some degree, some similarity between alterations in the skin and blood cells of SSc patients. Other measured ISGs, 6–16, and MxA were not differentially expressed between patients and controls (Figures 2(c) and 2(d)). The factors leading to this differential induction on ISGs are unknown, but could be due to cell exposure to various IFN subtypes abnormally expressed during the disease. The capacity of SSc sera to induce cytokines in treated cells [31] supports this observation, since this activation probably occurs due to cytokines and other molecules in the plasma.

Based on these findings, we hypothesize that the IFN system is modified in SSc. By still unknown mechanisms, at least IFNs alpha2 and IFN beta are induced in patients by a pathway that does not induce type III IFNs. Type I IFNs produced by patients' cells circulate and through activation of certain subsets of ISGs maintain the autoimmune status of SSc. These molecules are important links between innate and adaptative immune responses and can, among other actions, activate immune cells to improve autoantigens detection and autoantibodies production [32]. It has been already described that IFN alpha2 can induce TLR3 activation in SSc fibroblasts in culture [33]. This activation can also be happening *in vivo* by type I IFNs naturally produced by PBMCs, corroborating our hypothesis. IFN beta activation is a novel finding on the disease and can be of great importance to SSc altered immune homeostasis. Even though there is some redundancy between the activity of type I and type III IFNs, we could not link type III IFNs to SSc, at least when PBMCs are involved. Future experiments with higher sample size and using distinct cell populations isolated from total PBMCs are essential to further investigate these results.

These findings are of great importance to our understanding of the pathogenesis and pathology of SSc. Increased basal levels of type I IFNs can lead to abnormal activation of ISGs and undesirable side effects on the patients, even without interference by type III IFNs. The differential expression profile of ISGs detected in PBMCs is consistent with the profile found previously in fibroblasts [19] and could be a signature related to the disease. These unique modifications in the IFN response may be considered as candidates for SSc biomarkers and can be considered for further studies as diagnostic tools for SSc.

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Review Article

Endothelin Receptor Antagonists for the Treatment of Raynaud's Phenomenon and Digital Ulcers in Systemic Sclerosis

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Systemic sclerosis is a connective tissue disease characterized by fibrosis of the skin, internal organs, and widespread vasculopathy. Raynaud's phenomenon and digital ulcers are vascular manifestations of this disease and cause significant morbidity. Current treatments are only moderately effective in reducing the severity of Raynaud's in a portion of patients and typically do not lead to substantial benefit in terms of the healing or prevention of digital ulcers. Several studies have evaluated the efficacy of targeting the vasoconstrictor endothelin-1 for the treatment of systemic sclerosis-associated vascular disease. The purpose of this paper is to summarize the published studies and case reports evaluating the efficacy of endothelin receptor antagonists in the treatment of Raynaud's phenomenon and digital ulcers associated with systemic sclerosis.

1. Introduction

Systemic sclerosis (SSc) is a connective tissue disease characterized by fibrosis of the skin and internal organs and widespread vasculopathy. Raynaud's phenomenon (RP) is often the first manifestation of SSc, frequently preceding the onset of cutaneous sclerosis by several years particularly in patients with limited disease, and eventually occurs in 95% of patients with SSc [1]. Vasospasm of the digital arteries leads to the three characteristic phases of pallor, cyanosis, then erythema correlating with reduced blood flow, total loss of oxygen supply, and reperfusion. Episodes of RP are usually triggered by cold exposure or stress and can be associated with numbness and pain, resulting in significant disability [2]. Recurrent episodes of ischemia-reperfusion injury and the subsequent generation of reactive oxygen species can result in ischemic damage to distal tissue sites. Digital ulcers (DUs) are necrotic lesions that occur either at distal aspects of digits (fingers or toes) or over bony prominences and occur in up to 50% of patients with limited or diffuse cutaneous SSc [3]. These lesions are exquisitely painful, heal slowly, and interfere with activities of daily living often leading to substantial functional disability. Other complications associated with DU include scarring with loss of distal tissue, infection that can lead to osteomyelitis, and progression to gangrene requiring amputation [4, 5]. DUs that develop at distal aspects of digits are thought to be related to recurrent ischemia from various processes, including vasospasm from RP, thrombosis of digital arteries, calcinosis, and structural microvascular changes related to the underlying SSc [4, 6–8]. Recurrent trauma, particularly in patients with joint contractures, also contributes to the development of DU in patients with SSc. Ulcerations on the lower extremities proximal to the feet can occur in patients with SSc who likely have macrovascular disease as well. Current treatments for both RP and DU consist of vasodilators including calcium channel blockers (CCBs), alpha-adrenergic inhibitors, angiotensin converting enzyme (ACE) inhibitors, angiotensin receptor blockers, and nitroglycerin analogues. These medications are moderately effective in reducing the severity of RP in a portion of SSc patients [9], but typically do not lead to substantial benefit in terms of the healing and prevention of DU.

With the availability of powerful vasodilator therapies for the treatment of pulmonary arterial hypertension (PAH), options for the treatment of severe RP, DU, and progressive digital ischemia have increased. Prostacyclin analogues have been shown to accelerate the healing of DU, however, those agents found to be effective thus far require intravenous or

subcutaneous delivery [10–12]. Small studies have indicated that oral phosphodiesterase-5 inhibitors (PDE-5-I) are effective in reducing the severity of RP and promoting the healing of DU [13–15]. Large multicenter randomized controlled studies are underway to further evaluate the efficacy of PDE-5-I in the treatment of RP and DU. Several studies have evaluated the efficacy of targeting the vasoconstrictor endothelin-1 (ET-1) for the treatment of RP and/or DU. The purpose of this paper is to summarize the published studies evaluating endothelin receptor antagonists (ETRA) in the treatment of RP and/or ischemic DU associated with SSc.

2. The Role of Endothelin in the Pathogenesis of SSc-Associated RP and DU

The initial events leading to SSc vasculopathy are thought to involve endothelial cell injury [16] with subsequent loss of normal vasodilatory mediators such as prostacyclin and nitric oxide [17–20]. In addition, endothelial injury results in increased release of the vasoconstrictor endothelin-1 (ET-1) [21, 22]. ET-1 is a 21-amino acid polypeptide expressed primarily by endothelial cells, but has also been found to be expressed by epithelial cells, macrophages, fibroblasts, and cardiomyocytes among others [23, 24]. It acts locally, binding to the surface of smooth muscle cells and acts on the vascular endothelium itself in an autocrine manner. Levels of ET-1 have been found to be increased in the serum of patients with RP and SSc [25–27]. In addition to its role as a biomarker of vascular disease, ET-1 itself may be contributing to the fibrotic and vasculopathic aspects of SSc as it has been shown to stimulate fibroblast and smooth muscle proliferation [28, 29]. ET-1 signaling is mediated by two transmembrane G-protein-coupled receptors (ET_A and ET_B) with different binding affinity and physiologic effects [23, 30]. ET_A receptors are expressed on vascular smooth muscle cells and primarily mediate vasoconstriction whereas ET_B receptors are expressed on both endothelial cells, mediating vasodilatation, and on smooth muscle cells, mediating vasoconstriction [31].

3. Endothelin Receptor Antagonists

Endothelin receptor antagonists are a class of PAH-specific drugs that block the interaction of ET-1 with its receptors (Table 1). ETRAs can selectively act on ET_A receptors to varying degrees, thus interfering with the vasoconstrictive effects of ET-1. Those with a relatively low ET_A/ET_B selectivity are traditionally considered nonselective. Both nonselective and selective ETRAs have shown efficacy in the treatment of PAH and currently two are approved for this indication in the USA: bosentan and ambrisentan [32–35]. Sitaxsentan was approved in Europe, Canada, and Australia in 2006, but withdrawn from the market in 2010 due to concerns about severe liver toxicity. Case reports of patients showing improvement in their RP and DU while undergoing therapy with ETRAs for PAH have led to randomized controlled trials investigating the efficacy of these agents for the treatment of RP and DU in patients with SSc. As a result of two large randomized controlled trials, bosentan was approved for the prevention of DU in SSc patients in the European Union in June 2007. We will now review the published literature describing the use of ETRAs in the treatment of RP and/or DU in patients with SSc.

3.1. Case Reports. Table 2 summarizes the case reports describing the efficacy of ETRAs in the treatment of SSc-associated cutaneous ulcers. The first case report published in 2003 described a 50-year-old male with diffuse cutaneous SSc and severe PAH who was enrolled in a double-blind, placebo-controlled study investigating the efficacy and safety of bosentan in patients with PAH (the BREATHE-1 study [32, 36]). During the open-label extension phase of the study, he received bosentan 62.5 mg twice daily and within 4 weeks of this therapy, his leg and other small nonacral skin ulcers on his trunk and extremities had healed. In 2006, there were two published case reports describing the efficacy of bosentan in treating cutaneous ulcerations in sclerodermatous conditions. The first case described a 61-year-old female with limited cutaneous SSc and multiple DU refractory to CCB and IV prostacyclin therapy [37]. After 6 months of standard bosentan therapy (62.5 mg BID $\times$ 4 weeks then 125 mg BID), she experienced resolution of her DU correlating with a decrease in plasma ET-1 concentration. The second case reported a 4-year-old girl with pansclerotic morphea unresponsive to corticosteroids, methotrexate, CCB, ACE-inhibitors, and D-penicillamine [38]. Within the first months of bosentan therapy, both her widespread sclerotic skin lesions and her limb ulcers improved. Another case report published in 2007 described a 39-year-old female with limited cutaneous SSc and worsening DU despite IV prostacyclin therapy [39]. After 6 weeks of the standard approved dose of bosentan, her DU completely healed. In 2008, a report was published describing a 62-year-old female with long-standing SSc who experienced healing of a large pretibial ulceration after 6 months of standard bosentan therapy [40]. Finally, in 2009, a case report described a 39-year-old female with diffuse cutaneous SSc and recalcitrant DU treated with sitaxsentan 100 mg daily. After 6 months of therapy, her DU significantly improved and no new DU developed [41].

3.2. Open-Label Studies. Table 3 outlines the prospective studies investigating the utility of ETRAs in the treatment of RP and/or DU. The first prospective study published in 2006 described 3 patients with RP in the setting of prescleroderma (defined as RP associated with sclerodermatous nailfold capillaroscopic changes and SSc-specific autoantibodies) or limited cutaneous SSc independent of a history of DU [43]. The participants received the standard dosing of bosentan and at the end of the 16-week treatment course pain, RP disease activity and severity were noted to be reduced. A larger prospective observational study published in 2008 evaluated the long-term efficacy and tolerability of bosentan in 15 patients with SSc with current or a prior history of DU [44]. The patient population in the study was particularly heterogeneous with a wide range in age (11–72 years), 0 to 26 DU at baseline, and included 6 patients with interstitial lung

Table 1: Endothelin receptor antagonists.

Endothelin receptor antagonist	Oral dose	Relative ET_A/ET_B selectivity	Information
Nonselective			
Bosentan	Starting: 62.5 mg twice daily Maintenance: 125 mg twice daily	20x	FDA approved for use in the USA in November 2001 for WHO functional class III/IV PAH then extended to include WHO class II in 2009. Approved in the EU for WHO functional class III PAH in May 2002. In June 2007, the EU approved and extended the indication of bosentan as a therapy to reduce the number of new DU in patients with SSc and ongoing DU disease.
Selective			
Ambrisentan	Starting: 5 mg daily Maintenance: 5 or 10 mg daily	4000x	FDA approved for the once-daily treatment of WHO functional class II/III PAH in June 2007. It was later approved by the European Medicines Agency for the same indication in the EU in April 2008.
Sitaxsentan	100 mg daily	6500x	Approved in the EU in August 2006, then in Canada and Australia in March 2007 for the once-daily treatment of WHO functional class III PAH. On December 10, 2010, the manufacturer voluntarily removed sitaxsentan from the market and halted clinical trials due to concerns about liver toxicity.

FDA: Food and Drug Administration, EU: European Union, PAH: pulmonary arterial hypertension, DU: digital ulcer(s), RP: Raynaud's phenomenon, and SSc: systemic sclerosis.

Table 2: Case reports of the efficacy of endothelin receptor antagonists for systemic sclerosis-associated cutaneous ulcers.

Author date	Case report	Location of ischemic ulcer(s)	Prior treatments for SSc and/or ulcer(s)*	Results
Humbert and Cabane 2003 [36]	50-year-old male with diffuse SSc and PAH	Trunk Leg DU	IV prostacyclin	Received bosentan 62.5 mg twice daily and within 4 weeks of this therapy his leg and other small nonacral skin ulcers on his trunk and extremities had healed. After an additional 6 months of 125 mg twice daily, his DU completely healed.
Tillon et al. 2006 [37]	61-year-old female with limited SSc and pulmonary sarcoidosis	DU	CCB IV prostacyclin	Bosentan was initiated at the standard approved dose of 62.5 mg twice daily for a month then 125 mg twice daily. After 6 months, she experienced resolution of her DU correlating with a decrease in plasma ET-1 concentration. No new DU developed.
Roldan et al. 2006 [38]	4-year-old female with pansclerotic morphea	Ankles	Corticosteroids Methotrexate CCB, PUVA ACE-inhibitors D-penicillamine	Bosentan was started at an initial dose of 31.25 mg four times daily for 4 weeks, and then decreased to the standard dose for her weight of 31.25 mg twice daily. Within the first months of bosentan therapy, both her widespread sclerotic skin lesions and her limb ulcers improved.
Chamaillar et al. 2007 [39]	39-year-old female with limited SSc	DU	IV prostacyclin	Bosentan was initiated at the standard approved dose and after 6 weeks of this therapy her DU completely healed. No recurrence was noted over 2 years of continued therapy.
Ferreira and Scheinberg 2008 [40]	62-year-old female with diffuse SSc	Pretibial	CCB Antibiotics Antiplatelets IV prostacyclin Sympathectomy	Bosentan was initiated at the standard approved dose with improvement in the ulcer seen during the first few months of therapy. Complete healing of the large pretibial ulceration occurred after 6 months of therapy.
Gholam et al. 2009 [41]	39-year-old female with diffuse SSc	DU	Methotrexate Corticosteroids Cyclosporine CCB	Treated with sitaxsentan 100 mg daily; during the 6 months of treatment there was a decrease in pain and near complete healing of preexisting DU and no development of new DU.

*Treatments noted in the case report only; other treatments may have been used.
SSc: systemic sclerosis, DU: digital ulcers, PAH: pulmonary arterial hypertension, CCB: calcium channel blocker, ET-1: endothelin-1, ACE: angiotensin converting enzyme, and PUVA: psoralen plus ultraviolet A.

Table 3: Studies evaluating efficacy of endothelin receptor antagonists for systemic sclerosis-associated raynaud's phenomenon and/or digital ulcers.

Author date	Study type	Intervention	Patients enrolled/ completed	Duration	Primary endpoint for assessment of RP and/or DU	Results
Korn et al. 2004 *RAPIDS-1* [42]	R, PC, DB	(a) 62.5 mg bosentan BID × 4 weeks; 125 mg BID × 12 weeks (b) Placebo BID × 16 weeks	(a) 79/66 (b) 43/37	16 weeks	Number of new DU developing during the 16-week study period.	Patients receiving bosentan had a 48% reduction in the mean number of new DU at the end of the treatment period ($P = 0.0083$). No difference between groups in the healing of existing ulcers.
Selenko-Gebaue et al. 2006 [43]	Obs	62.5 mg bosentan BID × 4 weeks; 125 mg BID × 12 weeks	3/3	16 weeks	RP activity and pain severity.	Pain, RP disease activity, number and severity of Raynaud's attacks all decreased.
García dela Peña-Lefebne 2008 [44]	Obs	62.5 mg bosentan BID × 4 weeks; then 125 mg BID	15	4 to 36 months	Number and severity of DU.	There was a decrease in the number of DU. A trend towards efficacy was seen in the number of healed ulcers and in the severity of ulcers.
Funauchi et al. 2009 [45]	Obs	62.5 mg bosentan BID × 4 weeks; then 125 mg BID	15	40 to 96 weeks	Number and severity of DU and frequency and severity of RP.	After a median 8 weeks of treatment, 13 out of 15 patients had improved RP. DU also improved after a median 12 weeks' treatment in all of the 8 patients that had DU.
Tsifetaki et al. 2009 [46]	Obs	62.5 mg bosentan BID × 4 weeks; then 125 mg BID	26/23	36 months	Number of new and healed DU.	The mean number of DU per patient was reduced at 6, 12, and 36 months ($P < 0.001$).
Nguyen et al. 2010 [47]	R, PC, DB	(a) 62.5 mg bosentan BID × 4 weeks; 125 mg BID × 12 weeks (b) Placebo BID × 16 weeks	(a) 9/8 (b) 8/8	16 weeks	RCS, frequency, duration, and pain associated with RP attacks.	Compared with placebo, bosentan did not significantly reduce the severity, frequency, duration, or pain of RP attacks.
Kuhn et al. 2010 [48]	Obs	62.5 mg bosentan BID × 4 weeks; 125 mg BID	10/8	24 weeks	Healing of current DU.	Bosentan increased the number of healed DU from 42% at baseline to 88% at week 24 ($P < 0.0019$).
Giordano et al. 2010 [49]	Obs	62.5 mg bosentan BID × 4 weeks; 125 mg BID	14/14	48 weeks	Number and duration of RP attacks	Number and duration of RP attacks showed a statistically significant decrease at 12 weeks and maintained through 24 and 48 weeks ($P < 0.05$).
Mattuci-Cerinic et al. 2011 *RAPIDS-2* [50]	R, PC, DB	(a) 62.5 mg bosentan BID × 4 weeks; 125 mg BID × 20 weeks (b) Placebo BID × 24 weeks	(a) 98/75 (b) 90/73	24 weeks	Number of new DU and the time to healing of a preexisting DU.	Bosentan treatment was associated with a 30% reduction in the number of new DU compared with placebo ($P = 0.04$). No difference between groups in healing rate of preexisting ulcers.

R: randomized, PC: placebo controlled, DB: double blind, Obs: observational, DU: digital ulcer, RP: Raynaud's phenomenon, and RCS: Raynaud's Condition Score.

disease and 3 with PAH. They were treated with bosentan therapy at standard doses and were followed for a mean of 24.7 months (range 4–36 months). There was a significant decrease in the mean number of DU per patient from 5 at baseline to 0.4 at 12 months ($P < 0.05$). In 2009, an observational study was published on 15 patients with connective tissue disease associated PAH that specifically evaluated the effect of bosentan on DU and RP [45]. After a median of 8 weeks of treatment, 13 out of 15 patients had improved RP severity with 8 patients experiencing disappearance of all RP symptoms after a mean of 14 weeks. Healing of DU was observed after a median of 20 weeks (range 16–24 weeks) for 6 of the 8 patients who had DU at baseline. The longest prospective open label study of bosentan was a 3 year trial of 26 patients with DU refractory to CCB, ACE-inhibitors or sildenafil, published in 2009 [46]. Complete healing of DU occurred in 17 of the 26 participants (65%) after a median period of 25 weeks (range 8–26 weeks), and improvement

was noted in the DU of 4 additional patients. Overall, the mean number of DU per patient was reduced at 6, 12, and 36 months ($P < 0.001$). Additionally, healing of the DU was evidenced by complete reepithelialization on skin biopsy which was performed on 5 of the 26 participants. In a 24-week prospective open-label trial, 10 patients with SSc were treated with bosentan; skin fibrosis as assessed by the modified Rodnan skin score (MRSS) was the primary endpoint, but evaluation of DU was a secondary outcome assessment [48]. At each visit, examination of DU was performed by the same evaluator and categorized as present, indeterminate (>50% reduction in their surface area), or healed (total reepithelialization). 88% of DU were categorized as healed at the end of the 24-week treatment compared with 42% at baseline ($P = 0.0019$). Finally, in 2010, a 48-week observational study was published evaluating the effectiveness of bosentan for RP without DU in patients with SSc-associated PAH [49]. 14 patients who were on stable doses of other PAH-specific therapies (excluding patients treated with parenteral prostanoids within the previous 6 months) were treated with bosentan as add-on therapy. For patients with limited or diffuse cutaneous SSc, the number of RP attacks from baseline to 48 weeks decreased from 3.4 ± 1.8 to 2.1 ± 1.4 and 3.8 ± 1.7 to 2.1 ± 1.8, respectively ($P < 0.05$). Likewise, the duration of RP attacks decreased for patients with limited or diffuse cutaneous SSc from baseline to 48 weeks from 62.5 ± 43.7 to 22.1 ± 13.8 minutes and 61.0 ± 38.9 to 29.2 ± 13.7 minutes, respectively ($P < 0.01$).

3.3. Double-Blind, Randomized, Placebo-Controlled Studies. The first randomized placebo-controlled double-blind clinical trial evaluating an ETRA for the prevention of DU in patients with SSc was published in 2004 and is commonly known as the RAPIDS-1 (Randomized Placebo-controlled Investigation of Digital ulcers in Scleroderma) trial [42]. 122 patients with SSc and current DU or a history of at least 1 in the prior 12 months, enrolled across 17 centers in Europe and North America. 79 were randomized to receive bosentan and 43 to receive matching placebo during the 16-week treatment phase. The mean number of new ulcers during the treatment period was 1.4 for patients on bosentan versus 2.7 for patients on placebo ($P = 0.0083$) representing a 48% reduction in the number of new DU. However, there were no differences in the reduction of preexisting DU in the 63% of patients with active DUs at baseline. The most notable adverse event occurring in more patients on bosentan than placebo was elevated transaminase levels (14% versus 0%). 3 patients developed a marked transaminitis (>8x ULN), and 5 patients (6%) discontinued the study due to these laboratory abnormalities, but in all cases the transaminase values returned to normal when bosentan was discontinued. The results of a second randomized placebo-controlled double-blind clinical trial investigating bosentan for the treatment of SSc-related DU (RAPIDS-2) were recently published in 2011 [50]. This trial involved a longer 24-week treatment phase and all 188 patients enrolled across 41 sites in Europe and North America were required to have at least 1 active DU, the largest called the "cardinal ulcer," at baseline. The mean number of new DU over 24 weeks was 1.9 ± 0.2 for patients on bosentan versus 2.7 ± 0.3 for patients on placebo ($P = 0.035$) representing a 30% reduction in the number of new lesions. As with the RAPIDS-1 study, the RAPIDS-2 study failed to show a benefit in terms of healing of existing ulcers. Adverse events occurring in more patients on bosentan than placebo included peripheral edema (18.8% versus 4.4%) and elevated aminotransferases (12.5% versus 2.2%). Markedly increased aminotransferases (>3x upper limit of normal (ULN) and 1 case of >8x ULN) occurred in 10.5% of patients in the bosentan group, but these abnormalities resolved during continued treatment, after a decrease in dose, or following temporary or permanent treatment discontinuation. Only one placebo-controlled double-blind clinical trial evaluating an ETRA for the treatment of RP in patients with SSc has been published to date [47]. 17 patients without preexisting DU were randomized to either standard bosentan therapy or matching placebo during the 16-week treatment phase. Severity of RP was assessed via the Raynaud's Condition Score (RCS), a validated composite self-assessment of the severity of RP encompassing the number and duration of episodes, the associated symptoms, such as pain and numbness, and the degree of hand disability. The RCS is measured on a scale of 0–10 with 0 indicating no disability related to RP and 10 indicating extremely severe disability from RP. Patients recorded the frequency, duration, and severity of RP attacks in daily symptom diaries. The mean RCS score was reduced for both the bosentan and placebo groups (-31% and -36%) at week 16, but the improvements were not statistically significant compared with baseline nor were they different between the groups. Frequency of RP attacks significantly decreased in the bosentan and placebo groups, however, at week 16 only patients in the placebo group maintained a statistically significant decrease in RP frequency (bosentan: -30%; placebo: -57%, $P = 0.017$). In 16 weeks, a significant reduction in mean duration of RP attacks was observed for both bosentan (-26%; $P = 0.012$) and placebo (-60%; $P = 0.028$) compared with baseline, however, there was no significant difference between the groups. Interestingly, despite the lack of improvement on measures of RP activity and severity with bosentan, patients in the treatment group demonstrated statistically significant improvements in functional status as assessed by the scleroderma Health Assessment Questionnaire ($P = 0.03$ and $P = 0.01$ at weeks 12 and 20, respectively) and the UK functional score ($P = 0.04$ at weeks 8 and 16) compared with those treated with placebo. No serious adverse events were noted, and only 1 participant withdrew due to treatment-related peripheral edema. The authors did mention that DU developed in 1 patient on placebo and 2 on bosentan, but these resolved with similar healing times.

4. Conclusion

The findings from the literature reviewed here indicate that ETRAs may play a role in the treatment of RP and DU in addition to their indication for the treatment of PAH. The two large, randomized, placebo-controlled studies using bosentan have shown that this agent is useful in the prevention of new DU in patients with SSc (RAPIDS 1 and 2)

confirming findings in uncontrolled observational studies. However, both of these studies failed to show a benefit in terms of healing of existing ulcers [42, 50]. Although observational studies demonstrated an improvement in RP with ETRA treatment [43, 45, 49], the one randomized, placebo-controlled study using bosentan for the treatment of RP [47] did not show a statistically significant difference between bosentan and placebo. Although this was a relatively small study, the results highlight the importance of performing RCT in the assessment of potential treatments for RP and DU. In addition to the benefit of ETRA for DU, 3 of the case reports suggested benefit in nondigital ischemic ulcers in patients with SSc, but larger studies are necessary to verify these results [36, 38, 40]. This is important as nondigital ulcers are seen in up to 4% of patients with SSc [51]. The majority of studies published thus far have described the effects of the ETRA bosentan on RP and/or DU. It is unknown whether ETRAs with greater selectivity for the ET_A receptor, would show better tolerability and efficacy than bosentan in the treatment of SSc-associated RP and DU. Ambrisentan may be preferable to bosentan given the lower incidence of liver function test abnormalities, once daily dosing and lack of interaction with warfarin. Our center has recently completed the first open-label prospective study of ambrisentan for the treatment of SSc-associated DU and the results will soon be available. ETRAs may be preferable to prostacyclins given their oral bioavailability, but physicians must be cognizant of their teratogenicity and potential side effects including liver toxicity, edema, and anemia. Additional RCTs are necessary to better assess the role of ETRAs for the treatment of RP and DU in patients with SSc.

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Review Article

Vascular Changes in Bleomycin-Induced Scleroderma

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Systemic sclerosis (SSc) is characterized by vascular injury, immunological abnormalities, and fibrosis of the skin as well as various internal organs. Vascular impairment is the early manifestation and plays a fundamental role in the pathogenesis of SSc. Recent studies suggest that complex interactions among the endothelial cells, pericytes, smooth muscle cells, and fibroblasts are involved in the systemic vasculopathy in SSc, and histological feature of proliferation of vascular wall is seen in the lesional scleroderma skin at the late stage of disease. One of the most representative mouse models for scleroderma is the bleomycin-induced scleroderma; however, aspects of vascular alteration have not been described in detail so far. A number of studies have shown that bleomycin stimulates endothelial cells and fibroblasts to induce proinflammatory and fibrogenic cytokines, apoptosis, reactive oxygen species, and so on. This paper makes a focus on the vascular involvement in the bleomycin-induced murine scleroderma.

1. Introduction

Systemic sclerosis (SSc) is a connective tissue disease which shows fibrosis of the skin and various internal organs [1]. Although the pathogenesis of SSc has not been fully elucidated yet, it is characterized by vascular injury, immunological abnormalities, and excessive accumulation of extracellular matrix (ECM) proteins in the skin and various internal organs. In particular, systemic vasculopathy plays a fundamental role in SSc and is associated with various altered vascular dysfunctions in the lung, kidney, heart, and skin. Clinically, Raynaud's phenomenon, digital ulcers, and abnormal nailfold capillaries are seen in association with peripheral vasculopathy. Raynaud's phenomenon is caused by vasospasm, commonly seen in patients prior to the onset of sclerodactyly. Endothelial cells have been reported to play an important role in the initial inflammatory as well as subsequent fibrotic process. Histological analysis of the initial stage of scleroderma reveals perivascular infiltrates of mononuclear cells in the dermis, which is associated with increased collagen synthesis in the surrounding fibroblasts. T-cell interaction with vascular endothelial cells may lead to the subsequent cellular immune reaction, which may induce further vascular injury and tissue fibrosis. A number of studies have demonstrated the crucial role of several fibrogenic cytokines released from immunocytes in initiating the sequence of events leading to fibrosis.

Animal models are useful in providing clues for understanding various human diseases and for exploring new treatments. Although animal models which reproduce all the aspects of SSc are not currently available, bleomycin-induced scleroderma mouse exhibits definite dermal sclerosis mimicking human scleroderma [2]. In this model, features such as definite dermal sclerosis with dermal thickening, pulmonary fibrosis, and the presence of autoantibody in the sera are induced; however, vascular alteration in this model has not been remarked. In this paper, insights into the vascular pathogenesis in bleomycin-induced murine model are discussed.

2. Vascular Damages in Human Scleroderma

Vasculopathy in SSc is commonly seen in capillaries and small blood vessels. Raynaud's phenomenon is the common initial sign of SSc in the majority of cases, and digital ulcers, which are refractory and often impair quality of life of patients, are vasculopathies in which intima of vessels can be thickened and the lumen occluded. Vasculopathy in SSc

involves several types of cells such as endothelial cells, vascular smooth muscle cells, and pericytes, depending on different phases. Progressive thickening of blood vessel walls with proliferation of vascular intima is the typical feature of SSc [3]. Although the mechanism of intimal proliferation is uncertain, several factors such as chemical influence, virus, stress (e.g., oxidative or ischemia-reperfusion), immune-mediated cytotoxicity, apoptotic process, and antiendothelial cell antibodies (AECAs) are suggested as possible initial triggers. An abnormal response of microvascular endothelial cells to those direct or indirect stimuli may result in vascular injury. Proliferation of vascular smooth muscle cells and pericytes are suggested to lead to the vessel-wall thickening mediated by a Ras-depending manner [4] and occlusive changes by thickened intima. AECAs are frequently detected in sera of patients with SSc [5] and can activate endothelial cells to express cell adhesion molecules which alter leukocyte attachment and lead to endothelial cell damage and apoptosis. Kuwana et al. [6], however, proposed that insufficient vascular repair machinery due to defective vasculogenesis contributes to the microvascular abnormality in SSc. Although circulating concentrations of angiogenic factors are high in SSc, the levels of bone marrow-derived circulating endothelial precursors (CEP) are low [6], especially at late-stage disease [7], suggesting a complex dysregulation of vasculogenesis in SSc.

Endothelin-1 (ET-1) is a prototypical endothelial cell-derived product, and endothelial damage leads to increased production of ET-1. Since ET-1 is a vasoconstrictive agent, loss of normal vessel compliance and vasorelaxation may be induced by increased levels of ET-1. In addition, ET-1 promotes fibroblast synthesis of collagen [8]. ET-1 upregulates expression of adhesion molecules, which promote the homing of pathogenic leukocytes to the skin. Further, ET-1 can also induce myofibroblast differentiation in fibroblasts [9]. ET-1 can induce connective tissue growth factor (CTGF), and may mediate the induction of collagen synthesis by activation of CTGF [10]. Circulating ET-1 levels have been observed in patients with diffuse SSc with widespread fibrosis and those with limited SSc and hypertensive disease [11], suggesting that soluble ET-1 levels may be a marker of fibrosis and vascular damage. Thus, ET is suggested to significantly contribute to fibrogenesis, linking between vasculopathy, and fibrosis, and the blockade of ET signaling may lead to the reduction of fibrosis. *In vitro*, SSc fibroblasts synthesized increased amounts of ET-1, and further, bosentan reduced the contractile ability of the SSc fibroblasts [12]. Therefore, a blocking ET-1 might be expected as a benefit in reducing pulmonary fibrosis. Recently, bosentan is demonstrated to reduce the number of newly formation of digital ulcers associated with SSc [13]. Additionally, bosentan may reduce the sclerosis of the skin in a pilot study [14].

Nitric oxide (NO) is a strong vasodilator and inhibits the biochemical effect of ET-1. However, ET-1 induces inducible NO synthase (iNOS) expression in endothelial cells [15], and iNOS expression is detected in the endothelial cells in the lesional skin of SSc [16]. So far, several reports have shown impaired NO production in SSc [16, 17], which may contribute to the vascular pathogenesis of the arteriolar intimal proliferation in SSc. Thus, an imbalance between vasoconstriction and vasodilatation can lead to ischemia-reperfusion injury, endothelial damage and subsequent increased collagen gene expression *via* hypoxia. Hypoxia induces ECM proteins in cultured fibroblasts, and vascular endothelial growth factor (VEGF) overexpression may be caused in response to chronic hypoxia condition [18].

Reactive oxygen species (ROS) generated during various metabolic and biochemical reactions have multifarious effects that include oxidative damage to DNA. ROS can cause several abnormalities such as endothelial cell damage or enhanced platelet activation, leading to upregulation of the expression of adhesion molecules or secretion of inflammatory or fibrogenic cytokines including platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β); excessive oxidative stress has been implicated in the pathogenesis of scleroderma [19]. Indeed, scleroderma fibroblasts produce ROS constitutively [20]. Other effects of oxygen radicals include the stimulation of skin fibroblast proliferation at low concentrations [21] and the production of increased amounts of collagen [22], suggesting that low oxygen tension may contribute to the increased fibrogenic properties of scleroderma fibroblasts. Furthermore, several of the autoantigens targeted by scleroderma autoantibodies fragment in the presence of ROS and specific metals such as iron or copper [23]. The authors suggest that tissue ischemia generates ROS, which in turn induces the fragmentation of specific autoantigens. On the other hand, oxidative stress transiently induces CCL2 mRNA and protein expression in cultured skin fibroblasts [24], suggesting that ROS may play a regulatory role in inflammation by modulating monocyte chemotactic activity.

3. Vascular Changes in Bleomycin-Induced Scleroderma

Bleomycin has a number of biochemical properties, such as blocking the cell cycle at G2, cleaving the single-strand and double-strand DNA, degrading cellular RNAs, production of free radicals, and induction of apoptosis. Bleomycin exerts various effects on skin-constituted cells such as fibroblasts, keratinocytes, and endothelial cells, as well as immunocytes [25]. Bleomycin upregulates gene expression of ECM proteins as well as fibrogenic cytokines such as TGF-β and CTGF in cultured human skin fibroblasts [26]. Also, *in vitro* studies showed a dose-dependent stimulation of endothelial cell secretion of collagen synthesis by bleomycin, which was inhibited by the anti-TGF-β antibody [27].

Repeated local injections of bleomycin into the back skins induced dermal sclerosis in mice [28–33]. Histopathological examination revealed definite dermal sclerosis characterized by thickened collagen bundles, and the deposition of homogenous materials in the thickened dermis with cellular infiltrates, which mimicked the histologic features of human scleroderma. Dermal thickness gradually increased, up to twofold compared with control PBS injections, with the onset of the sclerosis. Cellular infiltrates were composed of T-cells, monocytes/macrophages, and mast cells, which are

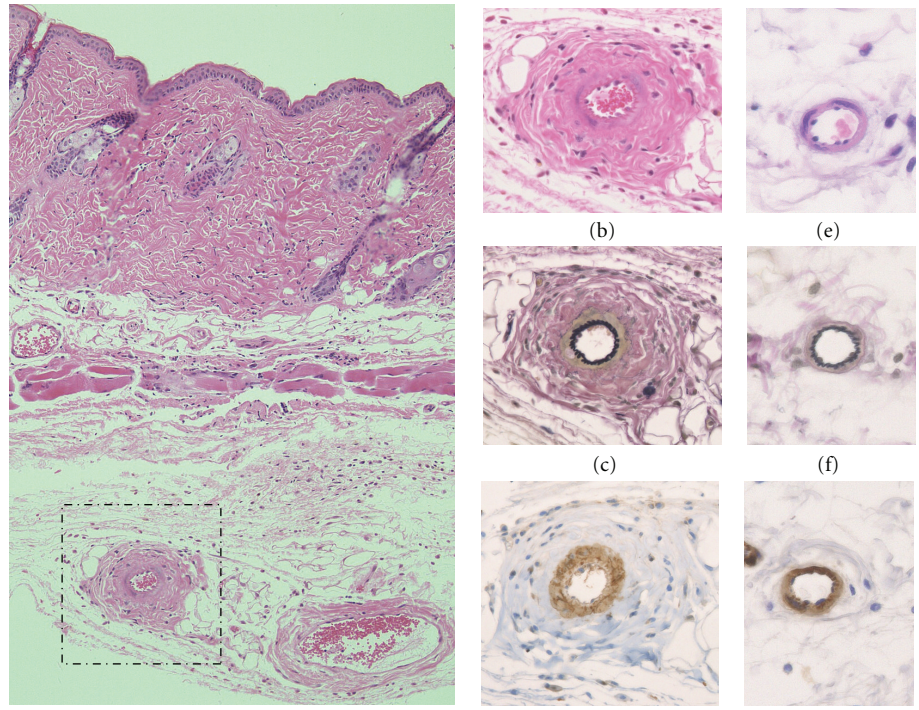

Figure 1: (a) Sclerotic skin induced by bleomycin injection. (b) Close-up view of the vascular lesions showing thickened wall. (c) Elastica van Gieson (EVG) stain showing proliferation of arterial intima. (d) α-SMA stain showing proliferation of vascular smooth muscle cells. (e)–(g) Vascular features of control mice treated with PBS were shown (e; H-E, f; EVG, g; α-SMA stain).

supposed to play an important role in the induction of dermal sclerosis. Increased production as well as upregulation of mRNA levels of type I collagen was observed in the bleomycin-treated skin. In the bleomycin-induced scleroderma, α-smooth muscle actin- (α-SMA-) positive myofibroblasts were observed in the dermis, and gradually increased in tandem with the induction of dermal sclerosis. In addition, significant thickness of vascular wall was also observed in the deep dermis (Figures 1(a) and 1(b)). Elastica van Gieson stain revealed proliferation of vascular intima (Figure 1(c)). Further, α-SMA stain suggests proliferation of vascular smooth muscle cells (Figure 1(d)). Those changes were distinct from control PBS-treated mice (Figures 1(e)–1(g)); however, whether the number of capillaries is reduced or not needs further detail investigation.

Recent studies have shown that apoptosis of endothelial cells induces resistance to apoptosis in fibroblasts largely through phosphatidylinositol-3-kinase-dependent mechanisms [33]. Furthermore, fibroblasts exposed to medium conditioned by apoptotic endothelial cells presented myofibroblast changes [34]. By contrast, cultured scleroderma fibroblasts were resistant to Fas-induced apoptosis [35, 36]. Although the effect of TGF-β on apoptosis differs according to cell type, stage of maturation, and other factors, TGF-β1 may play a role in inducing apoptosis-resistant fibroblast populations in SSc [36]. In scleroderma fibroblasts, Bcl-2 level was significantly higher, whereas the Bax level significantly decreased [36]. In primary pulmonary endothelial cells, bleomycin initiates apoptosis *via* the extrinsic pathway [37]. Also, involvement of the extrinsic apoptotic process in the bleomycin-induced scleroderma model has been investigated. DNA fragmentation revealed laddering of the bleomycin-treated skin, and increased expression of Fas and FasL was detected in the lesional skin. mRNA expression as well as activity of caspase-3 was also enhanced after bleomycin treatment. Administration of neutralizing anti-FasL antibody together with local bleomycin treatment reduced the development of dermal sclerosis, in association with the reduction of TUNEL-positive mononuclear cells and with the blockade of apoptosis. Caspase-3 activity was also significantly reduced after anti-FasL treatment. Excessive apoptosis, which cannot be treated by macrophages, may induce proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) or interferons (IFNs), and play a triggering role in the pathogenesis of bleomycin-induced scleroderma. In the bleomycin model, TUNEL-positivity was prominently detected on keratinocytes and infiltrating mononuclear cells, but not endothelial cell and fibroblasts in the sclerotic skin [38].

Vascular injury causes endothelial cell activation, dysfunction and altered capillary permeability as a primary event. These are followed by increased expression of adhesion molecules leading to mononuclear cell infiltrates in the skin. Cellular adhesion molecules (CAMs) are suspected of being responsible for the homing of pathologic inflammatory cells to the skin and are involved between immune cells, fibroblasts, endothelial cells, and ECM in the lesional skin of scleroderma. *In vitro*, bleomycin directly induces E-selectin

expression in endothelial cells through activation and nuclear translocation of NF-κB/Rel [39]. Also, *in vivo* studies show that intradermal injections of bleomycin into the normal human skin upregulate the expression of intercellular adhesion molecule-1 (ICAM-1) and E-selectin [40]. Those molecules play an important role in activation and migration of lymphocytes across the endothelium and basement membranes and in adherence to target tissues. The adhesion step may be important to the development of the initial pathologic changes of bleomycin-induced scleroderma.

4. Conclusion

In this paper, we have made a focus on vascular features of a bleomycin-induced murine scleroderma. Better understandings of the pathophysiology of collagen vascular disease in scleroderma are expected to contribute to the novel therapies specifically targeting vasculopathy of SSc.

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Review Article

Blocking TGFβ via Inhibition of the $\alpha v\beta 6$ Integrin: A Possible Therapy for Systemic Sclerosis Interstitial Lung Disease

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Interstitial lung disease (ILD) is a commonly encountered complication of systemic sclerosis (SSc) and accounts for a significant proportion of SSc-associated morbidity and mortality. Its pathogenesis remains poorly understood, and therapies that treat SSc ILD are suboptimal, at best. SSc ILD pathogenesis may share some common mechanisms with other fibrotic lung diseases, in which dysregulation of lung epithelium can contribute to pathologic fibrosis via recruitment or in situ generation and activation of fibroblasts. TGFβ, a master regulator of fibrosis, is tightly regulated in the lung by the integrin $\alpha v\beta 6$, which is expressed at low levels on healthy alveolar epithelial cells but is highly induced in the setting of lung injury or fibrosis. Here we discuss the biology of $\alpha v\beta 6$ and present this integrin as a potentially attractive target for inhibition in the setting of SSc ILD.

1. Introduction

Systemic sclerosis (SSc), also known as scleroderma, is a connective tissue disease of unknown etiology that is characterized by fibroproliferative changes in multiple organs, as well as microvascular and immunologic dysregulation. One of the most morbid conditions associated with SSc is interstitial lung disease (ILD), which occurs in 25–90% of SSc patients, depending on the detection methods used and the demographics of the population being studied [1, 2]. The pathologic mechanisms responsible for the initiation and maintenance of SSc ILD remain poorly characterized. Approximately 42% of patients with SSc ILD will die of disease progression within 10 years of diagnosis [3], and currently no curative therapies exist to combat this morbid complication.

2. Mechanisms of Fibrosis in SSc: A Role for Epithelial Cells?

Much of the research literature on SSc-associated fibrosis has focused on the roles of fibroblasts and myofibroblasts, the effector cells that are ultimately involved in the production of collagen and other extracellular matrix (ECM) proteins. However, the development of fibrosis in SSc is indeed a complex process involving crosstalk amongst multiple cell types, including epithelial, endothelial, immune, and mesenchymal cell types. In idiopathic pulmonary fibrosis (IPF), a progressive fibrosing lung disease that has a median survival of between two and three years [4], the principle defect is thought to be recurrent epithelial injury with resultant epithelial cell senescence and/or apoptosis. Epithelial injury can lead to the recruitment and activation of fibroblasts, which can be derived from resident fibroblasts, circulating fibrocytes, or the differentiation of epithelial cells, endothelial cells, or pericytes into fibroblasts. The best characterized of these changes in cell differentiation involves epithelial cells and has been termed epithelial-to-mesenchymal transition (EMT). Alveolar type II epithelial cell (AT2) injury has long been observed in lung biopsies from patients with ILD, and recent animal data suggests a causal relationship between AT2 injury and fibrosis. Sisson et al. recently demonstrated that targeted deletion of AT2 cells, using diphtheria toxin driven by a specific lung epithelial cell promoter leads directly

to lung fibrosis [5]. The most convincing evidence for the contribution of EMT to lung fibrosis came from studies by Kim et al., who used genetic fate-mapping methods to demonstrate the capacity of alveolar epithelial cells to undergo EMT in an established mouse model of lung fibrosis [6]. Based on these data and others, injured alveolar epithelial cells are viewed as potential drivers of pathologic pulmonary fibrosis.

Prior studies have provided evidence for increased epithelial cell damage in SSc ILD. Wells et al. measured the speed of clearance of technetium-labeled diethylene-triamine-pentaacetate (^{99m}Tc-DTPA) from the lungs in 53 patients with SSc ILD and found that rapid clearance, which suggested breach of epithelial barrier function, was associated with more dramatic clinical deterioration whereas normal clearance predicted stable disease [7]. Serum levels of the mucin-like glycoprotein KL-6, which is produced exclusively by lung epithelial cells and is associated with lung epithelial cell damage, are increased in ILD associated with connective tissue diseases [8].

Recurrent lung epithelial injury via chronic microaspiration has been proposed as a mechanism contributing to lung fibrosis. After the skin, the most commonly affected organ system in SSc is the gastrointestinal tract, affecting approximately 50–90% of all patients [9–11]. The esophagus is the most frequently involved site of the GI tract, leading to gastroesophageal reflux (GER). In a rodent model, chronic gastric fluid aspiration leads to a lymphocytic and obliterative bronchiolitis as well as parenchymal fibrosis, with increased TGFβ levels in bronchoalveolar lavage fluid [12]. Intriguingly, these histologic changes are independent of gastric fluid pH. The bile acid chenodeoxycholic acid stimulates TGFβ production in human airway epithelial cells and induces fibroblast proliferation *in vitro* in a TGFβ-dependent manner [13]. Correlative data support a relationship between chronic microaspiration and SSc ILD as well as other fibrotic lung diseases such as IPF [14, 15]. A strong association between GER and IPF has been recently reported in several studies, with an estimated prevalence of 67–88% for distal esophageal reflux and 30–71% for proximal esophageal reflux based on 24-hour esophageal pH monitoring. Interestingly, symptoms of reflux were poor predictors for the diagnosis of GER, implying a significant component of silent microaspiration [16–18]. Besides microaspiration, other mechanisms leading to lung fibrosis could also be at play in SSc ILD, involving not only epithelial cells but also endothelial, mesenchymal, and immune cell types. However, the hypothesis that microaspiration leads to SSc pulmonary fibrosis via recurrent epithelial injury is certainly an important one that needs to be strongly considered, especially given the prevalence of GER in SSc.

3. TGFβ: A Critical Mediator of Fibrosis

TGFβ is a pleiotropic cytokine that affects cell proliferation, differentiation, and apoptosis and is involved in a multitude of homeostatic functions. Importantly, TGFβ is regarded as the "master switch" of fibrosis in many tissues, including the lung [19]. The major effects of TGFβ include inhibition of epithelial cell proliferation, induction of fibroblast proliferation and the expression of genes encoding components of the ECM, and inhibition of the expression of metalloproteinase genes. TGFβ can stimulate fibroblast conversion into contractile myofibroblasts, which actively produce collagen and other ECM proteins, and may serve as an inducer of EMT, leading to fibrosis [20]. Mice that possess a gain of function mutation in the TGFβ pathway develop progressive fibrosis in multiple organs resembling SSc [21]. Global deletion of Smad3, a critical mediator of TGFβ signaling, or specific deletion of the TGFβ receptor II from lung epithelial cells affords resistance to bleomycin-induced lung fibrosis [22, 23].

Much data underscores the importance of TGFβ in SSc-associated fibrosis [24]. Increased expression of TGFβ1 or TGFβ2 is seen in early skin lesions and in lung tissue from patients with SSc ILD [25, 26], and TGFβ1 was significantly elevated in bronchoalveolar lavage fluid from SSc patients with pulmonary fibrosis [27]. A critical role for TGFβ in SSc has been highlighted by DNA microarray studies of SSc skin and fibroblasts. Recently, Sargent et al. generated a TGFβ-responsive signature in dermal fibroblasts comprised of 894 responsive genes [28]. Analysis of these genes in SSc skin biopsies revealed that this TGFβ-responsive signature occurred exclusively in a subset of skin biopsies from patients with diffuse SSc, and in particular, those who had a higher incidence of lung fibrosis. Importantly, these data suggest that a subset of SSc patients has disease that is predominantly driven by TGFβ.

4. Regulation of TGFβ by $\alpha v\beta 6$

There are three isoforms of TGFβ in mammals which are all bind to the same heteromeric receptor, leading to activation of the canonical pathway via phosphorylation of Smad proteins. In addition, noncanonical pathways are activated by TGFβ receptors, including several protein kinases (p38, JNK, Erk, c-Abl, TGF-β-activated kinase) and the lipid kinase PI3 kinase and its downstream target Akt. However, the phenotypes of mice lacking the different TGFβ isoforms are disparate, which could be explained by differences in isoform expression patterns or differential regulation of non-canonical signaling pathways.

Mice deficient in TGFβ1 exhibit uncontrolled tissue inflammation, autoimmunity, and premature death, demonstrating a critical role for TGFβ1 in immune homeostasis [29, 30]. These data suggest that general blockade of TGFβ should be approached with caution. A clinical trial of SSc patients utilizing an antibody directed against TGFβ1 showed no appreciable therapeutic effect [31], although the potency of this antibody has been questioned. Given its pleiotropic effects, TGFβ inhibition using strategies targeted to specific regions involved in fibrosis might be a better alternative. Most other approaches currently under consideration for targeting TGFβ block either TGFβ receptors or TGFβ itself. These approaches might lead to unwanted side effects by interfering with important homeostatic effects of TGFβ at

sites outside the organs affected by tissue fibrosis. Although mice lacking $\alpha v\beta 6$ do have mild inflammation in the lungs and skin, these effects are much less severe than those seen in mice lacking even a single TGFβ isoform. Additionally, the $\alpha v\beta 6$ integrin is highly upregulated in diseased tissue providing a mechanism for injury-induced TGFβ activation as compared to homeostatic control of TGFβ activity. By inhibiting only a subset of TGFβ activation, particularly in injured epithelial organs, targeting $\alpha v\beta 6$ could allow treatment of tissue fibrosis with substantially reduced risk of disrupting beneficial homeostatic control of inflammation and immunity.

The regulation of TGFβ activity involves multiple interactions of various proteins with the TGFβ cytokine. TGFβ is normally secreted as a complex which includes the bioactive peptide of TGFβ1, an amino terminal fragment of the TGFβ1 gene product called the latency-associated peptide (LAP), and the latent TGFβ-binding protein (LTBP) [32]. The TGFβ gene product is cleaved within the endoplasmic reticulum by the endopeptidase, furin, and it is assembled as a complex of two disulfide-linked homodimers formed from the shorter carboxy-terminal fragment (the active cytokine) and the longer amino-terminal fragment, LAP. These two homodimers associate noncovalently to form the small latent complex, which is unable to activate the TGFβ receptor because LAP shields the mature TGFβ homodimer from interaction with its receptor. In most cells, this small latent complex becomes disulfide linked to one of the latent TGFβ-binding proteins (LTBP). This large complex is secreted and attaches to components of the extracellular matrix and is covalently cross-linked to ECM proteins via the action of extracellular tissue transglutaminase. This preformed latent TGFβ complex exists at a high concentration in the ECM of most organs with little evidence of TGFβ activation [33]. Given the diverse and potent effects of TGFβ, its activity must be tightly regulated in a spatially specific manner.

Integrins are cell surface molecules comprised of alpha and beta chain heterodimers that regulate cell adhesion, survival, proliferation, and migration [34]. Pulmonary epithelial cells express at least 8 distinct integrin heterodimers. The $\alpha 3\beta 1$ and $\alpha 6\beta 4$ integrins recognize the epithelial basement protein, laminin 5 and play an important role in maintenance of epithelial integrity [35–38]. The other 6-lung epithelial integrins recognize ligands that are not present at baseline but are components of the provisional matrix that are upregulated in response to injury or inflammatory stimuli. The $\alpha v\beta 6$ integrin is the only integrin that is restricted in its expression to epithelial cells. This integrin, minimally expressed in healthy airway and alveolar epithelial cells at baseline, gets rapidly induced at these sites in response to a variety of insults, including lung injury [39]. Notably, and of possible relevance to the skin fibrosis of SSc, $\alpha v\beta 6$ is also upregulated on keratinocytes in the setting of wound healing but is minimally expressed at baseline [40]. *In vitro*, the $\alpha v\beta 6$ integrin binds to a number of ligands, including fibronectin, tenascin-C, and osteopontin [41] via interactions with an arginine-glycine-aspartic acid (RGD) tripeptide sequence, a sequence also recognized by several other integrins including those that share the αv subunit [42]. However, the *in vivo* relevance of $\alpha v\beta 6$ interactions with these ligands remains uncertain.

Mice completely lacking the $\beta 6$ integrin subunit, which pairs exclusively with the αv subunit, were viable with a near-normal life expectancy, but developed low-grade inflammation of the skin and lungs and late-onset emphysema [43, 44]. Following intratracheal delivery of bleomycin, a drug used to induce pulmonary fibrosis, $\beta 6$ deficient mice developed exaggerated inflammation in the lung but were remarkably protected from the subsequent development of pulmonary fibrosis [45]. These mice were also dramatically protected from radiation-induced pulmonary fibrosis [46]. These phenotypic findings suggested a role for the $\alpha v\beta 6$ integrin in regulating TGFβ, a key negative regulator of inflammation but a positive regulator of fibrosis. Amino acid sequence analysis revealed the presence of an RGD-binding sequence in the latency-associated peptide (LAP) of TGFβ1 and 3, and LAPβ1 and β3 were demonstrated to be bona fide ligands for $\alpha v\beta 6$ [47, 48]. Cells expressing the $\alpha v\beta 6$ integrin were shown to generate TGFβ activity that could be detected by an *in vitro* TGFβ reporter assay, and this activity was dependent upon cell-cell contact and could be specifically blocked with antibodies to $\alpha v\beta 6$ [45]. Microarray analysis of lungs from mice treated with bleomycin revealed a large group of TGFβ-inducible genes that were induced at much lower levels in the $\beta 6$ knockout mice compared with wild-type mice [49]. Collectively, these data provide strong evidence that the $\alpha v\beta 6$ integrin on lung epithelial cells is an important regulator of TGFβ activation. Activation could be inhibited by blocking actin polymerization [45] and by inhibitors of Rho kinase [50], suggesting a role for force generation by the actin cytoskeleton which presumably alters the conformation of latent complexes tethered to the extracellular matrix by matrix-bound LTBP, allowing for exposure of the active TGFβ cytokine and its interaction with TGFβ receptors.

Regulation of TGFβ activity in the lung was found to play an important role in the maintenance of alveolar homeostasis. Low-grade inflammation in the lungs of the $\beta 6$ knockout mice was characterized by increased numbers of alveolar macrophages, neutrophils, lymphocytes, and eosinophils, and this inflammation was reversed by transgenic overexpression of constitutively active TGFβ [44]. Microarray analysis of $\beta 6$ deficient lungs showed more than a 20-fold increase in the expression of matrix metalloproteinase 12 (MMP12) [49]. This protease, which is predominantly expressed by macrophages, preferentially degrades elastin, and has been implicated in the pathogenesis of emphysema. Emphysema was noted in older $\beta 6$ deficient mice, and crossing the $\beta 6$ deficient mice with mice lacking MMP12 completely rescued this phenotype. Expression of a wild-type form of the $\beta 6$ integrin prevented emphysema development, while expression of a mutant $\beta 6$ integrin subunit unable to support TGFβ activation did not prevent emphysema development. Studies have shown that the development of emphysema in $\beta 6$ deficient mice correlates tightly with the upregulation of MMP12, suggesting that MMP12 could serve as a surrogate biomarker to assess for this particular consequence [44].

5. Rationale for $\alpha v\beta 6$ Inhibition in SSc ILD

SSc ILD can be histopathologically classified as nonspecific interstitial pneumonia (NSIP) or usual interstitial pneumonia (UIP) [51–54]. NSIP is the more commonly encountered histopathologic subtype, comprised of varying degrees of inflammation and fibrosis, with some forms being predominantly inflammatory (cellular NSIP) and others primarily fibrotic (fibrotic NSIP). It remains unclear whether cellular NSIP and fibrotic NSIP represent a progression of one underlying disease process or rather two separate disease phenotypes, which in some cases can coexist within the same patient [55]. UIP is the pathologic pattern observed in idiopathic pulmonary fibrosis (IPF) and can also be seen in SSc ILD. UIP consists of interstitial fibrosis in a patchy pattern, honeycomb changes (both macroscopic and microscopic), and foci of fibroblastic proliferation. Although the UIP pattern in SSc is less commonly encountered clinically, it can be seen with increased frequency in patients with more severe fibrotic lung disease, including those with end-stage SSc ILD requiring lung transplant.

Currently, there are no highly effective agents for the treatment of fibrotic lung diseases. Several studies using anti-TGFβ agents have demonstrated protection from lung fibrosis in disease models [46, 56, 57]. Given the homeostatic roles of TGFβ in inflammation, immune regulation, and carcinogenesis, perhaps a better strategy for TGFβ inhibition would be to specifically target tissue-restricted activators of TGFβ such as the $\alpha v\beta 6$ integrin. In patients with IPF and SSc ILD with a UIP pattern, the $\alpha v\beta 6$ integrin is highly upregulated on lung epithelium, implicating this pathway in TGFβ activation [56]. In the only published report to date, upregulation of $\alpha v\beta 6$ was found on lung epithelium in seven out of seven SSc patients with UIP and in a single patient with SSc ILD who had fibrotic NSIP, but not in patients with cellular NSIP, however, the numbers of patients with NSIP analyzed were too small to draw meaningful conclusions [56]. It would therefore be important to better characterize whether upregulation of $\alpha v\beta 6$ specifically segregates with the UIP and fibrotic NSIP subsets of SSc ILD, and what role, if any, this integrin plays in the cellular NSIP subset. Anecdotal evidence and case series suggest that immunomodulators might more effectively target the cellular NSIP subset of SSc ILD, whereas the fibrotic NSIP and UIP subsets are thought to be more recalcitrant to currently available therapies [58]. Of particular interest, a mouse model of radiation-induced lung fibrosis identified a sharp upregulation of $\alpha v\beta 6$ expression by immunohistochemical analysis at 18 weeks following radiation challenge, with staining seen only in regions of fibrosis [46] and similar upregulation in fibrotic regions was found in lungs of IPF patients [56]. It thus appears that the induction of $\alpha v\beta 6$ correlates closely with fibrosis and that this integrin is often present at high concentrations in regions where active TGFβ could be contributing to disease progression.

A highly potent-blocking antibody to the $\alpha v\beta 6$ integrin was developed and shown to prevent fibrosis in mouse models of bleomycin- and radiation-induced lung fibrosis [46, 56]. In these studies, near maximal effects on collagen production were obtained at 1 mg/kg weekly dosing of the antibody. Importantly, a treatment (as opposed to prophylaxis) trial was performed in mice by giving the $\alpha v\beta 6$-blocking antibody at day 15 following intratracheal bleomycin administration, and decreased fibrosis at day 60 was observed using the hydroxyproline assay to measure lung collagen content. Given the finding of low-grade inflammation in the lungs of the $\beta 6$ deficient mice [43] as well as their late stage development of emphysema, a process that was dependent on MMP12 [44], a concerted effort was made to characterize whether a similar inflammatory phenotype with elevated MMP12 levels was observed in mice receiving the $\alpha v\beta 6$-blocking antibody. Transcript profiling of the lungs of mice treated with high doses (10 mg/kg) of the $\alpha v\beta 6$ blocking antibody paralleled the changes seen in $\beta 6$ integrin knockout mice, including upregulation of MMP12 levels. Importantly, at lower doses of the $\alpha v\beta 6$ blocking antibody (1 mg/kg or 3 mg/kg), MMP12 induction was greatly diminished [56], and BAL cell counts and inflammatory cytokines were not different than in saline-treated mice [46, 56]. At these lower doses of blocking antibody, significant inhibition of collagen production was still observed, as assessed by an *in vivo* collagen luciferase reporter system, suggesting that the antifibrotic effect of $\alpha v\beta 6$ inhibition could be uncoupled from the proinflammatory effect. Induction of TGFβ activation by bleomycin, as measured by phospho-Smad levels in lung lysates, was completely blocked at the 3 mg/kg but not by the 1 mg/kg dose of $\alpha v\beta 6$ blocking antibody suggesting that complete blockade of TGFβ signaling is not required to achieve antifibrotic efficacy and inhibition of TGFβ-induced fibrosis can be achieved without excessively perturbing the homeostatic functions of TGFβ.

Treatment of healthy, unchallenged mice with high doses of the $\alpha v\beta 6$ blocking antibody has been shown to lead to mixed cellular infiltrates (macrophages, lymphocytes, neutrophils) in lung tissue, not dissimilar to the inflammation seen in the $\beta 6$ knockout mice. However, long-term treatment of healthy primates with a humanized form of the same $\alpha v\beta 6$ blocking antibody leads to a minimal to mild increase in lung macrophages, which resolves completely following discontinuation of treatment, with no increase in mixed cellular inflammation (unpublished observations). These findings have suggested that inhibition of $\alpha v\beta 6$ does not induce the same degree of inflammation in primates as seen in mice. Additionally, no evidence of emphysema has been observed after 6 months of weekly dosing with high doses of $\alpha v\beta 6$ antibody in mice or primates and there has been no evidence of elevated MMP-12 expression in primates with $\alpha v\beta 6$ antibody treatment as observed in mice.

6. Conclusions

Inhibition of $\alpha v\beta 6$ as a means of locally dampening TGFβ activation by epithelial cells provides a rational therapeutic approach for conditions such as lung fibrosis. Importantly, the antifibrotic effect of $\alpha v\beta 6$ inhibition can be achieved at a dose that is uncoupled from its proinflammatory effect in mice [46, 56]. A phase II trial using a humanized $\alpha v\beta 6$ blocking antibody (STX-100) in IPF patients will soon

be underway, and these results should be of considerable interest to the SSc community. Evaluation of the utility of inhibition of $\alpha v\beta 6$-mediated TGFβ activation in SSc ILD, particularly the UIP and fibrotic NSIP subgroups, may be worth considering, especially if these early studies in IPF prove promising. In addition, recent data implicate an important role for epidermal keratinocytes in SSc skin fibrosis [59]. $\alpha v\beta 6$ is induced on injured keratinocytes in other settings, so the expression of $\alpha v\beta 6$ should be more closely evaluated in skin samples from SSc patients to determine whether a subset of these patients might also benefit from $\alpha v\beta 6$ blockade for treatment of skin fibrosis.

Given the known heterogeneity of SSc within and beyond the limited and diffuse subsets [60, 61], the inhibition of epithelial $\alpha v\beta 6$-mediated TGFβ activation may not address some of the other manifestations of SSc, in particular the vascular complications in which endothelial injury has been posited as an initiating mechanism. In fact, it is unlikely that any single treatment strategy will effectively combat the various pathologic manifestations of SSc. Whether the mechanisms leading to fibrosis of the skin and other internal organs in SSc are dependent upon $\alpha v\beta 6$-mediated TGFβ activation remains to be determined. Additional mechanisms involved in TGFβ activation, such as the integrins $\alpha v\beta 3$, $\alpha v\beta 5$, and $\alpha v\beta 8$, could be playing a contributory role, but discussion of this is beyond the scope of the current paper.

Importantly, when considering strategies that target TGFβ activity, potential side effects should be carefully monitored, such as the development of aberrant inflammation or cancer. However, in light of the morbidity and mortality associated with fibrotic lung diseases, especially IPF or the more fibrotic phenotypes of SSc ILD (UIP and fibrotic NSIP), perhaps these treatment risks can be justified given the lack of alternatives short of lung transplantation in some cases. TGFβ activity seems to be the "Achilles heel" of pulmonary fibrosis, and the ability to locally inhibit its activity presents an attractive strategy that may likely be met with clinical success.

Conflict of Interests

T. R. Katsumoto does not have a financial relationship with a commercial entity that has an interest in the subject of this paper. S. M. Violette is an employee of Stromedix and receives annual compensation of salary and stock. D. Sheppard is on the scientific advisory board of Stromedix, has sponsored research agreements with Stromedix (totaling $300,000), is coowner of the patents describing antibodies targeting the $\alpha v\beta 6$ integrin and the potential use of such antibodies for treatment of pulmonary fibrosis and acute lung injury and has received a portion of licensing fees related to one of these patents from 2002 to the present.

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Review Article

Tyrosine Kinase Inhibitors in the Treatment of Systemic Sclerosis: The Difficulty in Interpreting Proof-of-Concept Studies

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Tyrosine kinase inhibitors (TKIs) have emerged as a targeted therapy of interest for the treatment of systemic sclerosis (SSc). Recently, several groups have performed pilot or "proof-of-concept" studies to determine the feasibility of this approach for the treatment of the cutaneous and pulmonary manifestations of this multisystem disease. The conclusions drawn by these different studies have been conflicting, and some controversy has arisen as to whether tyrosine kinase inhibition is a treatment approach worthy of continued study. This paper summarizes this research to date with emphasis on the challenges in interpreting proof-of-concept studies in this patient group.

1. Introduction

Systemic sclerosis (SSc) is a heterogeneous, multisystem disorder characterized by vasculopathy, fibrosis, and autoimmunity in the context of both genetic and environmental factors [1]. Although the pathogenesis of SSc is not completely understood, there is strong evidence for a central role of abnormal signaling through the transforming growth factor beta (TGFβ) pathway [2]. The intracellular tyrosine kinase (TK) c-abl has been implicated in the downstream signaling pathways of TGFβ [3]. There is additional evidence for abnormalities in the platelet-derived growth factor (PDGF) axis, signaling through the receptor TK the PDGF receptor (R), as contributory to the pathogenesis of SSc as well [4].

Because of the roles of these pathways, there has been interest in evaluating the specific tyrosine kinase inhibitors (TKIs) which are able to block c-abl and the PDGFR in the treatment of SSc [5]. These include imatinib mesylate (Gleevec; Novartis Pharmaceuticals; Basel, Switzerland), dasatinib (Sprycel; Bristol Myers Squibb; New York, NY), and nilotinib (Tasigna; Novartis Pharmaceuticals) all of which inhibit c-abl and the PDGFR with different degrees of potency [6]. All three of these medications are FDA approved for the treatment of chronic myelogenous leukemia (CML), and imatinib is additionally FDA approved for the treatment of gastrointestinal stromal tumor (GIST) in addition to other hematologic and oncologic conditions [7–10]. Each medication is additionally capable of inhibiting other kinases. For example, imatinib inhibits c-kit, and dasatinib inhibits src kinases. It is possible that some of these different pathways could prove to be of biologic interest in fibrosing disorders. Each of these TKIs also has slightly different side effect profiles [11], which may also make one or another of them favorable for the treatment of SSc.

Imatinib, nilotinib, and dasatinib have been shown in *in vitro* models to decrease the TGF-β and PDGF-mediated production of extracellular matrix proteins and to abrogate skin thickening observed in mouse models of SSc [12, 13]. Imatinib has also shown antifibrotic properties in preclinical models of renal [14], hepatic [15], and pulmonary fibrosis [16]. Case reports of imatinib have shown promise to this treatment approach [17–21]. To date there have been several "proof-of-concept" (POC) approaches to determine the safety and potential efficacy of TKIs for dcSSc. The inclusion criteria and dosing of the various investigations have varied but the designs are similar as open-label and uncontrolled investigations to assess safety, efficacy, and effect on potential biomarkers of disease. The purpose of this paper is to review these investigations to date with a discussion of the various caveats that come into play with this type of study in SSc.

It is our opinion that these POC approaches can have great value in the search for improved treatment for this group of patients. However, POC trials pose challenges in study design and have important limitations to the conclusions that can be drawn from them. These POC approaches are appealing because they can utilize a smaller number of patients and provide insight into biologic effect, initial safety, and treatment effect *in vivo*. They can be used as an initial (but not definitive) test for clinical questions and are also an opportunity to test hypotheses with respect to biologic effect of treatment and biomarkers in SSc. The term "proof of concept" or "proof of principle" can hold several meanings. In general the term refers to animal or human studies which can provide evidence for or against a pharmacological or clinical effect. In industry-based drug development these POC trials are used for investment or "go/no go" decisions [22].

Multiple aspects of SSc make it difficult to fit into this "go/no go" mold. Firstly, SSc is a rare, heterogeneous, and multisystemic disease. Clinical endpoints and study design vary depending on whether the outcome of interest is cutaneous, pulmonary, vascular, or other. Patient recruitment is difficult because of the rarity of the condition, and the natural history of the involvement of each organ system is variable. Validated clinical endpoints exist for different outcomes, but some, for example, the modified Rodnan Skin Score (mRSS), may be open to bias in an uncontrolled trial, whereas others, for example, the forced vital capacity (FVC), are more objective. Many different biomarkers are under investigation and some have been validated in small populations or under certain circumstances [23]. However, these biomarkers have not yet been tested in clinical trials nor have they been shown to be prognostic at this point in time (with the exception of autoantibodies which have prognostic value at baseline but have not been shown to change with clinical status). The ability to define biomarkers has been further frustrated by the lack of unequivocably efficacious therapies in this condition. POC trials provide an initial insight into whether an agent seems to have acceptable safety and tolerability in the target population and whether there is a suggestion of efficacy that would make a larger double-blind randomized placebo-controlled trial worthwhile as well as how to power such an endeavor.

The investigation of TKIs in SSc is a case study of these issues.

1.1. Preclinical Studies Assessing Tyrosine Kinase Inhibitors for the Treatment of Fibrosis. Distler et al. showed that the treatment of cultured fibroblasts from both SSc patients and healthy controls with imatinib led to a dose-dependent inhibition of the synthesis of collagen Ia1, collagen Ia2, and fibronectin-1 when the fibroblasts were stimulated with TGF-β, PDGF, or left in their baseline state. Using the bleomycin-induced dermal fibrosis mouse model, this group also showed that imatinib treatment both prevented [12] and decreased established cutaneous fibrosis [24]. Using the tight-skin 1 (Tsk1) murine model a similar effect was shown [13]. Similar observations were made with respect to the effects seen in cultured fibroblasts and in the prevention of skin thickening in the bleomycin mouse model with dasatinib and nilotinib [13]. These well-executed studies were essential in establishing the rationale for the clinical study of TKIs in SSc, but it is important to note that both in vitro and animal models of fibrosis frequently fall short in predicting clinical response in SSc.

Following this work, TKIs have been used in several investigations delineating TGF-β signaling and its importance in the fibrotic manifestations of SSc. Pannu et al. observed that activation of TGF-β-mediated Smad1 signaling occurs in a subset of SSc patients and contributes to persistent activation of SSc fibroblasts [25]. This Smad1 pathway is inhibited by imatinib. Bhattacharyya et al. delineated a novel signaling mechanism of TGF-β involving a transcription factor called early growth response factor 1 (egr1) [26]. Recently, Bujor et al. have shown c-abl is positioned as an upstream regulator of the pro-fibrotic PKCδ/P-Fli1 pathway, via induction of PKCδ nuclear localization [27]. Li and Jimenez have shown that c-Abl and PKC-δ to be important in TGF-β-induced endothelial mesenchymal transformation [28]. These pathways are inhibited with imatinib. Additionally, microRNA29 (miR-29) has been observed to be downregulated in SSc fibroblasts, and treatment of these fibroblasts with imatinib reversed the downregulation of miR-29a [29]. Together this body of work presents a very compelling rationale for the investigation of these TKIs for the treatment of fibrosis in SSc and underlines the need for well-designed clinical investigations. These studies postulate specific biologic effects which would be well tested as investigational surrogate markers in a POC study, and collaboration between labs capable of performing such investigations with investigators performing the clinical trials needs to be a focus of the scleroderma research community.

1.2. Treatment of Other Fibrotic Conditions with Imatinib. Imatinib has been investigated for the treatment of various fibrotic diseases apart from systemic sclerosis. These include nephrogenic systemic fibrosis (NSF), sclerodermatous chronic graft-versus-host disease (cGVHD), morphea, and idiopathic pulmonary fibrosis (IPF). Kay and High observed decreased skin thickening and reduction in the mRSS in two patients with NSF [30]. The skin thickening recurred upon cessation of the drug. Olivieri et al. treated 19 patients with refractory cGVHD with fibrotic/sclerotic features and reported an overall response rate at 6 months of 79%, with 7 complete remissions and 8 partial remissions [31]. Similarly, Magro et al. reported a retrospective study of imatinib for sclerodermatous cGVHD where 7 patients responded, 4 of whom had mRSS improvements of 90% or more [32]. Moinzadeh et al. reported a case of one patient with therapy-resistant generalized morphea treated with imatinib with improvement [33]. Imatinib was studied for the treatment of IPF in a double-blind, placebo-controlled, randomized trial [34]. In this study 119 patients were treated with either imatinib or placebo at 600 mg daily (with dose adjustment to 400 mg daily for perceived drug toxicity). A difference was not detected between patients on imatinib compared with placebo with respect to the primary

endpoint: time to disease progression as defined as a 10% decline in percent predicted forced vital capacity (FVC) from baseline or time to death. Treatment had no effect on change in forced vital capacity or diffusion capacity of the lungs for carbon monoxide (DLCO) at 48, 72, or 96 weeks. More imatinib-treated patients discontinued study because of perceived drug-related AEs although this did not reach statistical significance (imatinib: 13 versus placebo: 6; $P = 0.073$). These IPF results were discouraging just as the data with sclerodermatous cGVHD and NSF seem encouraging. In both cases, the relation to SSc is uncertain.

1.3. Safety and Tolerability from Other Patient Populations. The side effect profiles of tyrosine kinase inhibitors have been described in patients with CML and other malignancies and are summarized in a recent review by Wei et al., and in general this is a relatively well-tolerated medication in this patient group [35]. With respect to imatinib in CML, the most common side effects include myelosuppression, nausea, fatigue, diarrhea, edema, and muscle cramps. Edema may be superficial and responsive to diuretics but also includes periorbital edema, which is less responsive to diuretics. Edema is seen less commonly with dasatinib and nilotinib when compared with imatinib. However, pleural effusion is a more prominent side effect of dasatinib treatment. Nilotinib has been associated with higher rates of dermatologic, hepatic, and pancreatic toxicity when compared with imatinib, but with lower rates of gastrointestinal intolerance, muscle spasm, and neutropenia.

Cardiotoxicity has been reported in patients with CML treated with imatinib, with some evidence for c-abl inhibition leading to this toxicity [36]. Retrospective, clinical experiences estimate the frequency of this to occur at a rate of 0.5–1.1% with association noted for typical risk factors for cardiovascular disease including age, diabetes, and hypertension [37, 38]. Patients in clinical practice with CHF are able to continue imatinib for malignancies with careful monitoring. In studies of patients with imatinib resistance or intolerance treated with nilotinib, sudden death was reported in 0.6% of patients, and an expanded-access program showed a similar rate of occurrence with concern for possible ventricular repolarization abnormalities because of timing of events [39]. Because of this, the package labeling for nilotinib requires careful EKG monitoring for QTc prolongation with repeat EKGs one week after dose adjustments and guidelines for dose adjustments based on QTc prolongation.

The side effects described above are important in an SSc population in particular because similar phenomena may be seen also as manifestations of disease activity. Patients with SSc may have occult or overt cardiac involvement with diverse manifestations ranging from conduction abnormality to cardiomyopathy. Not only is it important to carefully monitor for cardiac complications in SSc patients treated with TKIs, but also attribution of these side effects in open-label trials can be difficult. It would require a placebo arm to adequately understand if in SSc certain side effects can also be disease related. Edema, which is seen commonly with imatinib, can be particularly troublesome in a SSc population already uncomfortable with tight and thick skin. This side effect can be confounding in two ways: patients may have edema as an inherent part of early cutaneous manifestations of SSc which and edema can be difficult to distinguish from skin thickness and lead to an elevated measurement of the modified Rodnan Skin Score (mRSS). Myopathy is also common in scleroderma, often with low-grade creatine kinase (CK) elevations. Similar CK elevations have been seen in imatinib use in populations without underlying connective tissue disorders [40]. Attribution of myopathy, myalgias, or even asymptomatic CK elevations in imatinib-treated patients in uncontrolled clinical trials therefore can be similarly problematic.

Vascular issues are also an issue. PDGFR is on the pericyte and stabilizes blood vessels [41]. Alternatively, PDGF has been implicated in the abnormal proliferation and migration of pulmonary artery smooth muscle cells [42]. This effect in vivo needs to be carefully observed and could be postulated to lead to either efficacy or side effect. Indeed, Hatano et al. treated 5 patients with PAH (3 scleroderma-associated PAH and 2 idiopathic PAH) with imatinib (100 to 200 mg/day) for 24 weeks in addition to standard treatment [43]. However, there was no significant change in hemodynamics or exercise capacity. DLCO improved at 12 and 24 weeks of treatment ($P < 0.01$ and $P = 0.05$), and plasma PDGF-BB levels were significantly decreased after 12 weeks of treatment ($P = 0.04$). Two patients with high plasma PDGF-BB levels had a 15% decrease in pulmonary vascular resistance. Given the multitude of vascular issues in SSc patients, careful observation for vascular effect—positive or negative—needs to be performed carefully.

1.4. Clinical Data Summary—Systemic Sclerosis. There is a solid preclinical rationale for the use of TKIs in SSc; however, the clinical experience in this heterogeneous patient group is still in its early stages. Several case reports, which we have reviewed formerly [44], suggested the clinical benefit for imatinib treatment for the cutaneous and pulmonary manifestations of SSc, and three open-label clinical trials have also been recently reported. None of these were controlled clinical trials, limiting what can be said definitively about either the safety or efficacy of tyrosine kinase inhibition in scleroderma. Other studies have been presented in abstract form at scientific meetings. In addition to the studies that will be discussed below, there is an Italian study still ongoing and a French study completed but not yet reported to our knowledge. There is additionally one trial investigating dasatinib which is completed but not yet reported and one active and recruiting trial at our center investigating nilotinib [45].

The clinical trials reported to date are heterogeneous in terms of patient populations included, disease duration, imatinib dosing, and study duration. Difficulties in interpreting such open-label proof-of-concept trials with imatinib in scleroderma relate both to generic issues in terms of interpreting outcomes in a disease as heterogenous and variable in its natural history as scleroderma, as well as some issues more uniquely related to imatinib itself, namely the edema that often complicates treatment which can affect the mRSS.

Our group has recently reported an open-label experience treating 30 patients with dcSSc with imatinib for one year [46]. This was a 1-year, single-center, phase 2a, single-arm, open-label clinical trial. Two patient subgroups were prospectively targeted for enrollment: 20 patients with earlier disease (<4 years) and 10 patients with later disease (4–10 years) The mean disease duration based on the time since the first non-Raynaud's symptom attributable to SSc was 3.4 ± 2.3 years for the entire cohort. Mean disease duration in the earlier subgroup was 2.1 ± 1.2 years and in the later subgroup was 6.1 ± 1.6 years. All patients had an mRSS of at least 16, and the mean mRSS at baseline was 30.3 ± 8.7. ILD, which was not a requirement for entry, was present in 53% of patients. Concomitant administration of immunosuppressive agents was not allowed during the course of the trial or in the 3 months prior to enrollment, but 77% of patients had been on various other treatments for dcSSc previously. The target dose of imatinib was 400 mg/daily, but the mean dose tolerated was 300 mg/daily. 24 of 30 patients or 80% tolerated the medication well and completed the trial. Adverse events were common but mostly of grade 1 or 2. Of note, fluid retention which was seen in 80% of patients and GI side effects were similarly common. Neither of these AEs led to discontinuation of imatinib, but often dose adjustment or use of concomitant diuretics was employed. Twenty-four serious AEs were identified, the majority of which were not attributed to imatinib but to SSc itself or to complications from former therapies.

We observed a decrease in the mRSS by 6.6 points or 22.4% from baseline at 12 months in the 24 completers ($P = 0.001$). After 3 months of treatment there was no change in mRSS, but starting at 6 months of treatment this change started to become evident. ($\Delta = -4.5$; $P < 0.001$ at 6 months). This degree of improvement was seen in patients both early and late-stage diseases. In patients with disease duration of less than 18 months, there was an improvement in the mRSS by 29.5%, in those with disease duration less than 4 years the mRSS declined by 20.1%, and in those with 4 to 10 years of SSc the mRSS improved by 27.8%. There was an improvement of 6.4% predicted ($P = 0.008$) in the FVC, and the diffusion capacity remained stable after 12 months of treatment. The improvement in FVC was significantly greater in patients without interstitial lung disease. In patients with ILD both the FVC and DLCO remained stable over the one-year period of treatment: the mean change in FVC was +2.1% predicted (95% CI −2.9 to 7.0; $P = 0.36$) and the mean change in DLCO was +1.0% predicted (−8.0 to 10.1; $P = 0.81$). High-resolution CTs of the chest, although performed as part of standard of care in most of our patients, were not performed as part of the study protocol and so were not reported. The scleroderma Health Assessment Questionnaire-Disability Index (HAQ-DI) and the short form-36 (SF-36) physical component remained stable over the period of treatment. The SF-36 mental component, the patient global visual analogue scale (VAS), patient shortness of breath VAS, and pain VAS all improved significantly. Dermatopathological assessment demonstrated a significant decrease in skin thickness and improvement in skin morphology in a subset of patients. Histologic improvements paralleled improvements in mRSS.

Our group's experience is the most extensive published experience of imatinib in the treatment of dcSSc. Definitive conclusions regarding the efficacy of this imatinib for the treatment of dcSSc cannot be made based on this work because of the open-label design. Similarly, attribution of AEs is less certain in this sick patient population in the absence of a parallel placebo-controlled group. A criticism of this trial is that it included both early and later-stage patients, and not only early (<2 years disease duration) diffuse patients as based on the guidelines recommended by White et al. [47]. The rationale for including later-stage patients in this work were that the primary analysis was for safety and that this later-stage group is still very much in need of better treatments for fibrotic manifestations of both the skin and lung. Several facts were encouraging to us: (1) most of our patients were able to complete the trial despite side effects; (2) the improvement in mRSS held true across different disease durations including those with less than 18 months since the time of their first symptom of SSc apart from Raynaud's; (3) histopathology supported our clinical observations; (4) FVC and DLCO were stable in patients with ILD.

Pope et al. presented a 6-month POC trial of imatinib in which they found imatinib to be poorly tolerated at a dose of 200 mg twice daily [48]. Patients with active dcSSc as defined by an increase in mRSS, the presence of tendon friction rubs, or an elevated ESR were recruited. These patients had a mean disease duration of 3.1 years (range 0.3 to 6 years.) The trial was prematurely discontinued after enrollment of 10 patients because of concerns regarding 2 serious adverse effects—fluid retention and weakness, in one patient each. This was designed as a randomized controlled trial with a 4- to 1-randomization scheme and a planned enrollment of 20. Since the trial was terminated early, 9 subjects received active drug and only one received placebo, undermining the study power to compare the efficacy of active treatment versus placebo. Five patients stopped the study medication and needed to interrupt dosage due to adverse events which included fluid retention, weakness, nausea, vomiting, chest pain, worsening anemia, and hair loss. Four patients completed six months of active therapy. The mRSS at baseline was 32.3 and at 6 months was 30.5, which was not statistically different. No significant changes were noted with respect to echocardiogram, PFTs, or other parameters at six months' time. A number of exploratory translational studies from plasma and skin biopsies were reported as not changing significantly from 0 to 6 months other than sVCAM in plasma and sICAM-1 in skin, but data were not provided. An editorial pointed out that these inclusion criteria were somewhat unusual as well as the issue with using this medication in patients with myopathy [49]. This trial allowed concomitant use of methotrexate, which can have pharmacologic interaction with imatinib [50]. In summary, the short duration of the trial and small number of completers make the study inconclusive with respect to efficacy. This group found the tolerability to be poor.

Khanna et al. also reported on the use of a higher dose of imatinib (up to 600 mg daily with a mean dose tolerated of 445 mg) in 20 patients with SSc-ILD (14 dcSSc and 6 lcSSc) with treatment duration of 1 year [51]. Twelve of 20 patients discontinued treatment: 7 due to adverse events and 1 was lost to followup. Common AEs (≥20%) included fatigue, edema, nausea, diarrhea, and rash. Proteinuria of a range of 81 to 402 mg/dL was seen in 6 patients without concomitant increase in creatinine. These AEs are described in detail in this study. Adverse effects were felt to be dose related. The authors note that early in the study the first five patients who escalated their dose to 600 mg discontinued the study. The authors later allowed de-escalation or dose stabilization, and in the group that maximally titrated only to 400 mg/daily there were no discontinuations. Treatment with imatinib led to a nonstatistically significant increase in estimated FVC % predicted by 1.74%, TLC % predicted by 4.17%, and DLCO % predicted by 1.46% over 1 year ($P > 0.05$ for all). The mRSS improved by 3.9 units ($P < 0.001$). It is probable that the high level of adverse events noted in this trial relates to the increased dose, and only 30% of patients were able to reach that high of a dose. The improvements in indices of pulmonary function were modest but are worth comparing in magnitude to what was seen in the Scleroderma Lung Study where patients had very similar inclusion criteria where the FVC% predicted decreased by 1% point in the cyclophosphamide-treated arm [52]. Attributing this change to drug effect versus natural history is not possible without a control group, but as an exploratory POC trial this might be considered to justify further investigation in a randomized controlled trial.

Chung et al. reported in an interim fashion on nine patients treated with lower-dose imatinib at 100 to 200 mg daily [53]. Their cohort included 2 patients with limited SSc with ILD and 7 patients with diffuse SSc. The initial treatment period was 24 weeks. During this time 7 patients were able to complete the course of therapy, 1 patient dropped out secondary to SSc-related keratopathy, and 1 patient with severe ILD died from pneumonia and sepsis. Descriptive reporting of adverse effects included gastrointestinal complaints, edema, and infections. An improvement in mRSS of 32% was recognized in those patients with the diffuse subtype, $P = 0.005$. Evaluation of changes in gene expression by microarray showed a variable cutaneous molecular response to imatinib which might correspond to drug efficacy. This group had previously reported on 2 patients treated with imatinib 200 mg/d with significant improvement in pulmonary and cutaneous parameters [54]. Skin biopsies from these patients before and after treatment demonstrated decreased phosphorylation of PDGF receptor and Abl. Gene expression analysis with microarray on RNA extracted from the skin before and after treatment described the differential expression of 1,050 genes, which led to the hypothesis that there may be an imatinib-responsive gene signature in a specific subgroup of patients with SSc. We look forward to final publication of this experience. This work not only adds to the above experience with imatinib but raises the bigger questions of whether drug therapy influences gene expression in skin and whether we can use gene expression analysis with DNA microarray to predict response to treatment. It is important to emphasize that it will not necessarily completely answer these questions, but instead will provide information which can be considered hypothesis generating.

Distler et al. reported the results of an industry-sponsored multicenter study which recruited 27 patients to be treated with imatinib initially 200 mg daily to be titrated up to 600 mg daily for six months, then to be observed for an additional 24 weeks of study therapy. This was a more homogenous patient population with respect to disease duration and only patients with dcSSc with less than 18 months since the first non-Raynaud's symptom were enrolled. Only 16 of 27 patients completed 24 weeks of study, and 13 completed their 48-week study visit. The investigators prospectively defined a 25% decrease in mean mRSS at 24 weeks as a positive "proof of concept." This outcome was not met, and indeed at 24 weeks the mean mRSS increased by 9.9%. At 48 weeks, mRSS was recognized as decreasing by 21%. Levels of collagen Ia1 and fibronectin in skin were reduced with therapy, which might be seen as a positive effect on SSc biomarkers. We hope to see in the future publication of this paper a more extensive treatment of the biological samples obtained. The study utilized a high dose of imatinib, and the fluid retention and edema seen with this medication which is often dose related likely could have confounded interpretation of skin scores leading to an overestimate of skin scores related to edema rather than true skin fibrosis. Although the results at 48 weeks demonstrated decline in skin score, that was a small subgroup of patients, and in those ensuing several months other therapies were permitted making it difficult to know whether the improvement at 48 weeks was related to the earlier imatinib therapy, natural history disease, or effects of other medications. Moreover, with such substantial loss to follow up other patients, it is hard to say whether these results are biased by the greater likelihood of followup in patients who were doing well after exposure to the study intervention.

Denton et al. performed a retrospective exploratory analysis using these 27 patients from multiple centers as cases compared with well-characterized historical controls from a single-center database matched for age, sex, duration of disease, and baseline mRSS [55]. The change in mRSS observed in Distler et al. was not noted to be different from patients treated with various standard treatments, which in this group meant various active immunomodulatory agents including cyclophosphamide and mycophenolate mofetil treatments in 84% of the controls and no treatment in 16%. How to interpret this comparison is difficult, and there are obvious methodologic difficulties with the use of historical controls. Lumping both active treatments with immunomodulatory regimens with no treatment makes interpretation murky, and it is difficult to draw conclusions.

The authors of the above studies reach different conclusions regarding both tolerability and potential efficacy of imatinib. Some explanations for these diverse conclusions relate to the different populations studied: patients had different subsets of SSc with different organ manifestations, patients had different durations of disease, different dosages

of imatinib were used (in our experience, adverse events, in particular fluid retention, seemed to be dose related, with lower doses of the drug much better tolerated), and different durations of treatment were studied. Moreover, edema can confound the calculation of mRSS which may have been a consideration in the interpretation of the worsening of skin scores at 6 months in Distler et al. in which a high dose (600 mg daily) of imatinib was used. Additionally, it has been recently observed in a pooled analysis of 3 large RCTS that mRSS tends to improve after entering the clinical trials irrespective of the SSc disease duration [56].

2. Discussion

There is a strong rationale for the use of TKIs in SSc provided by the preclinical work. The early clinical work shows considerable adverse effects but potential efficacy in at least 3 of 5 the above-discussed trials. Definitive clinical investigation as from a well-designed randomized controlled-trial is yet to be done.

With respect to clinical outcomes, there is great difficulty in the interpretation of these pilot or proof-of-concept trials due to several factors. First and foremost, neither efficacy nor safety can be established with certainty from an uncontrolled experience. With respect to adverse effects, many of the symptoms attributed to medication side effects can also be attributed to the diverse manifestations of SSc, and without a placebo arm, it is not possible to truly interpret this. Secondly, the heterogeneous nature of dcSSc with respect to natural course of the disease further limits these studies' ability to determine efficacy especially if the effect size is small or moderate. If the response to treatment were comparable in magnitude, for example, to the effects of steroids on polymyalgia rheumatica, such conclusions would be clearer. However, even a modest clinical effect would still be welcome in the treatment of systemic sclerosis and is quite difficult to judge in an uncontrolled study. Additionally given the heterogenous nature of the disease, it is possible that a clinical effect would only be seen in a subset of patients, and such a hypothesis is put forth by the work of Chung et al.

Should open-label POC studies be performed in SSc? It is our opinion that there is value in these pilot studies. They afford initial data on safety in a population which may be very different from the original population tested. With respect to magnitude of response, this early data can be used to develop power calculations for larger, more definitive studies. When coupled to well-designed translational investigations these projects can illustrate important mechanistic concepts. Additionally an open-label trial with all patients receiving active treatment is substantially easier to recruit. Although the POC trials have value, they are inconclusive and specifically challenging in SSc for the reasons discussed above. For exploration of antifibrotic effect, it is unlikely for this to be observed in a short period of time given the half life of collagen, and this type of trial is usually performed over a short period of time. Additionally, there is a paucity of validated surrogate endpoints or biomarkers which can be used in SSc. In the work described above as well as in other early pilot studies in SSc, translational investigations are frequently utilized to explore potential mechanisms of action or to develop new biomarkers for disease activity. This type of work can be considered more hypothesis-generating mechanistic data rather than "proof-of-concept" of a clinical effect at this point in time.

Ultimately, these Proof-of-Concept open-label trials are inadequate to definitively address efficacy, safety, or even tolerability of TKIs in scleroderma. Those determinations will require the conduct of randomized, double-blinded, placebo-controlled trials. With respect to the use of TKIs in SSc, the available data, although imperfect, suggest that such studies are worth pursuing.

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Clinical Study

Skin Autofluorescence, as Marker of Accumulation of Advanced Glycation Endproducts and of Cumulative Metabolic Stress, Is Not Increased in Patients with Systemic Sclerosis

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Objective. To investigate whether advanced glycation endproducts (AGEs) in the skin are increased in patients with systemic sclerosis (SSc) and are related to the presence of disease-related and traditional cardiovascular risk factors. *Methods*. Skin autofluorescence, as a measure for the accumulation of AGEs, was assessed by measuring UV-A light excitation-emission matrices (AF-EEMS) in 41 SSc patients and 41 age- and sex-matched controls. Traditional cardiovascular risk factors and disease-related risk factors were recorded. *Results*. Skin AF-EEMS did not differ between SSc patients and controls (1.68 ± 0.58 a.u. versus 1.63 ± 0.41 a.u., $P = 0.684$). Skin AF-EEMS in SSc patients was associated with levels of CRP ($r = 0.44$, $P = 0.004$), Medsger's severity scale ($r = 0.45$, $P = 0.006$), and use of agents intervening in the renin-angiotensin system ($r = 0.33$, $P = 0.027$). When analysing SSc patients and controls together, in multivariate analysis, only age and use of agents intervening in the renin-angiotensin system were independently associated with AF-EEMS. *Conclusion*. These data demonstrate that skin AGEs are not increased in SSc patients.

1. Introduction

Vascular involvement is a key factor in major manifestations of systemic sclerosis (SSc), such as Raynaud's phenomenon (RP), myocardial dysfunction, pulmonary hypertension, and renal involvement. Microvascular involvement, in which endothelial injury is present, is the main characteristic of SSc [1, 2] Oxidative stress has been suggested as a major player in the process of endothelial dysfunction found in SSc. Endothelial damage may be induced by oxygen free radicals and reactive nitrogen species, generated locally by the inflammatory process and by periods of tissue ischemia followed by postischaemic reperfusion. This so-called ischaemic-reperfusion injury can be seen in RP [3, 4]. Increased levels of antibodies against oxidised low-density lipoproteins (LDL) [4–6] and increased serum levels of 8-isoprostane [7], being markers of oxidative stress, have, indeed, been observed in SSc.

Oxidative or carbonyl stress, leading to formation of so-called reactive carbonyl compounds, is an important source for the generation of so-called advanced glycation endproducts (AGEs) [8]. AGE generation as a result of oxidative stress has also been found in inflammatory diseases, such as rheumatoid arthritis and SLE [9–15].

Tissue autofluorescence (AF) is a marker of the accumulation of AGEs, validated in different patient groups and healthy controls [16–18]. Therefore, we assessed AGE accumulation in patients with SSc and hypothesized that

AGE accumulation is increased in patients with SSc compared to healthy controls based on the presence of oxidative stress and endothelial dysfunction in SSc. We related AGE accumulation to the presence of disease-related and traditional cardiovascular risk factors.

2. Material and Methods

2.1. Patients. Forty-one patients with limited cutaneous SSc from our university medical center out-patient clinic, fulfilling the ACR criteria for SSc [19] were included. Exclusion criterium was pregnancy. Forty-one age- and sex-matched healthy subjects were recruited as controls. The local research ethics committee gave approval for the study, and informed consent was obtained from all subjects. Clinical data were obtained by chart review and questionnaires. Diabetes mellitus was defined by the criteria from the American Diabetes Association. Dyslipidemia was diagnosed if plasma cholesterol exceeded 6.21 mmol/L, LDL cholesterol exceeded 3.36 mmol/L, and triglycerides exceeded 2.26 mmol/L or when the patient used lipid-lowering drugs [20]. Hypertension could not be categorized because of frequent use in SSc patients of vasodilating agents, such as calcium channel antagonists and ketanserin for other reasons than hypertension. Creatinine clearance (CrCl) was estimated using the Cockcroft-Gault formula. Smoking status and body mass index were also recorded. The SCORE risk estimation system was used, which was originally developed to obtain an estimation of total 10-year fatal cardiovascular risk in populations, using gender, age, total cholesterol level, systolic blood pressure, and smoking status [21]. Furthermore, we assessed disease-related factors that might influence skin AF and the development of atherosclerosis. Modified Rodnan Skin Score was assessed to determine skin thickness. To assess disease activity the preliminary European Scleroderma Study Group (EScSG) activity indices (a score ranging from 0 to 10) [22, 23] and the revised preliminary SSc severity scale (Medsger's severity scale) [24] were used. Immunosuppressive therapy was recorded.

2.2. Assessment of Skin AF. Tissue AGEs accumulation can be assessed as skin autofluorescence (AF), following the principles of the AGE Reader, which is a validated and noninvasive technique [16, 25]. Repeated measurements on one day in controls and diabetic patients showed an overall Alman error percentage of 5%. In this study, an adapted setup of the AGE Reader was used, namely, the Excitation Emission Matrix Scanner (EEMS), which is a technique to determine skin autofluorescence (AF-EEMS), which has the additional potential to discriminate between autofluorescence spectra from different fluorophores. This technique and setup has been described [26]. Measurement was performed at a skin site of approximately 4 cm^2 without evidence of fibrosis at the ventral site of the forearm, or other skin lesions. A series of measurements was obtained for each subject, and mean skin AF-EEMS was determined as described [14]. Skin pigmentation is also known to influence autofluorescence by light absorption. Therefore skin reflection should be >10% to perform an adequate measurement.

2.3. Laboratory Assessments. Lipid concentrations (total, high density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol and triglycerides), glucose, and creatinin were measured by routine techniques. CRP was measured using in-house enzyme-linked immunosorbent assays (ELISAs) as described [27].

2.4. Statistical Analysis. Values are expressed as mean ± SD when variables were normally distributed. In case of a nonnormal distribution, values are reported as median (interquartile ranges). For comparison between groups, continuous variables were analysed by Student's t-test or Mann-Whitney U tests, as appropriate. In case of categorical variables, the chi-square test was used. The univariate correlation between AF-EEMS values and other categorical variables was assessed by Pearson's correlation coefficient in case of normal distribution. Otherwise, Spearman's correlation coefficient was used. To assess the influence of tested parameters multiple regression analysis using the backward method was performed to assess the influence of demographic variables, outcome variables, and disease-related factors on skin autofluorescence (AF-EEMS). Variables which were significantly correlated in univariate analysis were used as independent variables in the multivariate analysis.

All analyses were performed using SPSS 14.0. A two-tailed P value < 0.05 was considered statistically significant.

3. Results

3.1. Characteristics of Patients and Controls. Characteristics of patients and controls are presented in Tables 1 and 2. Patients and controls were similar in age and gender and regarding (family) history of CVD, presence of diabetes mellitus, and renal function. Significant differences between SSc patients and controls were present in diastolic blood pressure and lipid levels, but also in medication used. Antihypertensive agents or vasodilating agents were used in 80% of patients compared to 2% of controls. Amongst these agents, 11 patients used angiotensin-converting enzyme (ACE) inhibitors and 3 patients used an angiotensin II receptor blocker. Also, statins were used more frequently in patients. BMI was slightly higher in controls, and none of the controls had a history of cardiovascular disease. Although the presence of traditional risk factors differed significantly between patients and controls, the total cardiovascular risk, SCORE, was comparable. CRP levels were significantly increased in patients compared to controls (3.4 mg/L (IQR 1.7–7.7) versus 1.4 mg/L (0.6–2.5), $P < 0.001$). Immunosuppressive agents, such as prednisolone, methotrexate, azathioprine, and cyclophosphamide were used in 15 (37%) SSc patients.

3.2. Skin AF-EEMS in Patients and Controls. No difference was found in AF-EEMS between patients and healthy controls (1.68 ± 0.58 a.u. versus 1.63 ± 0.41 a.u., $P = 0.684$, Figure 1). AF-EEMS was significantly higher in those with

Table 1: Clinical characteristics.

	Patients ($n = 41$)	Controls ($n = 41$)	P values
Age (years)	55.9 ± 11.0	55.4 ± 9.0	NS
Female, n (%)	33 (80%)	33 (80%)	NS
History of CVD, n (%)	4 (9%)	0	NS
Family history of CVD, n (%)	9 (22%)	13 (32%)	NS
Diabetes mellitus, n (%)	2 (5%)	0	NS
Glucose (mmol/L)	4.9 (4.3–5.4)	5.6 (5.3–6.2)	<0.001
BMI (kg/m^2)	24.1 (21.1–25.3)	25.0 (22.2–27.1)	0.019
Creatinine (μmol/L)	80 (67–93)	77 (59-84)	NS
Creatinine clearance (mL/min/1.73 m^2)	78 ± 23	87 ± 14	NS
Current smoking	3 (7%)	0	NS
Blood pressure			
Systolic (mm Hg)	120 (11–138)	128 (118–140)	NS
Diastolic (mm Hg)	75 (70–80)	78 (72–87)	0.023
Antihypertensive or vasodilating agents	33 (80%)	1 (2%)	<0.001
ACE inhibitors or ATII receptor blockers	14 (34)	1 (2%)	<0.001
Lipid levels			
Cholesterol (mmol/L)	5.0 ± 0.9	5.8 ± 0.9	<0.001
HDL cholesterol (mmol/L)	1.3 (1.2–2.2)	1.7 (1.4–2.0)	0.025
LDL cholesterol (mmol/L)	2.9 ± 0.8	3.5 ± 0.8	0.001
Triglycerides (mmol/L)	1.4 (1.2–2.2)	1.2 (0.9–1.7)	0.035
Statin use	7 (17%)	0	0.012
Dyslipidemia, n (%)	18 (44)	19 (46)	NS
Aspirin use	12 (29%)	0	<0.001
SCORE, %	1.0 (0.0–2.0)	1.0 (0.0–2.0)	NS
CRP (mg/L)	3.4 (1.6–7.7)	1.5 (0.6–2.5)	<0.001

Unless stated otherwise, data are expressed as mean ± SD when normally distributed and as median (25–75%) when nonnormally distributed.
CVD: cardiovascular disease; BMI: body mass index; ACE: angiotensin-converting enzyme; ATII: angiotensin II; HDL: high-density lipoprotein; LDL: low-density lipoprotein; SCORE: systematic coronary risk evaluation; CRP: C-reactive protein.

Table 2: Disease characteristics and disease-related factors in SSc patients.

Characteristic	Patients, n (%)
Duration SSc, yr	5 (3–11)
Duration RP, yr	11 (5–23)
EScSG activity index	0.5 (0.5–2.0)
MSS	6 (5–7)
mRSS	7 (5–14)

Data are expressed as median (interquartile ranges).
SSc: systemic sclerosis; RP: Raynaud's phenomenon; EscSG: European Scleroderma Study Group; MSS: Medsger's severity scale; mRSS: modified Rodnan Skin Score.

a history of CVD ($n = 4$) compared to those without a history of manifest CVD ($n = 77$) (2.22 ± 0.81 a.u. versus 1.62 ± 0.47 a.u., $P = 0.020$). In subjects using ACE inhibitors or ATII receptor blockers for hypertension or other reasons, AF-EEMS was significantly higher than in subjects not using these agents (2.01 ± 0.62 a.u. versus 1.57 ± 0.45 a.u., $P = 0.002$).

Univariate analysis between skin AF-EEMS and traditional risk factors and disease-related factors for CVD in SSc patients resulted in a positive correlation between skin AF-EEMS and CRP ($r = 0.44$, $P = 0.004$), as well as MSS ($r = 0.45$, $P = 0.006$) and use of ACE inhibitors or ATII receptor blockers ($r = 0.33$, $P = 0.027$). Univariate analysis of all subjects, patients and controls together, resulted in an association between skin AF-EEMS and age ($r = 0.28$, $P = 0.010$), CRP ($P = 0.25$, $P = 0.026$), and use of ACE inhibitors or ATII receptor blockers ($r = 0.275$, $P = 0.013$). All other clinical and biochemical variables did not show significant correlations with skin AF-EEMS.

Multivariate analysis revealed that age and use of ACE inhibitors or ATII receptor blockers were independently associated with skin AF-EEMS. Otherwise, no independent associations with skin AF-EEMS were present (Table 3).

4. Discussion

In this study, we demonstrated that skin AF as a marker of tissue AGE accumulation is not increased in SSc patients, while expected relations with age, prevalence of CVD, and CRP were found.

To our knowledge, only one study has been performed on the relation between AGEs and SSc. Kaloudi et al. [28] compared circulating levels of Nε-(carboxymethyl)lysine (CML), one of the AGEs which can be detected in vivo, in

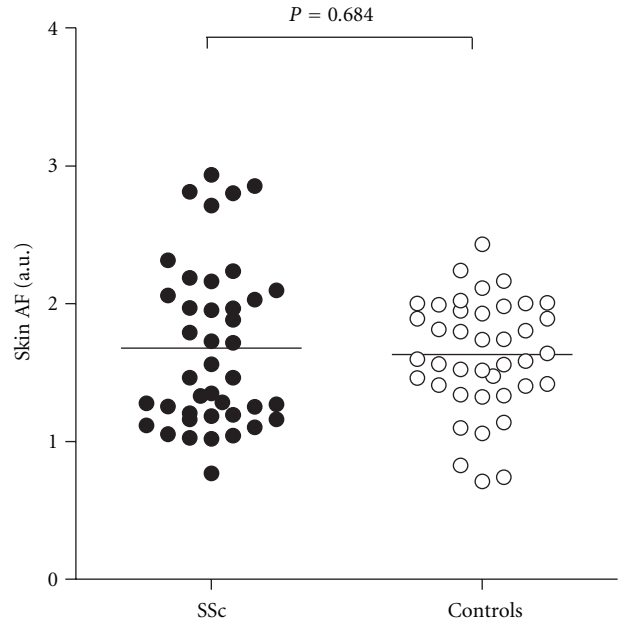

Figure 1: Skin autofluorescence (AF-EEMS) in patients (closed circles) and controls (open circles). The horizontal line represents mean skin AF-EEMS values.

Table 3: Multiple linear regression analysis with AF-EEMS as dependent variable in patients with systemic sclerosis and healthy controls ($n = 82$).

	B	β	P values
Constant	0.284		
Age	0.022	.400	0.006
Use of ACE inhibitors or ATII receptor blockers	0.613	.494	0.001

AF-EEMS: autofluorescence obtained by the Excitation-Emission Matrix Scanner; ACE: angiotensin-converting enzyme; ATII: angiotensin II.

SSc patients and healthy controls by means of ELISA. They found increased CML levels in SSc patients regardless of subset, with highest the levels found in SSc patients with an "early" disease pattern in nailfold videocapillaroscopy, suggesting that AGEs are involved in SSc microangiopathy. This is in agreement with the observation that the highest values of markers of oxidative stress are seen in early stages of the disease [4].

Several factors may be responsible for this discrepancy. We used noninvasive skin autofluorescence measurements for the assessment of accumulation of AGEs. This technique is simple, rapid, and noninvasive. Results from this technique were found to correlate strongly with levels of AGEs measured from skin biopsies [16, 29], although this validation was not extended to patients with SSc. Also, AGE detection in tissue with long-lived (years) proteins like the skin may better reflect the chronic accumulation of AGEs than measuring AGEs from serum or plasma with a relatively short (weeks) half-life of most proteins [25]. Since disease activity in SSc patients will wax and wane, we considered noninvasive skin autofluorescence measurements more useful in these patients. Our choice for the skin autofluorescence was also supported by the increased levels found in other autoimmune diseases with intermittent disease activity like rheumatoid arthitis and SLE in which skin AF was related to integrated disease duration and damage [14, 15]. Another consideration might be that skin AF was affected in the same emission range by other fluorophores (like NADH or tryptophan) to a different extent than in other conditions. Although we cannot exclude this, the expected relations with factors like age, CRP, and history of CVD still support skin AF as a marker of AGEs.

We expected to find more AGE accumulation in SSc patients with signs of inflammation and disease severity. Compared to controls, we found higher CRP levels in SSc patients, which might suggest a more active disease although absolute levels were not increased and EscSG activity index did not reflect active disease. We found a positive correlation between skin AF-EEMs levels and MSS, a measure of activity, severity, and damage in SSc patients, suggesting a relation between AGE accumulation and disease severity. Recently, doubts have been raised by Valentini and Cerinic on the weighting of the contribution of the different organ systems in the MSS [30]. Perhaps such a disbalance in the MSS explains why MSS, and also CRP, were not found to remain as predictors of skin AF-EEMS in multivariate analysis.

We found higher skin AF-EEMS levels in a substantial number of subjects using ACE inhibitors or ATII antagonists compared to subjects not using these agents, and their use was independently associated with skin AF-EEMS in multivariate analysis. This seems surprising because these agents have been found to reduce AGE accumulation in animal studies and in vitro studies [31–35]. In our SSc patients, these agents were used for a long period for several reasons, such as hypertension and Raynaud's phenomenon. Therefore, this treatment would have been expected to diminish AGE accumulation. We found higher skin AF-EEMS levels in subjects using these agents, but it cannot be ruled out that AGE accumulation was even more pronounced before agents intervening in the renin-angiotensin system were used.

Skin fibrosis in SSc could be a possible explanation of the lack of increase in AF-EEMS values that we had expected to occur, although we only included patients with limited cutaneous SSc, who had no significant fibrosis of the forearm. AGE accumulation and skin autofluorescence are strongly related to collagen-linked fluorescence and, thereby, to the usually very long half-time (15–20 years) of collagen. Previously reported increases in both collagen synthesis but especially increased degradation of skin collagen in SSc suggest that our results in SSc patients may be explained by accelerated skin collagen turnover [36–40]. In that case, the accelerated degradation of skin collagen could have prevented skin AGE accumulation and masked the effects of oxidative stress on AGE formation. Although assessment of skin AF-EEMS was performed at visible and palpable nonlesional skin to prevent influences by the presence of skin fibrosis, this influence cannot be ruled out completely. Also, increased collagen turnover may not be limited to affected skin only.

In conclusion, although oxidative stress seems present in SSc and is an acknowledged important factor in the generation of AGEs, increased levels of AGEs, as determined by skin autofluorescence, were not found. We cannot rule out that accumulation of AGEs in SSc patients was prevented by the use of ACE inhibitors or ATII receptor blockers, and by accelerated skin collagen turnover in clinically unaffected areas.

Conflict of Interests

R. Graaff and A. J. Smit are both founders of DiagnOptics BV, the Netherlands, manufacturing autofluorescence readers (http://www.diagnoptics.com/). The other authors have nothing to declare.

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Review Article

The Many Faces of Interleukin-6: The Role of IL-6 in Inflammation, Vasculopathy, and Fibrosis in Systemic Sclerosis

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Interleukin-6 is currently attracting significant interest as a potential therapeutic target in systemic sclerosis (SSc). In this paper, the biology of interleukin-6 is reviewed, and the evidence for interleukin-6 dysregulation in SSc is explored. The role of inteleukin-6 classical and trans signalling pathways in SSc relevant phenomena such as chronic inflammation, autoimmunity, endothelial cell dysfunction, and fibrogenesis is discussed. The existing evidence that interventions designed to block interleukin-6 signalling are of therapeutic relevance in SSc is evaluated.

1. Introduction

Systemic sclerosis (SSc) is a connective tissue disease characterised by fibrosis, vasculopathy, and immunological abnormalities. Over recent years, it has become clear that inflammation plays a crucial role in mediating the pathophysiological process underlying SSc, especially early in the disease. Endothelial cell activation and dysfunction are central to the disease pathogenesis, may be driven by a proinflammatory environment, and may result in the generation of a profibrotic phenotype.

Interleukin-6 (IL-6) is a pleiotropic cytokine. In addition to its role in the acute phase response, IL-6 has diverse roles in driving chronic inflammation, autoimmunity, endothelial cell dysfunction, and fibrogenesis. Therefore, it is currently attracting a great deal of interest in the rheumatology community as a potential therapeutic agent in SSc, a disease which at present lacks treatments directed at the underlying pathogenesis.

Recent evidence has suggested that IL-6 may play important roles in endothelial cell dysfunction and fibrogenesis in this disease, and clinical trials are currently being designed to further explore whether Tocilizumab, a monoclonal antibody directed against the IL-6 receptor, may be of therapeutic benefit to patients with SSc.

2. Interleukin-6 Biology

Interleukin-6 biology is complex. Few cells express the interleukin-6 receptor (IL-6R, gp80). This receptor is expressed on hepatocytes, monocytes, B cells, and neutrophils in humans. It is also found on a subset of T cells, but there is evidence that T cells respond to IL-6 predominantly through a process known as trans signalling [1].

Endothelial cells and fibroblasts do not express the IL-6R and are also thought to respond to IL-6 through trans signalling [2]. sIL-6Rs exist in the serum and bind to IL-6 forming an IL-6/sIL-6R complex. Soluble IL-6R (sIL-6R) is produced by two separate mechanisms, firstly by proteolytic cleavage from the surface of neutrophils and secondly by secretion from neutrophils and monocytes of an alternatively spliced version [3–6].

Although the regulation of the proteolytic cleavage of sIL-6R has not been fully elucidated, it is known to be stimulated by C-reactive protein (CRP). Cleavage from the surface of neutrophils, but not monocytes, is also stimulated by chemoattractants (interleukin-8 (IL8), C5a, leukotriene B4 (LTB4), and platelet activating factor (PAF)) [7]. Proteolytic cleavage can occur via a TNFα, converting enzyme-like enzyme although this does not account for all of the proteolytic cleavage [7].

We and others have shown that there is an increased concentration of the neutrophil chemoattractant IL-8 in SSc serum [8, 9], which may stimulate the release of sIL-6R from neutrophils. In addition, there are reports in the literature that LTB4 levels are elevated in the bronchoalveolar lavage fluid of patients with SSc lung disease [10], that may also contribute to the generation of sIL-6R.

The IL6/sIL6R complex can bind to the gp130 receptor, which is expressed ubiquitously on cells including endothelial cells and fibroblasts, to activate the signal transducers and activators of transcription protein 3 (STAT3) signalling pathway [1–11]. Endothelial cell activation via trans signalling results in an increase in the expression of adhesion molecules (intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1)), the release of chemokines (IL-8 and monocyte chemotactic protein-1 (MCP-1)), and the release of IL-6 [2–12] (Figure 1).

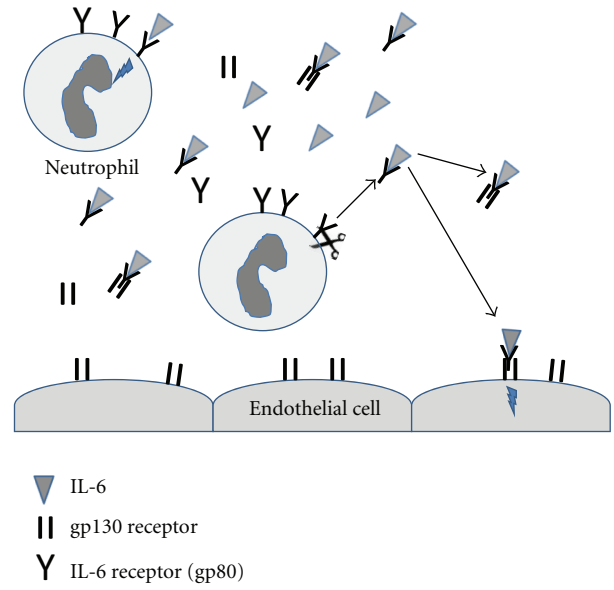

Figure 1: Interleukin-6 trans signalling. IL-6 receptors are expressed on leukocytes including neutrophils, but they are not expressed on tissue-resident cells, for example, endothelial cells. Endothelial cells can respond to IL-6 through the gp130 receptor only when the IL-6 is bound to a soluble IL-6 receptor (sIL-6R). sIL-6Rs are formed by secretion of an alternatively spliced version of the receptor or proteolytic cleavage from the surface of neutrophils. There is also a pool of soluble gp130 (sgp130) which can bind IL-6/sIL6R complexes and prevent them binding to cellular gp130. Therefore, the local concentrations of IL-6, sIL-6R, and sgp130 regulate IL-6 signalling.

3. Interleukin-6 in Systemic Sclerosis

IL-6 is a cytokine with several potentially important roles in the pathogenesis of SSc. It is elevated in the serum of patients with systemic sclerosis, especially those with diffuse skin involvement and early in the disease course [13, 14]. Immunocytochemistry studies have also demonstrated that IL-6 may be elevated in lesional tissue later in the disease, when other proinflammatory cytokines have dissipated.

Several other observations further support a role for this interleukin in SSc. Fibroblasts isolated and cultured from the lesional skin of patients with SSc constitutively produce higher levels of IL-6 than nonlesional or healthy donor fibroblasts [15]. This demonstrates the importance of considering local concentrations of cytokines in disease. Serum concentrations may not necessarily reflect local levels of a relevant cytokine at the lesional site. Hence, the use of *in vitro* models to explore local interactions between fibroblasts, endothelial cells, and immune cells, in the presence of locally elevated levels of cytokines, is of particular importance. Stimulated and unstimulated fibroblasts from lesional skin have also been shown to produce increased levels of IL-8 which may be implicated in local release of sIL-6R from neutrophils [16].

Previous research has shown that peripheral blood mononuclear cells from SSc patients, when cultured *in vitro,* produce higher levels of IL-6 and sIL-6R in the culture supernatants than control peripheral blood mononuclear cells, though levels of sgp130 were equivalent [17]. Furthermore, IL-6R levels were increased in the serum of patients with limited cutaneous SSc (lcSSc) compared to controls [18].

IL-6 transcription is under the control of a hypoxic response element via hypoxia-inducible factor-1-α (HIF-1-α). Measurements taken from the lesional skin of patients have demonstrated a persistent decrease in oxygen tension [19], down the equivalent of 3% O_2, sufficient to induce HIF-1α signalling [19].

In addition, it is important to note that hemodynamic flow may suppress IL-6-induced signalling in endothelial cells [20]. As such flow is dysregulated in SSc, this may play an important role in modulating the effects of IL-6 on endothelial cells in this disease.

4. Interleukin-6 Effects on B Cells

IL-6 also has a profound effect on B cells, promoting plasma cell differentiation and antibody production. This may explain the polyclonal B-cell expansion and hypergammaglobulinaemia which is frequently seen in SSc [11].

B-cell depletion using rituximab (monoclonal antibody directed against CD20) in 9 patients with progressive SSc skin disease, refractory to cyclophosphamide therapy, resulted in a clinical improvement in skin score after 3 months, which persisted up to 36 months. This was paralleled by a decrease in serum IL-6 concentration [21].

5. Interleukin-6 and Effects on Inflammation

IL-6 has been implicated in the generation and propagation of chronic inflammation. Initially in acute inflammation, proinflammatory cytokines promote neutrophil accumulation and the release of IL-6. Neutrophils then shed their IL-6Rs in response to chemokines such as IL-8. This promotes differential regulation of chemokine production by endothelial cells, promoting MCP-1 production and decreasing IL-8

production, therefore favouring monocyte accumulation. IL-6 trans signalling also increases the expression of endothelial leukocyte adhesion molecules (VCAM-1, ICAM-1), further promoting leukocyte accumulation [12–22]. In addition, IL-6 may have a role in promoting neutrophil apoptosis and therefore the resolution of acute (nonspecific) inflammation [23, 24]. Others however have reported an antiapoptotic effect of IL-6 on neutrophils [25], while Biffl et al. have shown that the effect depends on the neutrophil concentration [26]. We have been unable to reproduce any IL-6-specific effect on neutrophil apoptosis in our laboratory at concentrations of IL-6 ranging from 0.1 to 100 ng/mL (personal communication Helen Wright).

Conversely, IL-6 reportedly rescues T cells from apoptosis, which promotes a chronic inflammatory cell infiltrate [27–30]. IL-6 trans signalling also promotes the release of IL-6 from fibroblasts and endothelial cells in a positive autocrine feedback system. Therefore, it can be envisaged that IL-6 may have a role in propagating chronic inflammation, such as that seen in SSc. This is in keeping with immunocytochemical experiments which demonstrate that IL-8 and IL-6 are overexpressed in the lesional skin of patients with SSc, though in different patterns: the overexpression of IL-8 is associated with early disease (<1 yr), whereas IL-6 overexpression is associated with later disease [31].

IL-6 has also been implicated in autoimmunity. Evidence from patients with Crohn's disease indicates that autoreactive T cells are resistant to apoptosis due to protection by IL-6 trans signalling via the STAT3 signalling pathway [32]. IL-6 inhibits a Na^2+/K+ ATPase which regulates antigen internalisation and antigen presentation by dendritic cells to T cells, which may promote presentation of autoantigens [33, 34]. Finally, according to Matzinger's "danger theory," naïve T cells die if they receive a signal from proper antigen presentation that is not followed up by ligation of CD40 [35]. There is evidence that IL-6/sIL-6R complex can inappropriately substitute for this second signal and therefore lead to the persistence of autoreactive T cells [36]. Furthermore, autoimmune phenomena increase with age, in concert with an age-related increase in sIL-6R shedding [37]. Lissilaa et al. explored the role of IL-6 in the collagen-induced arthritis (CIA) and antigen-induced arthritis (AIA) models of autoimmune inflammatory arthritis. Using antibodies which specifically blocked classical IL-6 signalling and trans signalling pathways, they discovered that the classical IL-6 pathway was both necessary and sufficient for the development of pathogenic Th17 T cells which are implicated in autoimmunity and for the generation of antitype II collagen IgG responses which are associated with disease manifestations in the CIA model. They also demonstrated in the AIA model that IL-6 trans signalling was responsible for driving local inflammatory responses [38]. SSc is a disease associated with autoimmune phenomena. Many different autoantibodies are found in SSc (see Table 1), and the autoantibody profile in many cases correlates with clinical manifestations. There is, however, no convincing evidence for a direct role for autoantibodies in pathogenesis though some investigators have reported that antiendothelial cell antibodies, found in a proportion of patients, are associated with endothelial cell activation [39, 40].

Table 1: Systemic sclerosis-associated autoantibodies, potentially pathogenic antibodies which have been described in a proportion of patients with systemic sclerosis. Reviewed in [41]. ECM: extracellular matrix.

Autoantibody	*In vitro* activity
Antiendothelial cell	Endothelial cell apoptosis
Antifibrillin 1	Fibroblast activation, increased ECM production
Antimatrix metalloproteinase	Prevent degradation of the ECM
Anti-PDGFR	Induce collagen 1 production Convert fibroblasts to myofibroblasts
Antifibroblast	Increased expression of ICAM and IL-6
Anti-HSP47	Not known

6. Interleukin-6 and Effects on Fibrogenesis

Fibroblasts from patients with SSc are phenotypically unique. When isolated and cultured *in vitro* they continue to produce an excess of collagen [42, 43]. IL-6 is a profibrogenic cytokine. It has been shown to either increase or decrease fibroblast proliferation, increase fibroblast collagen, glycosaminoglycan, and tissue inhibitor of metalloproteinases-1 (TIMP-1) synthesis, and increase MCP-1 and IL-6 production [43–48]. IL-6 regulates the expression of vascular endothelial growth factor (VEGF), an important mediator of angiogenesis and fibrosis which is elevated in patients with SSc [49].

One case series has indicated that the use of tocilizumab, which blocks IL-6 trans signalling, in 2 patients with diffuse cutaneous SSc (dcSSc), one with renal involvement and the other with lung fibrosis, resulted in a decrease in skin thickening as measured by Rodnan skin score and Vesmeter (which measures viscoelasticity or hardness of the skin). In addition, skin biopsies taken before and after tocilizumab treatment indicated a reduction in collagen [50].

7. Interleukin-6 and Effects on Endothelial Cell Activation

Endothelial activation is thought to be central to the pathogenesis of SSc. There is also evidence for increased endothelial cell apoptosis though corroborative *in vivo* evidence for this is lacking [51]. The University of California at Davis line 200 chicken, an animal model for SSc, shows evidence of early endothelial cell apoptosis, preceding the inflammatory cell infiltrate and the development of fibrosis [39–52].

Serum markers of endothelial cell activation, for example, von Willebrand factor (vWF), sICAM-1, and sE-selectin are elevated in the serum of patients with SSc and appear to correlate with disease activity [53–55].

Previous studies have shown a role for IL-6 in endothelial cell activation. Endothelial cell activation via trans signalling results in an increase in the expression of adhesion molecules (ICAM-1, VCAM-1), the release of chemokines (IL-8 and MCP), and the release of IL-6 [2–12].

We have recently shown that SSc serum, in the presence of neutrophils, is capable of increasing endothelial cell activation and apoptosis in an IL-6-dependent manner [56]. It is postulated that in this circumstance the neutrophils are acting as donors of IL-6R. In our studies, spiking pooled control serum with IL-6 resulted in increased endothelial cell apoptosis and E-selectin expression in the presence of neutrophils, mimicking the effects of SSc serum. Complement inactivation did not abrogate the effects of SSc serum, neither did the addition of catalase to mop up reactive oxygen species. The serine protease inhibitor AEBSF partially blocked the effects of SSc serum on endothelial cell apoptosis but did not significantly affect the activation of endothelial cells by SSc serum [56]. Strategies to remove or block the effects of IL6 in SSc serum including immunodepletion of IL6 and the addition of an anti-IL6 blocking antibody reversed the effects of SSc serum on endothelial cell activation and apoptosis [56]. Most significantly, however, sgp130 which specifically blocks IL6 trans signalling abrogated the effects of SSc serum [56].

8. Conclusion

IL-6 blockade and specifically the blockade of IL-6 trans-signalling may have merit in the treatment of SSc, a disease that so far lacks treatment options directly targeting the pathogenic mechanism. IL-6 trans signalling is specifically implicated in driving local inflammation and inducing endothelial and fibroblast responses, and therefore targeting this IL-6 signalling pathway may be most profitable in SSc. However, SSc also has important and possibly pathogenic autoimmune phenomena, and targeting the classical IL-6 signalling pathway may be necessary in order to influence this important aspect of the disease. The currently available drug Tocilizumab targets both the classical and the trans signalling pathways. Other agents are in development which specifically block trans signalling, and they may be useful in mouse models of SSc to delineate which signalling pathway is most important for this disease.

IL-6 is increased in the serum of patients with SSc, especially in early dcSSc. In addition, it is also found in immunohistochemistry samples in both early and late disease and in both dcSSc and lcSSc. Fibroblasts and monocytes isolated from SSc patients autonomously produce IL-6 *in vitro*.

Early, small-scale nonrandomised controlled trials point to an important role for IL6 in SSc. B-cell depletion results in a decrease in serum IL-6 levels, reflected in a simultaneous reduction in skin score. More importantly, blocking IL-6 trans signalling with Tocilizumab has resulted in an improvement in skin score in 2 patients with diffuse disease. These data firmly establish IL-6 as an attractive candidate therapeutic target, especially in terms of preventing fibrosis.

However, in addition, new and exciting data imply that IL-6 has a role in the endothelial and inflammatory manifestations of this disease, which may make it a potential target in a much broader range of SSc patients with active vascular or inflammatory (e.g., joint) disease but relatively little fibrosis. Studies are being designed to address these important questions; the results are eagerly awaited.

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Research Article

Low-Dose Naltrexone for Pruritus in Systemic Sclerosis

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Pruritus is a common symptom in systemic sclerosis (SSc), an autoimmune disease which causes fibrosis and vasculopathy in skin, lung, and gastrointestinal tract (GIT). Unfortunately, pruritus has limited treatment options in this disease. Pilot trials of low-dose naltrexone hydrochloride (LDN) for pruritus, pain, and quality of life (QOL) in other GIT diseases have been successful. In this case series we report three patients that had significant improvement in pruritus and total GIT symptoms as measured by the 10-point faces scale and the University of California Los Angeles Scleroderma Clinical Trials Consortium Gastrointestinal Tract 2.0 (UCLA SCTC GIT 2.0) questionnaire. This small case series suggests LDN may be an effective, highly tolerable, and inexpensive treatment for pruritus and GIT symptoms in SSc.

1. Introduction

Systemic sclerosis (SSc; scleroderma) is an autoimmune disease characterized by vasculopathy and fibrosis of multiple organs including the skin, lungs, and gastrointestinal tract (GIT). This chronic disease process results in pain and pruritus, two distinct, but interacting phenomena [1]. Pruritus is most common in the early stages of disease and may subside as the disease progresses [2]. SSc patients that complain of pruritus have more significant skin involvement, more severe finger ulcers, worse respiratory symptoms, and a greater number of GIT complaints [3]. Of interest, pruritus is independently associated with GIT symptoms in SSc [3]. Although pruritus is associated with significant disability, management guidelines for pruritus in SSc do not exist [4].

Pruritus is also a feature of primary biliary cirrhosis (PBC), which occurs more commonly in SSc than the normal population [5]. There is a known association with PBC and oxidative stress as well as endothelial dysfunction [6]. Pharmaceutical management suggestions for treatment of pruritus in PBC include cholestyramine, rifampin, sertraline, and naloxone [7]. More recently, pilot trials of low-dose naltrexone hydrochloride (LDN), which is a pharmaceutical similar to naloxone, have recently gained increasing recognition for treating chronic pain associated with fibromyalgia, multiple sclerosis, and Crohn's disease [8–10].

Evidence supporting the hypotheses that increased opioid-mediated neurotransmission in the brain is a mechanism of pruritus and that central opioidergic tone is increased in cholestasis provides a rationale for treating the pruritus of cholestasis with opiate antagonists in PBC [11]. Another potential mechanism of action of LDN is through attenuation of the production of proinflammatory cytokines and superoxides potentially mediated by activity of toll-like receptor 4 [12]. Modulation of mitochondrial apoptosis has also been proposed as a mechanism of LDN [13]. In SSc,

oxidative stress may be important in disease pathogenesis [13]. Thus, an agent that potentially modulates oxidative stress is attractive as an emerging therapeutic in SSc. Given the putative mechanisms of action of LDN and the roles of these various pathways in SSc, our hypothesis is that LDN may be a reasonable agent to treat pruritus in SSc.

2. Patients and Methods

In this case series we report three SSc patients who presented to the University of Utah SSc Center with the chief complaint of pruritus. All were white females between the ages of 34 and 56. Two patients were of diffuse cutaneous SSc subtype (dSSc); the other one was limited SSc subtype (lSSc) [14]. One of the dSSc patients and the lSSc had tendon friction rubs. None had scleredema. The disease duration from the first non-Raynaud's phenomenon symptom varied from 1 year to 11 years. Raynaud's phenomenon onset was within the same year of diagnosis in two patients, and three years prior in one patient. All patients reported pruritus as an important feature of disease since onset of SSc and did not attribute it directly to change in skin thickening or GI manifestations. They all reported that "itchiness" of the skin had been present for >6 weeks and was unresponsive to antihistamines on all days. None had urticaria. They all denied a history of eczema or skin conditions other than SSc, any new product, or infectious exposures and attributed this symptom to SSc. A ten-point faces scale was used to assess pruritus. On this scale 0, is no symptoms, 1 very mild, 2 discomforting, 3 tolerable, 4 distressing, 5 very distressing, 6 intense, 7 very intense, 8 utterly horrible, 9 excruciating, 10 unimaginable/unspeakable [15]. It has been used in other clinical trials of pruritus [16]. Each of our participants ranked their pruritus >5.

All patients completed a University of Los Angeles Scleroderma Clinical Trials Consortium Gastrointestinal Tract Questionnaire (GIT 2.0). The GIT 2.0 is patient-reported outcome measure to assess health-related quality of life (HRQOL) and GIT severity in SSc [17, 18]. This 34-item instrument has seven scales: reflux, distention/bloating, diarrhea, fecal soilage, constipation, emotional well-being, and social functioning and a total GI score (a higher score denoted worse HRQOL). All scales are scored from 0.0 (better HRQOL) to 3.0 (worse HRQOL) except diarrhea and constipation scales that range from 0.0 to 2.0 and 0.0 to 2.5, respectively. The total GI score is the average of 6 of 7 scales (excludes constipation), and total GI score is scored from 0.0 (better HRQOL) to 3.0 (worse HRQOL). Each scale is further divided into 3 groups by severity: none-to-mild, moderate, and severe-to-very severe. These were calculated based on anchors included in the original published data and data collected in a recent National Scleroderma Foundation online survey and available online at http://uclascleroderma.researchcore.org. The instrument takes 6–8 minutes to be completed and has been shown to have acceptable reliability and validity [17, 19]. Additionally each patient had a modified Rodnan skin score (mRSS) as part of their routine care at baseline (Table 1).

Baseline laboratories were obtained in all patients as per routine care. All were antinuclear antibody (ANA) positive. A marker of inflammation (C-reactive protein) was normal in all patients. None of the patients had an eosinophilia (<0.4 k/μL) on peripheral blood smear. Anti-tissue transglutaminase and antimitochondrial antibody were negative in all patients. Two patients had skin biopsy performed as part of another clinical study. A computer-assisted image analysis of two of the biopsy specimens revealed that at baseline there was not an increase in mast cells greater than those present in a healthy control (Figures 1 and 2).

From a treatment perspective, all patients were RNA polymerase III positive, making a trial of corticosteroids for treatment of pruritus an unattractive option due to an increase risk of scleroderma renal crisis [20]. They all denied depression and were resistant to a trial of sertraline. None consumed alcohol or were taking narcotics for pain. One patient was on methotrexate for arthritis. The other two patients were not on immunosuppression medications. All patients denied respiratory complaints, had no evidence of pulmonary arterial hypertension on echocardiogram, and had normal pulmonary function tests. No patients had digital ulcers or met clinical criteria for fibromyalgia. All had normal colonoscopies without evidence of collagenous colitis.

Each patient was initiated on LDN 2 mg by mouth at bedtime for the first month. Using dosing recommendations from another trial and phone call monitoring for adverse effects, the dose was increased by 1 mg by mouth at bedtime each week (0.5 mg the final week) up to a maximum dose of 4.5 mg [9]. One patient requested to maintain the dose of 2 mg by mouth at bedtime, as she felt it was working well and did not wish to incur additional cost. The other two patients reached the 4.5 mg dose. All patients were seen in clinic for followup after two months of treatment. All patients repeated the GIT 2.0, and a mRSS was done at each visit. No other medication change, including use of over-the-counter agents, was allowed for any of these patients during the treatment period. However, nonmedicinal changes were allowed. One patient implemented head-of-the-bed elevation for reflux management, and two patients increased fluid and dietary fiber intake for symptoms of constipation.

3. Results

All patients reported an improvement in pruritus on a Faces Scale after initiating LDN (Table 1) [15]. In two different patients, pruritus completely resolved on LDN. In two patients a trend in improvement in mRSS was seen at two months, which is not clinically significant [21]. However, all patients reported feeling that skin was objectively softer. In all patients there was an improvement in total GIT 2.0 scores as well as in constipation and distention/bloating subscales. Repeat skin biopsy specimens were not obtained. No adverse drug effects were reported with the exception of two nights of insomnia reported in a single patient.

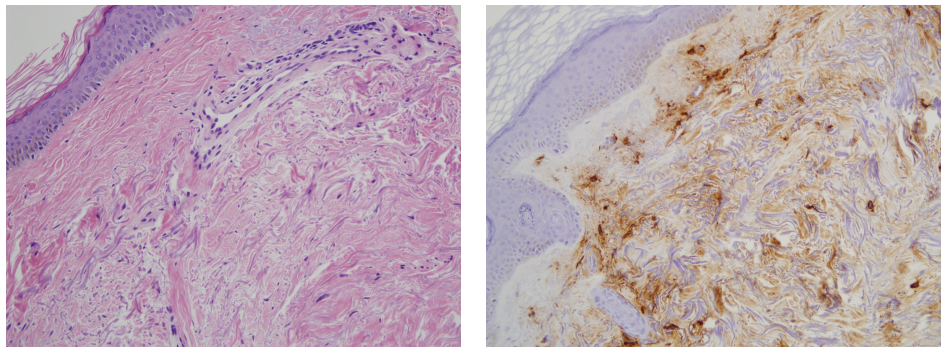
4. Discussion

Pruritus is a common symptom in SSc, with limited treatment options. The pathogenesis of chronic (>6 weeks

Table 1: Clinical features of systemic sclerosis participants.

Patient	Disease onset*, subtype	Pre-LDN pruritus	Post-LDN pruritus	Pre-LDN mRSS	Post-LDN mRSS	Pre-LDN UCLA SCTC GIT 2.0	Post-LDN UCLA SCTC GIT 2.0
1	2 years, diffuse	10	4	28	24	Total: 0.355 (i) Reflux: 0.63 (ii) Distention: 0.5 (iii) Soilage: 0 (iv) Diarrhea: 0 (v) Constipation: 1.25 (vi) Social: 0 (vii) Emotional: 0.11	Total: 0.302 (i) Reflux: 0.5 (ii) Distention: 0.5 (iii) Soilage: 0 (iv) Diarrhea: 0 (v) Constipation: 1 (vi) Social: 0 (vii) Emotional: 0.11
2	11 years, diffuse	6	0	19	17	Total: 0.395 (i) Reflux: 0.63 (ii) Distention: 1.75 (iii) Soilage: 0 (iv) Diarrhea: 0.5 (v) Constipation: 0.5 (vi) Social: 0.17 (vii) Emotional 0.22	Total: 0.216 (i) Reflux: 0.38 (ii) Distention: 0.5 (iii) Soilage: 0 (iv) Diarrhea: 0 (v) Constipation: 0.25 (vi) Social: 0.17 (vii) Emotional 0.22
3	1 year, limited	8	0	12	12	Total: 0.54 (i) Reflux: 0.25 (ii) Distention: 1.5 (iii) Soilage: 0 (iv) Diarrhea: 0 (v) Constipation: 1.25 (vi) Social: 0.33 (vii) Emotional 0.44	Total: 0.216 (i) Reflux: 0.13 (ii) Distention: 0.5 (iii) Soilage: 0 (iv) Diarrhea: 0 (v) Constipation: 0.5 (vi) Social: 0.17 (vii) Emotional 0.22

* First non-Raynaud's phenomenon symptom; LDN: low-dose naltrexone; mRSS: modified Rodnan skin score; UCLA SCTC GIT 2.0: University of California Los Angeles Scleroderma Clinical Trials Consortium Gastrointestinal Tract 2.0.

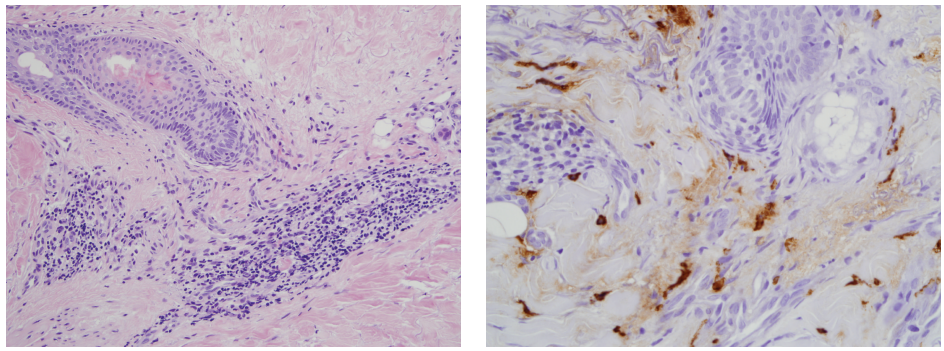


(a) (b)

Figure 1: Biopsy specimens from patient 1: H&E-stained section shows moderate perivascular and periadnexal inflammation and thickening of dermal collagen (20X). There are several mast cells in perivascular, interstitial, and periadnexal distribution with granular-appearing cytoplasm (IHC mast cell tryptase 20X).

duration) pruritus is complex and involves a network of resident cells (such as mast cells, keratinocytes, and sensory neurons) and transient inflammatory cells within the skin [22]. Pruritus in SSc is independently associated with GI symptoms [3]. Because of the association of pruritus with quality of life (QOL) in SSc, more attention to potential methods for intervention is warranted [23]. his small case series suggests a potential role for LDN for SSc with pruritus. Additionally, a possible improvement in mRSS and in GIT symptoms may also result from use of LDN.

Autoimmune gastrointestinal disorders such as inflammatory bowel disease (IBD), gluten-sensitive enteropathy, and PBC have also been associated with pruritus [24]. Evidence supports that increased opioid-mediated neurotransmission in the brain may be a mechanism of pruritus in PBC [11]. LDN has been found to improve QOL in IBD



(a) (b)

Figure 2: Biopsy specimens from patient 3: H&E-stained section shows scant perivascular inflammation and thickening of dermal collagen (20X). There are scattered mast cells in perivascular and interstitial distribution with granular-appearing cytoplasm (IHC mast cell tryptase 20X).

[10]. Although the real biological mechanism of LDN is not known, LDN may modulate pruritus through opioid-mediated actions or a reduction of inflammatory mediators [25, 26]. As such, a better understanding of the interaction of the GIT and pruritus is warranted.

SSc patients commonly have diarrhea, constipation, and distention/bloating that may mimic GIT hypersensitivity [27]. Whether the GIT is responsible for the pruritus in SSc or whether the GIT and skin are reacting to a similar stimulus or endogenous immune response is unknown. Mast cells have been suggested to have an important role in SSc as well as in functional bowel disorders [28, 29]. However, computer-assisted analysis of skin biopsy specimens in two of our patients did not reveal an abnormal percentage of mast cells.

Clearly a larger number of patients and a double-blind placebo-controlled trial are needed to define the therapeutic potential of LDN in SSc. The patients' subjective observation of "softer skin" was not validated by a significant change in mRSS and could be attributed to the natural history of disease rather than to naltrexone. Additionally, intraobserver variability could explain the improvement in mRSS. Psychological and physiological predictors of pruritus response as well as minimally important differences in the ten-point faces scale need to be defined. Gastrointestinal biopsy specimens would have been helpful for correlating to skin biopsy specimens for defining potential morphologic and histochemical change. Nonetheless, this series suggests LDN may be an effective, highly tolerable, and inexpensive treatment for pruritus in SSc and further supports a potential role for computer-assisted quantification of inflammatory cell types in skin biopsies to guide selection of therapeutics.

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Review Article

Apoptosis Modulation as a Promising Target for Treatment of Systemic Sclerosis

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Diffuse systemic sclerosis (SSc) is a fatal autoimmune disease characterized by an excessive ECM deposition inducing a loss of function of skin and internal organs. Apoptosis is a key mechanism involved in all the stages of the disease: vascular damage, immune dysfunction, and fibrosis. The purpose of this paper is to gather new findings in apoptosis related to SSc, to highlight relations between apoptosis and fibrosis, and to identify new therapeutic targets.

1. Introduction

Systemic sclerosis (scleroderma, SSc) is a heterogeneous disease which develops into three forms: limited, intermediate, and diffuse [1]. The limited form only affects skin of the limbs. In addition to cutaneous disorders, the diffuse one also affects internal organs such as lungs, heart, and kidneys. After a debilitating phase, the most severe form leads to death. This disease is characterized by a strong autoimmune reaction, although it is not clear whether this is a consequence of the disease or a causal factor. Nevertheless, autoantibodies, principally raised against nuclear epitopes, are used like prognostic markers.

In the United States, the disease strikes more African American people and females than Caucasians and males [2]. Besides, the disease appears more prematurely in the African American group and hits them more severely than the Caucasians [3]. The mortality rate for SSc in the group of women has increased by seventy percent over the last twenty years without convincing explanations. The cost of the medical care associated with this disease was estimated at more than $20,000 per patient per year in Canada [4]. The disease is particularly devastating because it strikes people during the most productive period of their professional life.

The causes of SSc are not clearly identified. Genetic factors could not be excluded [5, 6], but environmental influence seems to be more important. Notably, chronic exposition to chemicals, such as organic solvents [7] and silicone [8], viral infection by cytomegalovirus (CMV), a member of herpesviruses family [9, 10], and microchimerism [11], could all play a role in the disease.

It is thought that SSc begins with vasculopathies through massive endothelial cells death that would lead to obliteration of small arteries and arterioles. It is however unclear if autoantibodies are produced before vascular damages and/or in response to it. Subsequently, cell-to-cell communications are substantially altered, notably by cytokine and growth factor secretion dysregulation. The consequence of these biological changes is an excessive extracellular matrix (ECM) deposition, fibrosis, in tissues followed by their loss of function [12]. SSc varies during the progression of the disease, showing noteworthy changes in fibroblasts phenotype [13]. At the early stage of the disease, cells respond to TGFβ, but become totally insensitive at the late stage for the most affected patients, explaining why treatments targeting this cytokine remain mostly ineffective.

Over the last decades, various models had been used to study SSc [14]. Some animals develop a disease similar

to this pathology such as UCD-200 chicken, Tsk-1 and -2 mice. Normal mice could also show some symptoms of the disease after a treatment with Scl-GvHD, Bleomycin, or growth factor injection. Nevertheless, several observations made on these animal models are quite different than those ascertained in humans. Primary cultures of cells isolated from patients are also widely used, and, more recently, a new tissue-engineered reconstructed skin model was shown as a powerful tool to assess the mechanisms involved in the progression of human SSc [13].

2. A Brief Overview of the Mechanisms of Apoptosis

From the development of the embryo up to its death, apoptosis plays a crucial role in the induction and the maintenance of several physiologic functions, at several steps of both normal and atypic development steps during life. By eliminating cells during the development of the embryo, it assists at leading the modeling patterns of the body. It also contributes to eliminating the nonfunctional neurons and allows the selection of the adequate synaptic connections. Later, it allows the elimination of unsuitable lymphocytes (AICD, activation-induced cell death) [15], preservation of the homeostasis [16, 17], contributing to the maintenance of the functional status of the immune system by eliminating deviant or infected cells. However, an unbalanced ratio between the various factors involved in apoptotic pathways can lead to excessive cell proliferation and potentially to cancer or, on the contrary, to neurodegenerative diseases (Alzheimer, Parkinson), autoimmune diseases (rheumatoid arthritis), or immunosuppressive diseases (AIDS).

Apoptosis, or programmed cell death, should not be confused with necrosis. The apoptotic process occurs according to specific and sequential steps that have been documented. In contrast, necrosis results from passive mechanisms that lead generally to an inflammatory process while apoptosis does not [18]. Apoptotic cells are truly destroyed from the inside and quickly removed from the tissues, a situation that seriously delayed the discovery of this physiologic process [19]. Apoptosis is regulated through a sequence of events that have been described. First, the cellular morphology changes with the formation of structures in bubbles, rounding of cells probably related to a disorganization of actin filaments, a reduction of the cytoplasmic volume, a weakening of mitochondrial membrane, facilitating the liberation of various factors. The chromatin condenses, and the DNA is cleaved at the level of nucleosomes, before the fragmentation of the cell nucleus. Finally, the cells are fragmented into apoptotic bodies that are mainly cleared by macrophages.

Several mechanisms are described as playing a role in the induction of apoptosis [20]. Following a proapoptotic stimulus, a cascade of specific proteases is activated in the cell. These proteases are called caspases, cysteinyl aspartic acid proteases [21]. Caspases are produced as proenzymes which must be cleaved to become active. Protease specifity is defined for each caspase by an aspartic acid containing consensual sequence in target proteins. Caspases involved in apoptosis could be classified in 2 groups: the initiator or apical caspases, like caspase-8 and caspase-9, whose role is to activate the other caspases, and the effector or downstream caspases, like caspase-3, which generally cleave vital targets. Apoptotic pathways differ according to the nature of the inductor and the cell type, making their study very complex, especially in vivo. Nevertheless, two main pathways can be distinguished: the mitochondrial, or intrinsic, pathway and the cell death receptors, or extrinsic, pathway (Figure 1). There are numerous connections between these two pathways, then it could be difficult to separate them. Other pathways have also been described, but would be of lesser importance.

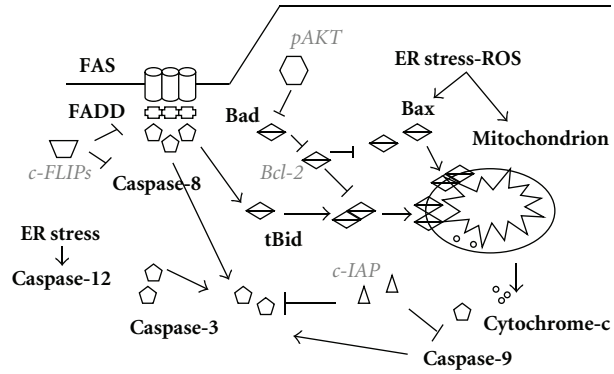

Figure 1: Major apoptotic pathways. Schematic representation of intrinsic, mitochondrial, and extrinsic, death receptor, pathways. Proapoptotic molecules are **in bold** and antiapoptotic are *in italic*.

The mitochondrial pathway seems to be the most common apoptotic mechanism, and it is well documented [22]. Under the influence of several stimuli (ultraviolet irradiations, chemical damage, etc.), proteins of the Bcl family act independently or in complexes on the mitochondrial membrane [23, 24]. Bcl family proteins could be separated in proapoptotic proteins (Bax, Bak, Bok, Bcl-Xs), which destabilize the mitochondrial membrane, antiapoptotic proteins (Bcl-2, Bcl-XL, Bcl-W, Mcl-1, Bcl-2A1), that protect this membrane and proapoptotic BH3-only proteins (Bad, Bid, Bik, Bim, Puma, Noxa), which inhibit the antiapoptotic function of the Bcl-2-like proteins [25]. Under Bcl proapoptotic protein influence, mitochondrial pores open and lead to depolarization of the membrane releasing proapoptotic factors such as cytochrome C. The latter associates with the adaptor Apaf-1 (apoptosis-associated factor-1) and procaspase-9. In presence of dATP, this complex, named apoptosome, leads cleavage and activation of procaspase-9 which responds by cleaving procaspase-3 to activate it. Caspase-3 degrades its substrates such as ICAD/DFF45, an inhibitor of the endonuclease CAD/DFF40 (caspase associated DNAse), responsible for the fragmentation of the DNA. Various inhibitors exist for this pathway such as some antiapoptotic proteins of the Bcl family [23], preventing the depolarization of the mitochondrial membrane. Furthermore, when phosphorylated by AKT/PKB, Bad, a proapoptotic member of this family could be sequestrated by the

protein 14.3.3 [26]. This action prevents Bad to bind to Bcl-xL and inactivate its protective function. IAP (inhibitor of apoptosis protein) could also block mitochondrial apoptosis by inhibiting the cleavage of procaspases 3, 7, and 9 [27, 28]. Other pathways that are dependent from mitochondria but independent from caspases can also be activated, notably by the factor AIF (apoptosis inducing factor) released from mitochondria [29].

The second best known apoptotic pathway, the cell death receptor (DR) pathway, has also been extensively studied [30–32]. Until now, about fifteen DRs from the TNF (tumor necrosis factor) receptor family were identified. Some are well known, especially TNFR-1/p55 and Fas/Apo-1/CD95. They are characterized by an extracellular domain consisting of cysteyl-rich regions (from 1 to 6 repeats), a transmembrane domain, and an intracellular domain carrying a death domain region (DD). Inserted in the plasma membrane of the cells, monomeric or trimeric forms of receptors are clustered into microdomains called lipid rafts [33–35]. The DR ligands are mostly transmembrane proteins that must be matured by proteolytic cleavage [36]. Once released, they could bind their receptors although it has been shown that Fas ligand (FasL) and the membrane form of the TNF (mTNF which is especially bound to TNFR-2 [37]) can also act in a transmembrane form. Activation of TNFR and Fas [38] induces a wide range of functions such as cellular proliferation, cell survival and apoptotic cell death, cell differentiation, inflammation, and defense against microorganisms (viral, bacterial, fungal, and parasitic infections) [39]. Output of ligand binding is determined by cellular environment and adapter molecules availability.

According to the cells, mechanisms to induce apoptosis via DR pathway are different. In type-I cells, proapoptotic cascade is fast and unaffected by a Bcl-2 overexpression, while, in type-II cells, apoptotic cascade is slower, demonstrates an activation of procaspase-9, and is sensitive to Bcl-2 antiapoptotic activity [40]. In type-I cells, such as dermal fibroblasts, Fas activation by FasL leads to the recruitment of FADD (Fas-Associated DD), an adapter protein [41]. FADD shares homologous domains with its partners: DD with receptor and Death Effector Domain (DED) with apical caspases. FADD allows the recruitment and self-activation of procaspase-8, which can consequently cleave and activate procaspase-3. This pathway is tightly modulated by c-FLIP (cellular-flice inhibitory protein) [42, 43], a caspase homolog without any proteolytic activity. cFLIP can bind FADD or procaspase-8 and prevents its activation. Once more, IAP could inhibit caspase-3 activity.

Mechanisms associated to apoptosis of type-II cells seem, at least partially, common with the mitochondrial pathway. In these cells, caspase-8 activity is weak, and one of its targets is Bid, of which the truncated form, t-Bid, acts on mitochondria [44].

Mitochondrial and DR pathways could be regulated by the activation of the MAPK (Mitogen-Activated Protein Kinase) cascade [45]: p38 MAPK, ERK (Extracellular signal-Regulated Kinase) and JNK (Jun N-terminal Kinase) or by those of NFκB (Nuclear Factor-κB) [46]. MAPK activation could lead to survival or apoptosis, depending on the strength and duration of the signal. The antiapoptotic function of these kinases mostly results from transcription factor activation, leading to antiapoptotic proteins synthesis, notably from the Bcl family. NFκB exists on an inactive form linked to inhibitor IκB. Phosphorylation and subsequent degradation of this protein release a phosphorylated form of NFκB which translocates to the nucleus and activates not only the expression of c-FLIP [47], but also the expression of c-IAP1 and 2, XIAP, and other various antiapoptotic proteins.

Although less studied, other apoptotic pathways are documented. Cytolytic granules are specialized secretory lysosomes mainly composed of perforin and granzymes. Granules are secreted by killer cells, such as natural killer (NK) or cytotoxic T lymphocytes (CTLs) to control viral infection, intracellular pathogen dissemination, and tumorigenesis. After granule is released in immunological synapses, perforin opens cell membrane allowing entry of granzyme into the cell using a still unclear mechanism. Granzyme B cleaves and activates not only procaspase-3 but also Bid in its t-Bid form and ICAD to release DNAse CAD. Granzymes A and C do not activate procaspases but act on mitochondrial membrane to induce apoptosis. Granzyme A could also target SET complex in endoplasmic reticulum which in turn induces DNA damage [48, 49]. Endoplasmic reticulum (ER) cell responses are important to appropriately answer to unfolded proteins but, when unresolved, they could induce apoptosis [50, 51]. ER stress induces apoptosis through mitochondria or activation of procaspase-12 and subsequently procaspase-3 [52].

Our aim was not to give an exhaustive description of apoptosis, and we voluntary restrain it to major pathways. Very interesting reviews could be read on other forms of cell death, especially necrosis, oncosis [53], and necroptosis, a well-organized death [54].

3. Fibrosis and Apoptosis

Links between apoptosis and fibrosis emerged with force during the late 1990s. The level of the soluble form of Fas, sFas (a receptor antagonist for the proapoptotic protein Fas [55]), in sera from affected patients is higher than the one of normal volunteers in idiopathic pulmonary fibrosis [56] and silicosis [57]. This increased sFas serum level could play a role in preserving a subset of dysfunctional lymphocytes. Activated but unwanted lymphocytes which are normally eliminated by Fas activation could remain and induce fibrosis. They could also increase the fibroblastic cellularity and consequently the collagen deposition. The Fas/FasL system in T cells is also altered after silica exposure and leads to silicosis [58]. Whereas pulmonary fibrosis involves an increased resistance to apoptosis [59, 60], Fas is downregulated in fibrotic fibroblast membrane [56] and the proapoptotic Bid was shown to be required to induce fibrosis [61]. As in silicosis, the Fas/FasL system was thus thought to play a key role in several other pulmonary fibrotic pathologies [62, 63].

Similar results were observed in SSc. For instance, an increased level of sFas in SSc serum [64, 65] and a higher

resistance of pathological fibroblasts to Fas apoptosis [66–69] have been reported. Some studies with bleomycin-treated mice indicated that the Fas/FasL pathway is critical for the development of SSc pathology. Animals devoid of Fas or FasL genes [70] or treated with anti-FasL antibodies [71] show a decrease of apoptosis and a concomitant decrease of collagen accumulation. SSc affects the immune system of the patients since changes in Th1/Th2 response were detected resulting in an inadequate profibrotic activation [72–77]. Several authors attribute the antifibrotic effect of the Fas pathway deletion to a decreased selection of altered profibrotic lymphocytes subset. The selection of these abnormal lymphocytes would mainly result from a deficient apoptotic process [78, 79]. It could also be noted that Th cells have different sensitivity from Fas-induced apoptosis and that difference could be explained by cFLIP expression level. Th2 and Th17 are naturally more resistant to Fas-induced cell death than Th1 [80]. Fas activation could then lead to selection of a Th2/Th17 response to the detriment of Th1. This is the immunologic pattern generally expected in SSc. Overexpression of FLIP decreases sensitivity of all these cells [81] and could reverse autoimmune disease in animal model [82]. sFas levels could then be an attempt to patient to stop the Th1 to Th2/Th17 fibrotic switch.

SSc also affects the vascular network and a lot of evidences pointed out apoptosis as an effector. Sera from patients have been shown to induce apoptosis according to several mechanisms. Antiendothelial cells antibodies (AECAs), a heterogeneous group of antibodies directed against proteins and molecules specifically present on surface and inside of endothelial cells, induce apoptosis by stimulating Fas [83] or activating procaspase-3 [84]. Apoptosis could also be amplified when a patient is infected by CMV, one of the putative cause of SSc. This amplification was done via viral UL94 protein [85]. In SSc patients, sera could also induce endothelial apoptosis via secretion of IL-6 by monocytes (or fibroblasts) and E selectin expression [86]. In healthy people, a break in the vascular network results in a quick repair and the reconstitution of small vessels via angiogenesis. In SSc patients, this repair is blocked by the death of both, endothelial progenitor cells (EPCs) and circulating angiogenic cells (CACs). Death of EPC results from factors that are present in SSc patient sera such as AECA [87]. In EPC exposed to SSc patient sera, pAKT is reduced and could not inhibit the phosphorylation of FOXO3a and Bim, a proapoptotic Bcl-2 family member, is increased [88]. Furthermore, authors have demonstrated that CAC are killed by microparticules (MPs) released by apoptotic endothelial cells. These MP membranes are rich in arachidonic acid, which induces mitochondrial death of CAC [89, 90]. Tweak, known for its capacity to preserve and develop vascular network, is decreased in SSc patient sera with pulmonary affection [91], but not in sera of SSc patients with unaffected lungs [92]. This impairment of vascular endothelial repair results in hypoxia, which, in turn, could induce apoptosis in immune system cells and fibroblasts. Together with profibrotic IL4 cytokine, widely expressed in SSc, hypoxia also results in an increase in Lysyl-hydroxylase-2 and an alteration of the type-I collagen crosslinking [93]. Hypoxia also helps to stimulate ECM deposition, and, in a vicious loop, such tissue fibrosis induces more hypoxia [94]. Apoptosis of endothelial cells has other effects, notably on fibroblasts, discussed in the next paragraphs.

Some evidence indicates apoptotic epithelial cells could play a role in fibrosis, especially in lung of bleomycin-treated mice [95, 96] as well as in a new SSc mouse model [97] and human Idiopathic Pulmonary Fibrosis [98]. Epithelial cell death is clearly mediated through Fas/FasL pathway. Activated T cell and fibrotic fibroblasts could express FasL and induce apoptosis in lung epithelium [99]. During repetitive cycles of epithelial injury, epithelial cells release cytokines and growth factors, which promote fibroblast activation such as in wound healing process. These chronic phenomena lead to fibrosis.

Recent data indicate that SSc patient epidermis is abnormal and play a role due to its interaction with dermis [100, 101] like it was shown previously in skin wound healing [102]. SSc keratinocytes promote release of TGFβ, a fibrosing agent, from fibroblasts and secrete themselves CTGF, which stabilize the fibrotic phenotype of fibroblasts. Change in epidermis-dermis interaction in SSc is thought to result from chronic epidermis injury. The exact cause of this injury remains unclear but a limited apoptosis could not be excluded at this level, especially due to results obtained in lung fibrosis.

Fibroblasts, the major ECM secreting cells, also play a role in the evolution of SSc. Many studies focus on the regulation of fibroblast apoptosis in relation with fibrosis. It is thought that profibrotic cells are more resistant to apoptosis than others, maintaining their presence in situ despite immune system endeavour to remove them. This has been observed in various types of fibrosis such as hypertrophic scars [103, 104], pulmonary fibrosis [59, 60], and SSc [66–69]. Proteins from the mitochondrial pathway seem to be involved in the apoptotic resistance as well as death receptors proteins and several elements connecting both pathways. Some studies highlight proteins of mitochondrial pathway. Bax expression is decreased in SSc dermal fibroblasts [69] but Bcl-2 could be increased [105]. pAKT is increased in SSc fibroblasts [105, 106] and then could inhibit Bad proapoptotic function. The Fas pathway is also repressed in SSc with c-FLIPs and through c-IAP overexpression [67]. In other studies, protective potential of SSc fibroblast is explained by modulation of transcription of antiapoptotic proteins through kinase cascade. MAPK pathway, including ERK, is also activated by pFAK [107, 108]: the result of such activation could lead to the expression of antiapoptotic proteins. MIF [105], an ERK activator inducing Bcl-2 expression, and AKT, or PKCε [66], responsible for MAPK activation, are both reported to be involved in the modulation of apoptosis in SSc fibroblasts. Involvement of ROS in SSc is known for a long time [109]; they result mainly from vascular damage and inflammatory process. Besides protein and lipid oxidation, they induce DNA damage in fibroblasts [110] but seem to play little or no role in SSc-related vasculopathy. It is however well known that DNA damage could, in turn, induce apoptosis.

4. Selection of Profibrotic Fibroblast Populations: Role of Apoptosis

Several groups have postulated that lesional SSc fibroblasts could have been selected through unknown mechanisms from a subpopulation already present in situ, prior to the emergence of the first lesions.

Fibroblasts are heterogeneous in terms of their collagen secretion pattern [111] that they retain for several passages in vitro [112]. In SSc lesions, there is an increase in fibroblasts producing high levels of collagen and this phenotype is also retained in vitro [111–113]. A clonal selection of high-collagen-producing fibroblasts had been proposed as a mechanism for scleroderma-associated fibrosis onset [114]. It is postulated that the increase of the high collagen producing cells in SSc tissues could result either from a higher proliferative capacity or from a higher resistance to apoptosis of these cells. Some authors have shown that an exposure of fibroblasts to SSc sera increases the proportion of fibrotic fibroblasts [112], but others do not [115]. Lesional SSc fibroblasts do not grow at higher proliferation rates than normal ones in monolayers [67] or in three-dimensional tissue engineered cultures [13]. Recent evidences point out apoptosis resistance as the main mechanism from which profibrotic/apoptosis-resistant cell subpopulations emerge at the detriment of healthy cells leading to the development of fibrotic lesions. Fibroblasts from nonlesional area of late stage SSc patients exposed to FasL show an increased resistance to Fas-induced apoptosis and a decrease of their MMP secretion, which could result in higher ECM deposition. A similar mechanism was described for metastatic cancer cells [116].

5. Are Apoptosis Resistance and Profibrotic Potential of Fibroblasts Related to or Resulting from Independent but Concomitant Mechanisms?

During SSc development, fibroblasts are surrounded by numerous proapoptotic and profibrotic stimuli [76, 115, 117]. It could be interesting to relate both events. After vascular damage, thrombin is released in order to form a fibrin clot, but it also activates PAR-1, inducing the modulation of the fibroblast phenotype from quiescent to fibrotic. At the same time, thrombin promotes fibroblast apoptosis resistance through the effect of p21Cip1/WAF1. This protein induces PKCε activation that inhibits Fas/FasL signaling and reduces or slows down apoptosis [66]. Endothelial cell death leads to the local diffusion of several mediators including not only CTGF [118], a protein involved in the stimulation of fibrosis [119], but also some antiapoptotic factors that may promote the maintenance of lesional SSc fibroblasts on site [120]. Various types of cells died through apoptosis during SSc, probably due to patient sera composition, FasL exposition, or hypoxia. Apoptotic bodies released from such dead cells may contribute to the survival of fibroblasts that exhibit a profibrotic phenotype in response to macrophages TGFβ secretion [121]. It is interesting to note that Thrombospondin-1 (TSP-1) is increased in SSc and activates the release of TGFβ from latent complex [122]. TSP-1, released from apoptotic fibroblasts, is also responsible of the activation of apoptotic body phagocytosis by macrophages [123]. Some microRNAs recently discovered also seem to play a role in the potential interactions between apoptosis and fibrosis. Mir-29b decreases the expression of Mcl-1, a Bcl-2 family member [124], sensitizing cells to apoptosis. Mir-29a has been demonstrated to repress collagen synthesis, and it is significantly poorly detected in SSc fibroblasts [125]. Finally, Mir-29a/b transcription is modulated by NFκB [126], a protein with dual roles in apoptosis.

Nevertheless, the protein that seems to be the most relevant to link apoptosis and fibrosis is IL-6. IL-6 is strongly overexpressed in SSc [76, 86, 117, 127–131]. This cytokine is known to induce a relocalization of receptors outside lipid rafts (Figure 2). This change of compartmentalization increases TGFβ signaling and collagen synthesis [132]. Interestingly, in response to IL-6, fibrotic cells become more resistant, and normal fibroblasts become more sensitive to apoptosis [133]. It is not known if IL-6 can change Fas localization outside lipid rafts in fibrotic cells and inside the rafts in normal cells. However, several authors have demonstrated that translocation of Fas in lipid rafts increases cellular response to apoptosis [134–139].

6. New Therapeutic Targets

No treatment is currently available to help SSc patients who deal with a major loss of function and a poor quality of life. A deeper knowledge of the mechanisms underlying SSc is thus required to identify new therapeutic targets to establish a therapeutic strategy to control or cure SSc [140]. As the primary causes of the disease are unknown, it is necessary to target the secondary causes. Apoptosis is the heart of the SSc. In every stage, establishment and maintenance of the disease, this phenomenon plays a crucial role. So, it should be possible to modulate apoptosis to block the development of the disease and, perhaps, to go back towards a healthy status especially for early stage of the disease.

6.1. Targeting Immune System Cells Apoptosis. In SSc, Th balance is in favor of Th2–Th17 cells allowing secretion of profibrotic cytokines as TGFβ and IL6 rather than Th1 cells, TNFα secreting cells, which are less harmful. Sensitivity of Th cells to Fas-induced apoptosis favors clonal selection of Th2–Th17 cells, which express more cFLIP than Th1 cells. Overexpression of cFLIP in a transgenic mouse model leads to restoration of Th balance [82]. Nevertheless, cFLIP accumulation should be restrained to lymphocytes because cFLIP presence also promotes fibroblast resistance to Fas-induced apoptosis and selection of fibrotic dermis cells [67]. Gene therapy could achieve this goal but no safe and efficient gene transfer protocol is available for now.

It had been observed that SSc patient peripheral blood mononuclear and T cells secrete more sFas, the soluble form of Fas, to trap FasL, and thus to reduce apoptosis in tissue. It is believed that this strong sFas secretion could result in

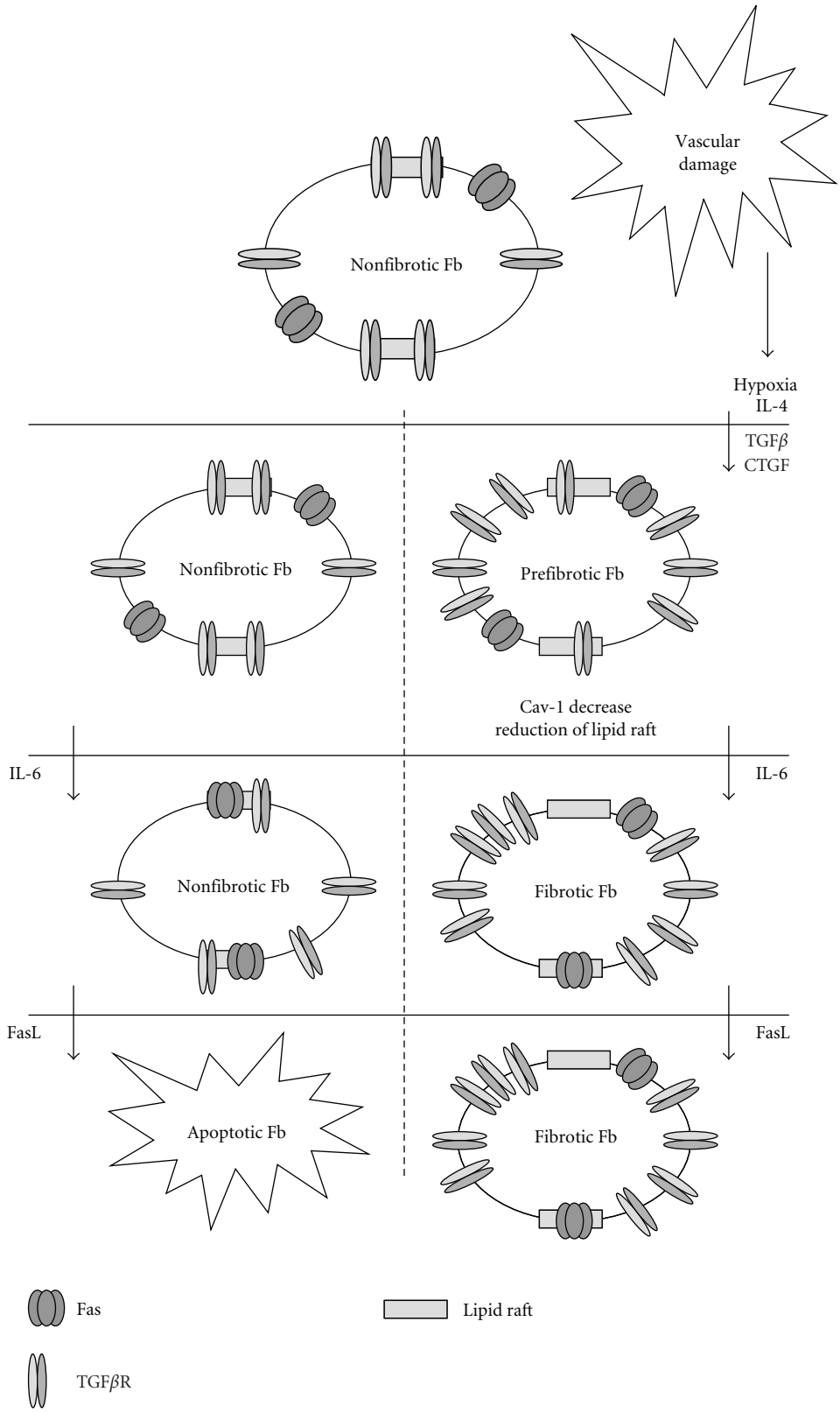

Figure 2: Hypothetic mechanism of FasL/IL6 selection of prefibrotic fibroblast in scleroderma lesions. After vascular damage, various factors are directly secreted by apoptotic endothelial cells or are secreted by macrophages, T cells, and fibroblasts in response to endothelial cell deaths and subsequent hypoxia. More proximal cells become prefibrotic by increasing their response to TGFβ. IL-6 secretion induces a more fibrotic phenotype for cells and increases apoptosis-resistant feature of profibrotic ones. FasL secreted by infiltrating cells kills nonfibrotic fibroblast with low or none effect on fibrotic cells. ECM is then deposited in excess and organ fails.

a selection of an unwanted subset of profibrotic-activated lymphocytes, but in the light of the results mentioned below, it also could be an unsuccessful attempt to restore Th balance. In this case, treatment with recombinant sFas could favor Th1 cells. Injection of blocking but not activating anti-Fas antibodies or anti-FasL could also be envisaged [71]. In the same vein, downregulation of Fas exposition at the surface of the cells [141] or a decrease of the Fas concentration in lipid rafts could also be tested [138, 142]. As it has been shown in transgenic mouse model where Fas or FasL genes were deleted, Fas/FasL pathway abolition could thus result in an abrogation of fibrosis development [70].

Finally, several studies link breast cancer and scleroderma [143]. Tamoxifen was known to restore Th1/Th2 balance [144] and could be a candidate to potentially block the disease evolution.

6.2. Targeting Endothelial Cells Apoptosis. Vasculopathy is an early event in the development of SSc, and a lot of patients received their SSc diagnostic when they consult for Raynaud-like symptoms. Then, endothelial cell apoptosis managing could be a valuable strategy. At this level, protecting endothelial cells or their precursors is a need. As previously described, SSc pathologic process targets not only endothelial cells from microvessels but also the mechanism to repair the vascular damage.

Endothelial cell apoptosis is induced by pathologic serum exposition especially AECA. Two strategies could be developed: to determine what are the antigens recognized by these antibodies and to inject recombinant peptides to block their action or to design antibodies raised against AECA to inactivate them. The initial vasculopathy blocking should then stop the disease at a very early stage.

In order to allow an efficient repair of microvessel, restoration of microcirculation, and reversion of fibrosis, it is also required to prevent EPC and CAC death. Arachidonic acid containing MP could be counteracted by Oltipraz and 1,2-dithiole-3-thione congeners [145]. VEGF and/or PDGF treatment could also been envisaged to maintain or restore microvasculature. This therapy should however be coupled with another one that can block endothelial cell apoptosis.

6.3. Targeting Epithelial Cells Apoptosis. Apoptosis modulation in the lung epithelial compartment could be achieved by reducing exposition of FasL to the surface membrane of activated fibroblasts or to decrease Fas at the surface of the lung epithelium. Epithelia are very accessible tissue to therapy due to their localization in direct contact with outside. Gene therapy assay to reduce Fas expression should thus be easily driven.

6.4. Targeting Fibroblasts Apoptosis. Because fibroblasts secrete ECM responsible of fibrosis, these cells should be targeted as soon as first fibrotic symptoms are obvious. The challenge of this step is the very heterogeneous molecular results at the origin of fibroblast resistance to apoptosis. The diversity of the mechanisms, and then of the antiapoptotic proteins involved, could result from the various disease aetiologies, the ethnic patient origins, or the model used. Nevertheless, a precise definition of the apoptotic mechanism involved for each patient is clearly needed.

Hyaluronan could be a valuable molecule to treat this pathology. Hyaluronan decreases collagen synthesis by reverting localization of TGFβ receptors in nonsignaling membrane domain [146]. It also induces translocation of Fas into lipid rafts and then sensitizes cells to apoptosis [136]. Similar effects on Fas recruitment in lipid rafts are obtained with edelfosine, an inhibitor of PI3K [33, 134, 135, 137].

In the same way, restoring expression of Caveolin-1 could be of a great help. Cav-1 expression is reduced in SSc [147], as well as in some breast cancers [148], and this decrease favors localization of TGFβ receptors outside lipid rafts and towards signalling domains. Besides, Fas is effective to induce apoptosis when presents in lipid rafts. So Cav-1 underexpression could explain, at least partially, the resistance of fibrotic fibroblasts to Fas-induced apoptosis. Restoration of Cav-1 expression level could thus help to reverse fibrosis.

Curcumin could also provide a promising strategy for the treatment of SSc. Curcumin has been shown to induce apoptosis in fibrotic cells only [149]. This molecule is widely used in traditional south-east Asian cooking and for cancer treatment [150–154]. It is a safe drug but its optimal effect is obtained when administered in combination with other drugs. Another natural product, Resveratrol, has proapoptotic properties which could be used to sensitize fibrotic cells to apoptosis [155, 156]. This molecule inhibits Mcl-1 and Bcl-XL which are both known to play a role in apoptotic resistance of fibroblasts in SSc. Resveratrol also counteracts AKT and MAPK pathways. Regulation of oxidative stress has been extensively previously described and may help to define antioxidant therapy to restore normal function of organs including skin [157].

In order to increase Fas sensitivity of fibrotic cells, thalidomide could be used. Thalidomide has been shown to increase Fas number [158]. Presence of infiltrating T cells producing FasL in SSc tissues could thus result in the elimination of fibrotic cells. Thalidomide is currently used for the treatment of autoimmune diseases [159] inducing apoptosis through an unknown mitochondrial pathway [160]. Restored levels of mir-29a/b could also be of a great help to control scleroderma but further studies based on microRNA seem to be necessary. Sexual hormones such as oestradiol and testosterone could also modulate the expression and the sensitivity of cells to the FasL/Fas pathway [161]. As the majority of SSc patients are women who have reached the end of their active reproductive period, the potential adverse effects of hormonal treatment could be minimized, with a rigorous clinical monitoring. Control of cFLIP and cIAP expression using siRNA or antisense oligonucleotide could also be of interest [67]. Anticancer therapy targets these proteins [162]. In the case of fibroblasts, cFLIP expression level need to be decreased in fibrotic cells or increased in their nonlesional counterparts. cIAP is a more complex protein to modulate due to its redundant isoforms but could not be eluded due to its numerous biological functions and its potential role in SSc.

7. Concluding Remarks

Apoptosis plays a key role in emergence and maintenance of SSc. Apoptosis is involved in immune system response by changing the subset of infiltrating cells, in vascular damage and its consequences, and finally in selecting fibroblasts with profibrotic phenotype, responsible of loss of function of organs and fatal outcome. Depending on the patient, molecular mechanisms could be different and involve different proteins. Nevertheless FasL/Fas seems to play a key role. A deeper knowledge of how apoptosis modulates fibrosis could allow the development of new therapies adapted to the apoptotic profile of patients and, thus, to cure the disease with the least adverse side effects.

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Review Article

B-Cell Depletion Therapy in Systemic Sclerosis: Experimental Rationale and Update on Clinical Evidence

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Systemic sclerosis (SSc) is a systemic rheumatic disease with poor prognosis since therapeutic options are limited. Recent evidence from animal models suggests that B-cells may be actively involved in the fibrotic process. B-cells from tight skin mice, an animal model of scleroderma, display a "hyperresponsive" phenotype; treatment with rituximab (RTX) significantly attenuates skin fibrosis in this animal model. In humans, B-cell infiltration is a prominent finding in most lung biopsies obtained from patients with SSc-associated interstitial lung disease. Several open label studies have assessed the clinical efficacy of RTX in SSc. In most patients skin fibrosis improved; lung function either improved or remained stable. Definite conclusions regarding the clinical efficacy of RTX in SSc cannot be drawn but further exploration with a multicenter, randomized study is warranted.

1. Introduction

Systemic sclerosis (SSc) is a systemic rheumatic disease characterized by vasculopathy, autoimmunity, and fibrosis. The available therapeutic options are extremely limited and prognosis is variable. Cyclophosphamide (CYC) has shown modest efficacy in the treatment of SSc-associated interstitial lung disease (ILD) [1] but its long-term use is accompanied by significant toxicity. Therefore, novel therapeutic approaches are desperately needed. During the last decade, B-cell depletion by rituximab (RTX), a monoclonal antibody that targets B-cells, has emerged as a promising therapy for a wide range of systemic autoimmune diseases. It has been approved for the treatment of rheumatoid arthritis but it has also been tried in systemic lupus erythematosus [2], systemic vasculitides [3], and multiple sclerosis [4], among others. An expanding body of experimental evidence suggests that B-cells play a role in the fibrotic process, raising the question of whether B-cell depletion might be a potential therapeutic approach in SSc [5–8]. During the last 2 years, 4 small-scale, open-label studies and a few case reports have addressed this question to some extent, reporting encouraging results. In this paper we provide the experimental evidence supporting the active role of B-cells in fibrosis and summarize all the available clinical evidence regarding the use of RTX in patients with SSc.

2. Methods

We performed a literature search in PubMed from 1995 and onwards. We used the following key words: systemic sclerosis, scleroderma, rituximab, B-cells, fibrosis, ILD, and therapy in various combinations.

3. Results

3.1. The Role of B Cells in Fibrosis: Experimental Evidence

3.1.1. Animal Models. Research in SSc has been problematic due to the low prevalence of the disease and the lack of an animal model that possesses all features of the human disease. Nevertheless, the tight skin mouse (TSK) and the

bleomycin (BLM) induced mouse model of SSc have been extensively used as animal models of the disease. TSK mice are characterized by extensive skin fibrosis and immunological abnormalities including the presence of autoantibodies to topoisomerase-1, both reminiscent of those observed in the human disease [9]. A lot of research has been done on the potential role of B-cells in this animal model. It has been reported that TSK B-cells exhibit enhanced CD19 signaling compared to WT B-cells, although the expression of this molecule was similar in TSK and WT B-cells [10]. CD19 is a membrane glycoprotein of the immunoglobulin superfamily and part of the hetero-oligomeric complex comprising the complement receptor type 2, which positively regulates BCR activation. The authors reported enhanced CD19 tyrosine phosphorylation by 45% compared to WT B-cells. Tyrosine phosphorylation of Vav and Lyn kinase, both of which are important downstream steps of CD19 signaling, was also found to be enhanced by 3.4-fold and 22% respectively, compared to WT B-cells. Cytoplasmic Ca^{+2} responses, generated by CD19 ligation, were also significantly augmented in TSK B-cells compared to WT B-cells. The crucial role of CD19 signaling in this animal model is underscored by the fact that CD19 deficiency normalizes the "hyper responsive" phenotype of TSK B-cells and leads to a significant improvement of skin fibrosis compared to control TSK mice. The above data suggest that B-cell hyperactivity and fibrosis are somehow linked in this animal model and raise the question of whether targeting B-cells might be an effective way of attenuating fibrosis.

In another study by Asano et al., the effect of enforced CD19 overexpression on B-cells in the TSK mouse model was assessed [11]. CD19 transgenic TSK mice exhibited increased autoAb production compared to control TSK mice. More specifically, CD19 transgenic mice that expressed 20% higher levels of CD19 (CD19 $TG4^{+/-}$) and 200% higher levels (CD19 $TG1^{+/+}$) exhibited increased antitopoisomerase-1 levels by 7.9-fold and 20-fold, compared to control TSK mice, respectively. Despite this increase in autoAb production, CD19 overexpression did not lead to worsening of skin fibrosis compared to control TSK mice. The authors also focused on gaining further insight into the hyperactive phenotype of the TSK B-cell and found that this can relate to a defective CD22 signaling, an important negative BCR response regulator. Upon stimulation, CD22 tyrosine phosphorylation was 58% lower in TSK B-cells compared to WT B-cells. The hypophosphorylation of CD22 coincided with the hyperphosphorylation of CD19, which led the investigators to suggest that defective CD22 signaling leads to increased CD19 signaling which is, at least partially, responsible for the hyperresponsive phenotype of the TSK B-cell.

Recently it was reported that TSK mice have autoantibodies agaist CD22 which were found to be functional and able to attenuate CD22 activation [12]. Since CD22 inhibits B-cell activation, these autoAbs may contribute to the "overactivated" phenotype of TSK B-cells. In other words, TSK B-cells produce an autoAb that enhances their own activation, something that may have pathogenic implications.

Once the link between B-cell hyperactivity and fibrosis had been established in the TSK mouse, the next logical step would be to assess the effect of B-cell depletion in this animal model. RTX administration to newborn TSK mice led to a significant attenuation of skin fibrosis by 43% compared to control TSK mice accompanied by a significant reduction in autoantibody production [13]. When the same treatment was applied in 56-day old TSK mice, which are characterized by established skin fibrosis, skin thickening was not reduced, compared to TSK control mice. These data suggest that B-cells may be more important in the early phase of the disease but less so in established disease. It is noteworthy that B-cells are not present in the skin of TSK mice, indicating that their contribution to skin fibrosis may be indirect. Despite the improvement of skin thickening, B-cell depletion had no effect on the lung disease of TSK mice, indicating that different manifestations are probably mediated by different pathogenic mechanisms in this animal model. Another study explored the effects of B-cell survival factor (BAFF) inhibition in TSK mice and reported improvement of skin fibrosis and attenuation of autoAb production [14]. All these data underline the key role of B-cells in the fibrotic process in this animal model.

The role of B-cells in fibrosis has also been explored in the BLM-induced mouse model of SSc. Besides skin involvement, lung fibrosis is a prominent feature of this animal model, making it suitable for the assessment of a given treatment on ILD. The investigators reported that BLM administration to CD19 knockout mice led to diminished skin thickening compared to BLM-treated WT littermates [15]. More importantly, CD19 deficiency led to attenuation of BLM-induced lung fibrosis, indicating that B-cells may be actively involved in the fibrotic process not only in the skin but in the lung as well.

Recently, the role of CD19 during the development of pulmonary fibrosis in the BLM-induced model has been extensively assessed [16]. Mice either lacking or overexpressing CD19 were treated with intratracheal injections of BLM. It was reported that CD19 knockout mice exhibited less lung fibrosis in sharp contrast to mice overexpressing CD19 which showed augmented lung fibrosis compared to WT littermates. Interestingly, CD19 expression correlated with the number of B-cells in the bronchoalveolar lavage fluid; CD19 deficiency inhibited the accumulation of B-cells in the alveolar compartment following BLM challenge. The above data indicate that CD19 plays a crucial role in pulmonary fibrosis in this mouse model.

Research on animal models of scleroderma has provided evidence indicating a potential link between B-cell hyperactivity and fibrosis. However, we should note that SSc is a far more complex disease and therefore these data cannot be directly extrapolated to humans.

3.1.2. Humans. Only a few studies have addressed the potential contribution of B-cells in the pathogenesis of SSc. In one such study, it was reported that B-cells from patients with SSc had 20% higher CD19 expression compared to B-cells from healthy subjects [17]. Detailed phenotypic characterization of B-cells from patients with SSc revealed that peripheral blood B-cells were increased in patients with SSc compared to healthy subjects [18]. Naive B-cells

were reported to be increased in patients with SSc whereas memory B-cells and plasmablasts were reduced, compared to healthy subjects. Memory B-cells from patients with SSc had increased expression of several activation markers, including CD95 and were prone to spontaneous apoptosis. These data indicate that B-cell homeostasis in SSc is disturbed.

Since patients with SSc express higher levels of CD19 on B-cells and since CD19 is a key molecule in the regulation of signaling thresholds in these cells, something that may relate to break of tolerance and induction of autoimmunity, investigators explored the mechanisms involved in CD19 overexpression in SSc B-cells. Tsuchiya et al. explored the potential association of CD19 polymorphisms with SSc and the level of CD19 expression on B-cells [19]. They reported a significant association between the −499T allele in the promoter region of the CD19 gene with SSc, with an odds ratio of 2.18; carriers of this allele exhibited significantly higher CD19 levels on B-cells compared to noncarriers. These data raise the question of whether CD19 upregulation in SSc B-cells is genetically determined.

It is not clear why SSc B-cells exhibit such an "overactivated" phenotype; B-cell survival factors may be implicated. It has been reported that BAFF serum levels are higher in patients with SSc compared to healthy controls ($P < 0.001$) [20]. Furthermore, patients with the diffuse form had significantly increased levels compared to patients with the limited form of the disease. Nevertheless, this was not a disease-specific finding, since patients with SLE or DM had similarly increased levels. Patients with SSc, exhibiting increased serum BAFF levels tended to have more severe skin fibrosis as assessed by the MRSS tool ($P < 0.01$), worse FVC values ($P < 0.05$) and higher ESR ($P < 0.05$). Furthermore, decreasing serum BAFF levels were associated with attenuation of skin fibrosis, whereas increasing levels correlated with clinical worsening. The expression of BAFF in the skin at the mRNA level was found to be increased in early disease. Moreover, upregulation of the BAFF receptor was reported on B-cells from patients with SSc compared to healthy subjects. It is also worth mentioning that B-cells from patients with SSc stimulated with BAFF produced 38% more IL-6, a cytokine able to stimulate fibroblasts, compared to B-cell from healthy subjects.

In a recent study it was found that peripheral blood mononuclear cells (PBMCs) from SSc patients produced significantly more APRIL (a proliferation inducing ligand), a B-cell survival factor, compared to PBMCs from healthy subjects ($P < 0.01$) [21]. This increase was associated with more severe disease manifestations. These data indicate that upregulation of B-cell survival factors may contribute to B-cell hyperactivity and autoimmunity in SSc. BAFF and APRIL emerge as two interesting therapeutic targets; inhibition of these molecules may modulate B-cell function in SSc and potentially lead to clinical benefit.

An interesting study, performed by Whitfield et al. examined gene expression profile, using microarrays in scleroderma skin compared to normal [22]. The authors found that genes characteristic of B-cells, fibroblasts and endothelial cells were differentially expressed in scleroderma compared to normal skin. Interestingly, the same expression pattern was evident in patients with SSc in both clinically involved as well as uninvolved skin, underscoring the systemic nature of the disease. Since endothelial cells and fibroblasts are considered key players in SSc, these data point towards a potentially active role of B-cells in skin fibrosis.

The potential role of B-cells in SSc-associated ILD has been inadequately investigated. It has been reported that B-cells are present in lung biopsies from patients with SSc-associated ILD [23] and that plasma cell infiltration of the alveolar walls is an early finding [24].

The above studies have documented the presence of B-cells in both skin and lung of patients with SSc. However direct evidence of a pathogenetic role is lacking.

3.2. B-Cell Depletion in SSc: Update on Clinical Evidence. There are 4 small-scale, open-label clinical studies exploring the potential clinical efficacy of RTX in SSc, including one from our research group and a few case reports. In the first study by Smith et al., eight patients with early (defined by disease duration of <4 years from the first non-Raynaud's disease manifestation) diffuse SSc received a single course of RTX (consisting of 2 infusions, 1000 mg each, at day 1 and 15) [25]. Patients were evaluated clinically at 24 weeks and subjected to skin biopsies at baseline and 12 weeks. Five patients had evidence of mild ILD; patients with severe ILD were excluded from the study. Improvement of skin thickening was reported as assessed by the MRSS tool (mean ± SD: 24.8 ± 3.4 versus 14.3 ± 3.5 at baseline versus 24 weeks resp., $P < 0.001$). It is worth mentioning that improvement of skin thickening was verified at the histological level, since both collagen and myofibroblast score were reduced significantly following treatment ($P = 0.014$ and $P = 0.013$, resp.). Half of the patients had evidence of B-cell infiltration in their skin; treatment effectively depleted these cells. Lung function tests, systolic pulmonary artery pressure, left ventricular ejection fraction, creatinine clearance, and HAQ score remained stable throughout the study. Two serious events were reported (one patient underwent coronary artery bypass surgery and another was hospitalized due to low-grade fever that spontaneously subsided) but were considered to be probably unrelated to study drug. This is the first study that provides clinical as well as histological evidence that RTX treatment may favourably affect skin fibrosis in SSc. It should be noted however, that this study has potential limitations which are the relatively small number of patients recruited and the lack of a control arm. Resolution of skin fibrosis is associated with the natural course of the disease, therefore uncontrolled studies are difficult to interpret. Nevertheless, the improvement of skin fibrosis reported in this study was quite significant, which makes it rather unlikely to have occurred spontaneously, especially within the limited time frame of the study.

Another study reported the effects of a single course of RTX treatment in 15 patients with early (as defined by disease duration of <18 months from the first non-Raynaud's disease manifestation) diffuse SSc [26]. Similar to the study by Smith et al., only 7 out of 15 patients had evidence of mild ILD, since the existence of moderate or severe ILD was an exclusion criterion. Patients were

clinically evaluated at 24 weeks and 1 year. In contrast to the study by Smith et al., no improvement of skin thickening as assessed by the MRSS, tool was reported (mean ± SD: 20.6 ± 4.4, 20.2 ± 5.5 and 21.1 ± 5.2 at baseline, 24 wks, and 48 wks, respectively, P = ns). However, histologic improvement was found; myofibroblast score declined from 49.5 to 36.6 ($P < 0.05$). Skin infiltrating B-cells were significantly increased in patients with SSc compared to healthy controls who had no B-cells and were eliminated posttreatment. Pulmonary function tests remained stable at 24 wks compared to baseline; it is noteworthy though that FVC values increased by an average of 3.5% at 24 wks compared to pretreatment values, but the 95% CI was wide and thus results were not statistically significant. The authors do not report PFTs at 1 year posttreatment. No evidence of new or progressive major organ involvement was reported. Treatment was well tolerated.

Our research group has performed an open label, randomized controlled, 1-year pilot study, assessing the effect of RTX in SSc [27]. We recruited 14 patients with SSc randomized as follows: 8 patients in the treatment arm and 6 patients in the control arm of the study. All patients had diffuse disease, were anti-Scl70 positive, and had evidence of ILD. There were no differences in terms of disease duration, baseline MRSS and baseline PFT's between the treatment and the control group. Patients in the treatment arm received 2 cycles of RTX at baseline and 24 weeks (each cycle consisting of 4 weekly RTX infusions (375 mg/m^2)). We found a significant improvement of both FVC (mean ± SD: 68.13 ± 19.69 versus 75.63 ± 19.73% of predicted values, at baseline versus 1 year resp., $P = 0.0018$) and DLco (mean ± SD: 52.25 ± 20.71 versus 62 ± 23.21% of predicted values, at baseline versus 1 year, resp., $P = 0.017$) in the treatment group whereas no change was noticed in the control group. The median (upper and lower quartile values) percentage of improvement of FVC in the RTX group was 10.25% (6.19–18.65) whereas in the control group FVC deteriorated (median percentage of deterioration (upper and lower quartile values) 5.04% (4.11–11.6)). Direct comparison of FVC changes recorded at 1 year, revealed that the RTX-treated group improved significantly ($P = 0.002$) compared to the standard-treatment (control) group. The median (upper and lower quartile values) percentage of improvement of DLco in the RTX group was 19.46% (3.7–30.8) whereas in the control group the median percentage of deterioration was 7.5% (1.4–26.57) ($P = 0.023$).

Skin thickening, assessed with the MRSS, was similar in the two treatment groups at baseline ($P = 0.50$). However at the 1 year evaluation, there was a significant decrease of MRSS in the RTX group compared to the baseline score (mean ± SD, 13.5 ± 6.84 versus 8.37 ± 6.45 at baseline versus 1 year, resp., $P = 0.0003$). On the contrary, no significant change in skin scores was noticed in the control group at 1 year when compared to the baseline MRSS (mean ± SD, 11.50 ± 2.16 versus 9.66 ± 3.38 at baseline versus 1-year, resp., $P = 0.16$). The median (upper and lower quartile values) percentage of improvement in the RTX-treated group was 39.25% (27.33–64.95) compared to 20.80% (10.78–39.28) in the control group. Statistical analysis revealed that differences tended to be but were not statistically significant ($P = 0.06$). Improvement of skin fibrosis was also documented at the histological level. We found a significant reduction of collagen deposition in the papillary dermis at 24 wk compared to baseline in patients treated with RTX; histologic improvement correlated with skin B-cell depletion. Histological data matched the clinical data, since all patients showing histologic improvement also improved clinically. One serious adverse event was reported (respiratory tract infection); the patient recovered fully following short-term hospitalization.

Even though this is the first and only so far, randomized, controlled study assessing the efficacy of RTX in SSc, several limitations exist. Firstly, the small number of patients recruited does not provide the study with sufficient statistical power to prove efficacy. Additionally, most patients had long-standing disease since no disease duration restriction was applied and was heterogeneous in terms of disease duration, severity, and previous treatments.

Recently, one more study assessing the clinical efficacy of RTX in SSc was published [28]. Nine patients with SSc were recruited and received one course of RTX (consisting of 2 infusions, 1 gr each). All patients had severe cutaneous involvement and had experienced worsening of skin score despite treatment with CYC. A significant reduction of skin thickening was reported with patients showing a median decrease of skin score of 43.3% at 6 months compared to baseline. Disease activity and severity index also declined. PFTs remained stable throughout the study. A significant decline in IL-6 levels following treatment was also reported; the authors hypothesized that this may have contributed to attenuation of skin fibrosis.

Three case reports have also appeared in the literature regarding the use of RTX in SSc. The first case describing the beneficial effect of RTX on SSc-associated ILD was reported by McGonagle et al. [29]. PFTs, musculoskeletal manifestations and functional status improved following treatment. However treatment effect waned over time and a second course of RTX was administered. PFTs improved again; DLco increased from 34.3% to 48% of predicted values. These data are in agreement with ours, showing that consecutive treatment courses may be needed to augment and sustain the effect of RTX on pulmonary function.

The beneficial effect of RTX on SSc-associated ILD has also been documented in a case report by our group [30]. Our patient was treated with 4 consecutive RTX courses every 6 months and completed a followup of 2 years. PFTs significantly increased; FVC and DLco reached values of 35%, and 33% respectively, compared to 30% and 14% of pretreatment values. Quantification of ground glass lesions using a computer-aided diagnosis system showed a 14% reduction. Skin thickening improved as indicated by a decline in MRSS from 20 to 9. Clinical improvement coincided with histologic improvement with reduction of collagen accumulation and myofibroblast score; skin infiltrating B-cells were eliminated post treatment. The functional status of the patient improved as indicated by a decrease in HAQ score and an increase in 6-minute walking distance. This was the first report of long-term RTX treatment in SSc. Recently, another case of

Table 1: Studies assessing the efficacy of RTX in SSc.

Study	Number of participants	Study type	Evaluation time point(months)	Number of RTX courses	Outcomes				
					Skin			Lung function tests	Functional status
					Clinical assessment	histologic improvement (yes/no)	Skin B-cell depletion		
Smith et al.	8	Open label, uncontrolled	6	1	Improved	Yes	In 4 patients	Stable	Stable
Lafyatis et al.	15	Open label, uncontrolled	6/12	1	Stable	Yes	Yes	Stable	Stable
Daoussis et al.	8	Open label, randomized controlled	12	2	Improved	Yes	Yes	Improved	Improved
Boselo et al.	9	Open label, uncontrolled	6–36	1 (3 patients receive a second cycle)	Improved	Not reported	No	Stable	Improved
McGonagle et al.	1	Case report	—	2	Not reported	Not reported	—	Improved	Improved
Daoussis et al.	1	Case report	24	4	Improved	Improved	Yes	Improved	Improved
Yu	1	Case report	—	1	Not reported	Not reported	—	Improved	Improved

successful treatment of CYC-resistant SSc-associated ILD was reported [31]. Finally, RTX seems to favourably affect ILD in the context of other systemic rheumatic diseases such as the antisynthetase syndrome as indicated by several reports [32, 33]. All the available published data regarding the clinical efficacy of RTX in SSc are summarized in Table 1.

4. Discussion

SSc is perhaps the most challenging disease for rheumatologists. So far treatment is based on nonspecific immunosuppression, with agents such as CYC [1] or mycophenolate mofetil [34], with modest results. RTX has been used with varying degrees of success in most systemic autoimmune diseases and this is why, one may argue that its effect on a severe, incurable systemic autoimmune disease such as SSc, is worthwhile exploring. Furthermore, there is a strong rationale for the use of RTX in SSc; an expanding body of evidence from basic research points to the direction that B-cells may be active players in the fibrotic process. On clinical grounds, until now, 43 patients with diffuse SSc have been treated with RTX (available published data). What conclusions can be made regarding the use of RTX in SSc? First of all, this agent seems to be well tolerated in SSc since only few adverse events have been reported. But is RTX clinically effective in SSc? Based on the available clinical evidence, definite conclusions cannot be drawn; however results are encouraging. In 3 out of 4 studies skin thickening significantly improved and, even in the single study where no clinical benefit on skin thickening was found, histologic improvement was documented. These data may suggest a disease modifying role of RTX in skin fibrosis.

The most fearful manifestation of SSc is lung disease which is nowadays the leading cause of mortality. The clinical evidence on the efficacy of RTX in SSc-associated ILD is limited, since most patients recruited either did not have ILD or had only very mild ILD. With the exception of our study, the other 3 studies were not designed to test the potential clinical efficacy of RTX in SSc-associated ILD. Nevertheless, in these three studies PFTs remained stable, even though most patients had early disease and therefore were most likely to exhibit declining PFTs during their followup. In our study, a significant improvement of lung function was reported in contrast to other studies where PFTs remained stable at 6 months compared to baseline. We should note, however, several differences in the design of the studies that could potentially explain these diverse findings. In the other studies most patients did not have ILD in contrast to our study where the presence of ILD was an inclusion criterion. Furthermore, the higher dose of RTX used ($4 \times 375\ \text{mg/m}^2$ instead of $2 \times 1000\ \text{mg}$), consecutive treatments, and the longer evaluation period could also be potential explanations. The significant improvement in lung function tests observed in our study indicates that RTX may favourably affect SSc-associated ILD. Long-term treatment with RTX may either improve or stabilize lung function over time in patients with SSc. All 8 patients recruited in the treatment arm group of our study remained on RTX treatment and received two additional courses; their PFTs continued to improve during the second year of followup [35].

If RTX turns out to be an effective treatment for SSc, which could be the potential mechanisms of action? Several possibilities exist. First, B-cells appear to be actively involved in the fibrotic process, as indicated by data derived from both animal models and humans. Elimination of skin infiltrating B-cells by RTX has been documented, albeit not consistently. Taking into account that B-cell infiltration is a prominent finding in lung biopsies from patients with SSc-associated ILD, depletion of this population may be a potential explanation for the clinical improvement. It would therefore be of great interest to study the effect of RTX on lung disease at a histological level, but this may be inherently difficult and challenging. We have also recently shown that RTX-induced improvement of skin fibrosis associates with a decrease in PDGFR phosphorylation (which corresponds to activation) in scleroderma skin [36]. Finally, another mechanism may involve indirect effect(s) of RTX on other cells, such as T cells [37].

Both experimental and clinical evidence regarding B-cell depletion in SSc is encouraging and certainly points to the direction that further exploration of its clinical efficacy is warranted. The best way forward would be a multicenter, randomized, double blind, placebo-controlled study. If such a study is performed we propose that it should focus on ILD rather than skin disease and that it should be designed in such a way, that patients are treated for at least one year and evaluated thereafter; in this way, a treatment effect on ILD (if any) would be more easily depicted. To our knowledge, up until now, no such study has been registered. Interestingly, however, a phase II study assessing the clinical efficacy of RTX in SSc-associated pulmonary arterial hypertension has already been launched (http://clinicaltrials.gov/ identifier NCT01086540). Moreover, a phase I study of MEDI-551 (mAb against CD19) has also been launced (http://clinicaltrials.gov/ identifier NCT00946699). We believe that a large-scale multicenter, randomized study assessing the potential clinical efficacy of RTX in SSc is highly needed.

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