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Progress in Understanding the Pathogenesis of Systemic Sclerosis with Lung Involvement: The Contribution of Proteomic Studies

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Abstract

Systemic sclerosis (SSc) is a chronic inflammatory disease involving the skin and various internal organs. SSc is characterized by microvascular dysfunction, activation of the immune system and tissue fibrosis. Endothelial cell damage seems to be the initiating factor, but the precise triggering events that underlie the development of the disease remain unclear.

Lung involvement is a frequent complication, which causes increased morbidity and mortality in patients with SSc. In particular, pulmonary arterial hypertension and interstitial lung disease are the two major clinical diseases that affect SSc patients, but the current treatments appear to have no satisfying effects on these pulmonary complications, until yet.

Recently researches using innovative technologies have highlighted several peptides that might contribute to better understand the underlying pathogenic mechanisms of pulmonary injury and could promote more effective therapeutic strategies for SSc patients. Here, we focus on the major proteomic studies on biological fluid from patients with SSc.

Keywords: Proteomics; Pulmonary arterial hypertension; Interstitial lung disease; Systemic sclerosis

Introduction

Systemic sclerosis (SSc) is an autoimmune disease that affects skin and internal organs.

The incidence of SSc is approximately 20 per million and the cause of it remains poorly understood [1].

Current hypotheses in SSc pathogenesis suggest that several exogenous agents activate an abnormal cellular and humoral immunity, in genetically predisposed subjects [2]. In this way, products of the immune activation cause vascular damage possibly through the production of autoantibodies and inflammatory peptides that induce vascular permeability, up-regulation of adhesion molecules and endothelial cell (EC) apoptosis. These vascular dysfunctions enhance the infiltration of mononuclear cells in affected organs and cause tissue fibrosis.

SSc is clinically diverse in terms of skin and organ involvement. Normally, it is distinct in two major clinical subtypes: the diffuse form, that typically leads to a rapid sclerosis of the whole skin and it is associated with important body organs fibrosis, including lungs, heart, digestive tract and kidneys; the limited form, that is characterized by sclerosis of the distal parts of the skin and it is associated with a much slower progression of visceral fibrosis.

In general, two major pulmonary syndromes are associated with SSc: the pulmonary arterial hypertension (PAH) and the interstitial lung disease (ILD) [3,4].

Both PAH and ILD have a great impact on morbidity and mortality of SSc patients, despite the rate progression is variable through SSc patients population [5,6]. As current therapy appears to have modest effects on these conditions, it is necessary better understanding their underlying pathogenic mechanisms.

The basic science studies, integrated with clinical evaluation of SSc patients, are direct to identify either new biological disease marker(s) suitable for clinical practice or new signaling pathways involved in the pathogenesis of SSc disease [5,7].

Although progresses have been made in the pathogenesis of lung involvement in SSc patients, much still remains to be investigated. The recent development of innovative technologies for massive protein analysis allows the researchers to shed light on SSc disease. In SSc patients with lung involvement, proteomic studies mainly focus on two biological liquid interfaces: the bronchoalveolar lavage fluid (BALF) and the serum.

In the present review, we discuss the more significant proteomic studies on BALF and serum in SSc patients with lung disease.

Proteomic High-Throughput Approaches in BALF Analysis from SSc Patients with Pulmonary Involvement

BALF is a suitable way to evaluate lower respiratory tract abnormalities in clinical setting and to sample the biological components such as proteins of the epithelial fluid [8].

Recently, Rottoli et al. [9,10] have conducted a 2-D analysis of BALF from SSc patients with ILD. The Authors have reported quantitative rather than qualitative differences in protein profile in the comparison between SSc patients with lung involvement and patients with other ILD forms, such as idiopathic pulmonary fibrosis or sarcoidosis [9].

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The differently expressed proteins belong to the classes of proteases, cytokines and antioxidants (Table 1). In addition, increased protein carbonyl content, as measurement of protein oxidation, was observed in SSc patients with lung involvement compared to patients with other ILD forms, suggesting a redox imbalance between oxidant generation and antioxidant mechanisms in SSc patients with lung involvement [10,11]. Higher values of complement C3b, the cleavage product of C3, were also found in BALF from SSc patients with lung damage [10]. Despite the etiopathogenic role of complement in the development of interstitial lung disease is still under investigation [12-15], a genetic study on complement system in SSc reported a strong association between SSc and the null alleles at the C4A locus in the major histocompatibility complex (MHC) [16]. In particular, the aplotype comprising C4AQ0, DR3/DR52a and DQA2 had been associated with pulmonary fibrosis in SSc patients [17].

Fietta et al. [18] compared BALF from SSc patients with and without lung disease by 2-D with MALDI-TOF and HPLC MS analysis. Among these proteins, a fragment of mtDNAtopo 1 was found only in BALF from SSc patients with ILD, while glutathione S-transferase P (GST) and cystatin SN were detectable only in SSc patients without ILD (Table 1). In addition, increased levels of calgranulin B (S100A9), a molecule promoting extracellular matrix deposition and lung fibrosis, were found in BALF from SSc patients with ILD compared to SSc patients without lung involvement, similarly to what observed in BALF from patients with idiopathic pulmonary fibrosis.

In agreement with this study, Shirahama et al. [19] identified two groups of proteins which expression was either increased as α2 magroglobulin, α1 antitripsin and pulmonary surfactant protein A (SPA) or decreased as heat shock proteins (HSPs) and GST. Disregulation of these proteins levels might participate in the pathogenesis of lung fibrosis in SSc patients. In fact, abnormal levels of α2 magroglobulin, α1 antitripsin have been related to development of alveolitis in SSc patients [20], while SPA seems to play an important role in immunological surveillance [21]. In BALF from SSc patients, the reduction in HSPs and GST levels might indicate impaired protection in SSc lungs since HSPs are molecular chaperones involved in cell protection from injury and GST is an anti-oxidant protein [22].

A proteomic analysis using Reverse Phase-High Performance Liquid Chromatography-Electrospray-Mass Spectrometry in BALF of 46 scleroderma lungs and 15 controls, allowed us to reveal the abnormal presence of Thymosin β4, β4 sulfoxide and β10 in several SSc patients (Table 1). Thymosin β4 levels were significantly higher in SSc patients than in controls, but patients experiencing a worsening in the alveolar score had relatively lower BALF thymosin β4 levels [23]. These data are consistent with the ability of thymosin β4 to down-regulate a number of key inflammatory cytokines, like the tumor necrosis factor-α. On the other hand, Thymosin β4 sulfoxide levels were higher in smokers and SSc patients with alveolitis and analyzing the progression of lung disease at one-year follow-up, we found that higher thymosin β4 levels seem to have a protective role against lung tissue damage. Another component of the thymosin family, the thymosin β10, was also present in BALF of SSc patients, yet not different from controls, but the negative correlation between thymosin β10 and the diffusion lung capacity for carbon monoxide, given the anti-angiogenic properties of thymosin β10, suggests a potential inhibiting role of thymosin β10 on the alveolar-capillary barrier function [24]. To shed further light on the angio-biologic milieu, the levels and the alveolar macrophage expression of the vascular endothelial growth factor (VEGF) have been investigated in BALF of SSc patients. Patients with high PAH have capillary loss at the capillaroscopic analysis and high circulating serum-plasma levels of VEGF, suggesting that loss of capillaries and dysregulated angiogenesis both contribute to the tissue damage [25,26].

We found a statistically highly significant fall of VEGF levels in SSc patients compared to controls, a profound decrease of VEGF levels in alveolitis patients compared to the non-alveolitis ones, a strong direct correlation with the fibrotic score and an inverse correlation with neutrophil and eosinophil counts, confirming the hypothesis that the alveolo-capillary barrier can really be compromised by the lack of angiogenic factors [27].

In another study, Bargagli et al. [28] identified the macropage inhibitory factor (MIF) as multi-tasking cytokine in SSc patients with pulmonary involvement, using 2DE analysis of BALF validated by ELISA assay. The MIF expression was increased in bronchiolar epithelium and in area with active fibrosis, suggesting a possible role of MIF in progressive lung fibrosis.

Author(s)	Biological material	Proteomic approach	Differently expressed proteins
Rottoli P et al. [9]	BALF	2-DE	Protease, anti-protease Coagulation system Cytokines Anti-oxidant proteins Calcium binding proteins
Rottoli P et al. [10]	BALF	2-DE	Transferrin Immunoglobulin(s) Complement protein
Fietta AM et al. [18]	BALF	2DE MALDI-TOF HPLC-MS	Glutathione S-transferase P Cystatin SN
Bargagli E et al. [22]	BALF	2-DE	Calgranulin B (S100A9)
De Santis M et al. [23]	BALF	reverse phase HPLC coupled to quadrupole ion-trap MS -ESI	Thymosins β
Bargagli E et al. [28]	BALF	2-DE, ELISA	Macrophage Inhibitory Factor
Shirahama R et al. [19]	BALF	2-DE MALDI-TOF MS	α2 macroglobulin α1 antitripsin Pulmonary surfactant protein A Heat shock protein Glutathione S-transferase P
Bogatkevich GS et al. [29]	fibroblasts from BALF	2-DE/MS	Vimentin Tropomyosin Actin Disulfide isomerase Glutathione S-transferase

Table 1: Proteomic Studies on Bronchoalveolar Lavage Fluid from Patients with Ssc and Pulmonary Involvement.

In a proteomic study on fibroblasts from BALF of SSc patients had been also identified 24 differently expressed protein spots, including cytoskeletal proteins (vimentin, tropomyosin and actin associated proteins) and proteins involved in redox imbalance (disulfide isomerase and GST), which may contribute to EC damage, fibroblast trafficking and tissue injury (Table 1) [29].

Serum Interface for Proteomic Analysis of Soluble Biomarkers in SSc Patients with Lung Involvement

Serum represents an attractive biological fluid to study, since it is easily accessible and contains an enormous amount of proteins, which might be involved in the pathogenesis of SSc. In addition, serum sampling is less invasive than BALF collection.

In sera from SSc patients with lung involvement, the proteomic approach allowed the identification of new targets for anti-endothelial cell antibodies (AECA), which further support the role of EC damage as central event in the pathogenesis of the disease [30-32]. In particular, Negi et al. [32] observed a significantly higher incidence of PAH in AECA positive patients. Moreover, Tamby et al. [33] identified antigens from micro and macro-vascular endothelial cells, using 2DE and immunoblot analysis of the protein extracts from serum of patients with PAH. Recently, Dib et al. [34] identified other new target antigens for AECA, such as lamin A/C, tubulin β chain and vinculin, using the same proteomic approach.

The 2DE combined with immunoblot analysis of the sera from SSc patients with PAH allowed the identification of α-enolase as target for anti-fibroblast antibodies, which might result from cross-reactivity after contact with either α-enolase from microorganisms or tissue damage [35-37]. Another possible explanation is the reduced clearance of α-enolase, which might be immunogenic but might also represent a substrate of caspase-1 that participates to activation of inflammasome (i.e.: IL-1β, NF-kB) [37,38].

In a study by 2DE combined with mass spectrometry and immunoblot analysis of sera from SSc patients with lung disease, Bussone et al. [39] identified new antigens involved in TGF-β1 pathway, which participate to fibroblast dysregulation and accumulation of extracellular matrix. Moreover, the recombinant antibody microarray permitted to identify mucin-1 and monocyte chemoatraction protein-4 (MCP-4) in serum of SSc patients, which might be the link between link inflammatory events and tissue fibrosis [40]. MCP-4 has been related to other different pathological contexts characterized by mononuclear infiltration, tissue remodeling and atherosclerosis [41,42]. Further functional studies should evaluate MCP-4 in a larger SSc patient population.

Using SELDI -TOF MS proteomic technological approach, van Bon et al. [43] identified increased levels of S100A8 (or calgranulin A) in sera from SSc patients with lung involvement, which might be a possible biomarker of chronic inflammation and progressive vascular injury.

By peptidomic approach, that combines a microamount peptide-separating method with magnetic beads and mass spectrometry analysis, Xiang et al. [44] detected in sera from SSc patients with ILD a group of short peptides with mass/charge values of 1,865, 1,778, 1,691, 1,563 and 1,450. These peptides had been identified as family members of C3f-des arginine (DRC3f) derived from C3b that might be a sign of complement system activation. Elevated levels of DRC3f and its degraded smaller fragments had been linked to vascular involvement and disease activity. In particular, the frequencies of ILD, sicca syndrome and esophageal involvement were significantly higher in patients with elevated DRC3f levels than those with normal DRC3f value. Interestingly, C3 and C4 serum levels were negatively correlated with DRC3f levels. Caccavo et al. [45] confirmed previous studies and reported an inverse correlation between high resolution CT scan (HRCT) score and serum levels of C3/C4 in SSc patients. These data had been documented in patients with autoantibodies directed against carbonic anhydrase I and/or II (CAI, CAII) and with HRCT score ≥10. CA is a ubiquitous metalloenzyme that catalyzes the reversible hydration reaction of carbon dioxide to bicarbonate and hydrogen ions. In the lungs, CAII is mainly expressed by alveolar epithelium and is involved in respiratory gas exchange and pulmonary capillaries pH/PCO_2 balance [46]. Antibodies anti-CAII were significantly increased in patients with ILD comparing to patients without ILD and healthy controls [47].

Recently, we carried out a comparative proteomic analysis of sera from SSc patients, that permitted us to identify 14 differentially expressed proteins mainly involved in EC protection and immune response [48]. We found increased concentrations of complement factor H in SSc subjects compared to healthy controls. Factor H is an important regulator of the alternative complement pathway, which normally protects self cellular surface from complement cascade. We documented a defective capacity of factor H to bind ECs and to protect them from complement mediated damage, especially in patients with pulmonary involvement. An aberrant expression of complement regulatory proteins had been already demonstrated in the endothelium of SSc patients [49] and it had been suggested that an inadequate protection from complement activation on cellular surface may be very important in the early phase of SSc disease. Complement activation leads to the formation of molecules that promote recruitment of inflammatory cells, generation of radical oxygen species and release of cytokines and chemokines, resulting in enhanced expression of EC adhesion molecules, apoptosis and tissue damage (Figure 1). The recruitment of circulating fibroblasts progenitor cells and their activation into the tissue are events that may also be facilitated by microvascular dysfunction [48,50].

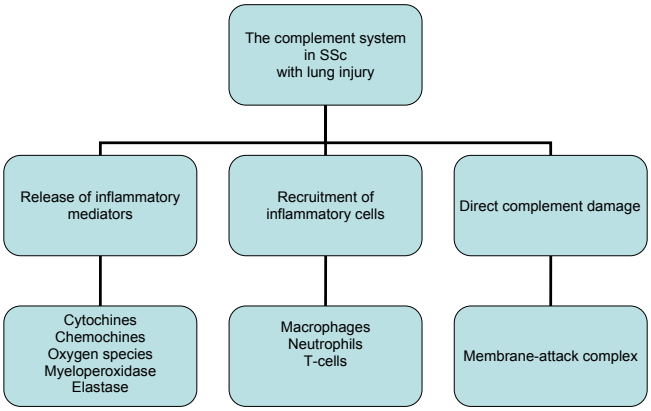

Figure 1: Role of complement system in SSc with lung involvement. Complement bioactive molecules, such as C3a and C5a anaphylatoxins, trigger a series of events that culminate in the recruitment of phagocytic cells, release of inflammatory molecules, activation of T-cell and augmentation of antibody response at the site of inflammation. The membrane-attack complex (MAC), resulting from distal complement component activation, can directly contribute to tissue damage through the lysis of cells, that are not properly protected by complement regulators.

Conclusions

The presented studies highlight complex and diverse pathways that intersect with each other and lead to tissue injury in the two major pulmonary syndromes associated with SSc. In general, these studies suggest an imbalance between aggressive and protective mechanisms, resulting in EC damage, amplified inflammatory response and fibroblast dysfunction in patients with lung involvement.

Although it has been provided important evidence on complicated pathogenetic mechanisms involved in SSc lung damage, further studies should be carried out to evaluate the effective role of the identified molecules, especially in early stages of the disease, in order to identify new therapeutic targets.

Rheumatology Key Messages

- Complex pathogenic pathways are involved in SSc with lung damage.
- Proteomic analysis provides a better understanding of pathogenesis through the identification of new molecules.

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