Lysyl Oxidase in Systemic Sclerosis: Getting Under the Skin

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Systemic sclerosis (SSc) is an autoimmune disease characterized mainly by vascular abnormalities and fibrosis of the skin and other organs [1]. The pathophysiology of fibrosis is complex. Despite our growing understanding of this process and the many targets available, our therapeutic success in ameliorating fibrosis in SSc is minimal [2,3]. Moreover, even today, skin fibrosis is usually assessed by the modified Rodnan skin score (mRSS), a score that is based on clinical inspection of 17 parts of the body, has a significant inter-observer variability, and is rather subjective [4]. Therefore, other objective and specific markers for assessing fibrosis are needed.

Current research is focusing on the discovery of useful biomarkers reflecting ongoing inflammatory or fibrotic activity in the skin and internal organs, as well as being predictive of the future disease course. Recently, remarkable progress has been made toward a better understanding of numerous mechanisms involved in the pathogenesis of SSc. This has opened new possibilities for the development of novel biomarkers and therapy.

THE ROLE OF LYSYL OXIDASE IN CONNECTIVE TISSUE

Collagen I is the most abundant structural protein of connective tissues such as the skin. The formation of collagen is an active process that reflects a balance between degradation and synthesis and involves disintegrin and metalloprotease bone morphogenic protein 1 (BMP-1). Under physiological conditions, the chemical cross-linking of collagen molecules incorporated in collagen fibrils is essential for the mechanical stability of these fibrils. Moreover, the presence of chemical cross-links makes fibril-incorporated collagen molecules more resistant to proteolysis. Formation of cross-links is an enzymatic process catalyzed by lysyl-oxidase (LOX) [5].

LOX is a copper-dependent amine oxidase that initiates the covalent cross-linking of collagen and elastin by catalyzing oxidative deamination of lysine and hydroxylysine residues to aminoadipic semi-aldehydes. These highly reactive semi-aldehydes can spontaneously condense to form intra- and inter-molecular covalent cross-linkages that assure extracellular matrix stability. LOX activity is a requisite to maintenance of the tensile and elastic features of connective tissues of the skeletal, pulmonary and cardiovascular systems, among others. LOX is synthesized as a pre-pro-LOX and is secreted into the extracellular environment, where it is proteolytically processed by BMP-1 and other procollagen C-proteinases to release the mature and active 32 kDa form and its pro-peptide [6].

THE ROLE OF LYSYL OXIDASE IN FIBROSIS

LOX has been evaluated in several states of fibrosis, including primary myelofibrosis (PMF) [7], hepatic [8] and myocardial fibrosis [6], and was found to be over-expressed in the relevant tissues. Data on LOX in patients with SSc is scarce. In 1995 Chanoki et al. [9] reported increased expression of LOX in the skin of patients with SSc. Ten years passed before Rajkumar and colleagues [10] published their observations on skin biopsies of four patients with SSc and suggested that LOX is increased in interstitial fibroblastic cells, but only in SSc patients without skin atrophy. In 2007, Meyringer et al. [11], using RNA arbitrarily primed-polymerase chain reaction, were able to show that the gene of LOX was over-expressed specifically in fibroblasts from the skin of patients with SSc.

These histological observations encouraged us to study LOX further and to try to understand its clinical correlates in SSc. In 2013, we published our initial results regarding LOX in SSc [12]. We evaluated 26 SSc patients and compared them to healthy controls as well as to primary myelofibrosis (PMF) patients as a disease control. Ten SSc patients had diffuse disease, 8 of them with lung fibrosis, and 16 had limited cutaneous disease. We found elevated concentrations of LOX in the peripheral blood of patients with SSc compared to healthy controls but similar to levels of PMF disease controls. LOX was significantly higher in patients with diffuse SSc when compared to limited disease, and in a subgroup of patients without lung fibrosis LOX correlated with mRSS [Figure 1]. This study was the first clinical study with evidence to suggest a role for LOX as a biomarker for fibrosis in SSc. Encouraged by these results, we decided to further examine the skin and lung biopsies from 11 patients with SSc. LOX

Figure 1. Comparison of serum lysyl oxidase (LOX) concentrations: **[A]** between systemic sclerosis (SSc) patients, primary myelofibrosis patients (PMF) and healthy controls (con). **[B]** between systemic sclerosis (SSc) patients with limited disease and diffuse disease. **[C]** Linear regression between LOX concentration and modified Rodnan skin score (mRSS) in patients with systemic sclerosis

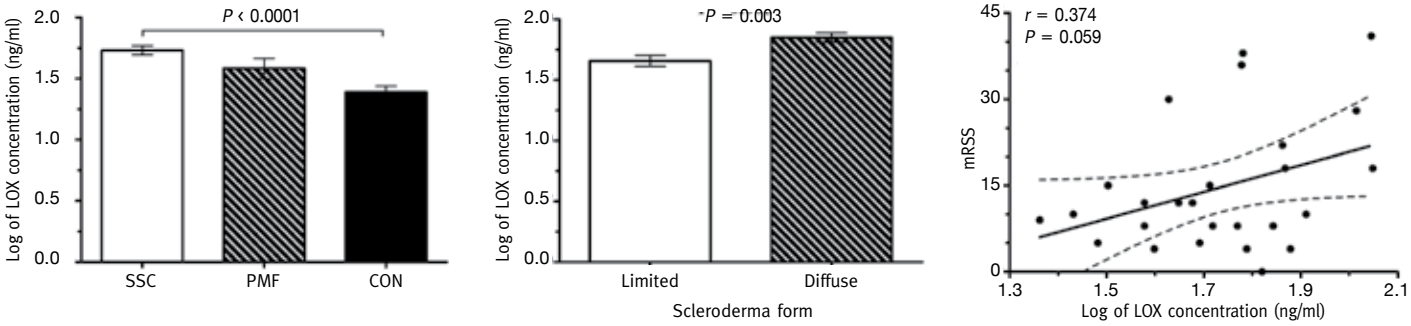
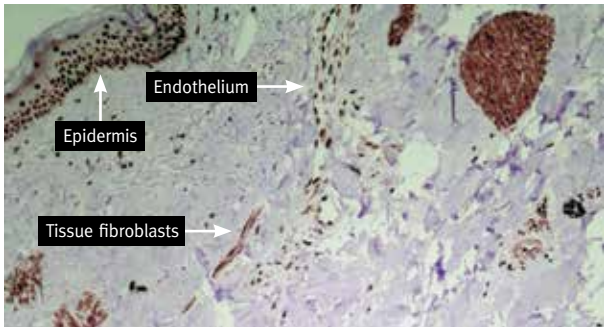


Figure 2. Hematoxylin and eosin x100 staining and lysyl oxidase immunostaining of skin biopsy from a patient with diffuse systemic sclerosis showing localization of lysyl oxidase to fibroblasts mainly in the epidermis and near blood vessels in the dermis



was found to be located in the epidermis in the skin and in the endothelium of blood vessels within the dermis and lung tissues [Figure 2]. This observation raised the question as to whether the source of elevated LOX level in patients with SSc is derived solely from fibroblasts in fibrotic skin and lung tissue, or also from damaged endothelium in an associated vasculopathy. In order to validate the earlier finding of elevated LOX blood levels in SSc and to delineate its source we have undertaken a second, larger scale, multicenter study, comparing 86 patients with SSc to 110 patients with very early diagnosis of SSc (VEDOSS), 86 patients with primary Raynaud's phenomenon and 80 healthy controls. Preliminary results from this study (unpublished data) confirm the increased level of LOX concentration in patients with SSc. LOX was not found to be elevated in the early stage of VEDOSS nor in patients with primary Raynaud's phenomenon, suggesting a specific role for LOX in the pathogenesis of SSc. Thus, LOX is a potential marker that may help facilitate the diagnosis of SSc and differentiate it from primary Raynaud's phenomenon and early disease. Furthermore, a negative correlation of LOX with DLCO observed in this study suggests damaged endothelium as an additional source for the increased expression of LOX in SSc and further supports the possible role for LOX as a biomarker for lung fibrosis and disease severity. In line with our findings is a recent report that found LOX mRNA to be up-regulated in lung fibroblasts of patients with systemic sclerosis, correlating with the degree of lung fibrosis [13].

OTHER BIOMARKERS FOR FIBROSIS IN SSC

The complex cascade of cytokines, chemokines, as well as collagen production and degradation products involved in SSc is a fruitful resource in the search for markers of fibrosis and disease severity. Some of the most important markers are delineated here.

- The enhanced liver fibrosis (ELF) test, developed as a clinical grade serum test for chronic liver diseases, includes procollagen-III aminoterminal-propeptide (PIIINP), tissue inhibitor of matrix metalloproteinase-1 (TIMP-1), and hyaluronic acid (HA) in its algorithm. Recently, the ELF test was tested in SSc patients and showed significant correlations with both disease activity and severity [14]. The ELF score also correlated with mRSS, Health Assessment Questionnaire-Disability Index (HAQ-DI), and inversely correlated with DLCO, but did not correlate with vasculopathy features such as PAH.
- Cartilage oligomeric protein 1 (COMP) is a non-collagenous glycoprotein, mostly synthesized by chondrocytes, osteoblasts, synovial fibroblasts, and dermal fibroblasts. This protein, highly regulated by transforming growth factor-beta (TGFβ), is not detectable in the healthy skin but is highly over-expressed in skin biopsies and fibroblasts of SSc patients. COMP was found to be increased in SSc sera and correlated with the extent of skin involvement, as assessed by mRSS and ultrasound [15].
- Matrix metalloproteinases (MMPs), responsible for the degradation of collagens and other extracellular matrix (ECM) proteins, are involved in the release and activation of many cytokines and growth factors. MMP-9 and MMP-12 have been found to be potential markers for skin fibrosis. MMP-

9, whose substrates include type IV collagen in basement membrane, has been found in to be excessively expressed from fibroblasts isolated from SSc patients compared to healthy controls. Furthermore, the serum level of MMP-9 was elevated in SSc, with higher concentration in diffuse SSc compared to limited SSc, and correlated well with mRSS [16]. MMP-12, also known as macrophage metalloelastase (MME), has broad substrate specificity for matrix macromolecules, recognizing elastin, type IV collagen, fibronectin, or vitronectin. In SSc patients, MMP-12 is expressed and released from dermal fibroblasts [17] and serum levels are significantly increased, correlating with skin fibrosis [18].

- Finally, a strong relationship between inflammation and fibrosis in SSc is supported by the up-regulation of both pro-inflammatory and pro-fibrotic markers in the serum as well as in skin. The role of various cytokines and chemokines has been analyzed in skin fibrosis of SSc in several studies. For instance, IL-6 and IL-10 serum levels were found to be elevated in SSc patients and significantly correlated with skin fibrosis assessed by mRSS [19]. CXCL4, largely viewed as a pro-inflammatory chemokine, in addition to its chemoattractant activity regulates an array of immune cells, including T cells, monocytes, dendritic cells, as well as non-immune cells like endothelial cells. Circulating CXCL4 levels strongly correlate with the extent of skin fibrosis, more with diffuse SSc subsets than the limited form. In a prospective cohort study, elevated CXCL4 in the serum of SSc predicted a more rapid progression of skin fibrosis [20].

LOX AS A PUTATIVE TARGET FOR TREATMENT IN SSC

Considering the data presented here, it would seem that LOX could be a reasonable target for treatment in SSc. LOX enzymatic activity is inhibited irreversibly by β-aminoproprionitrile (BAPN), which has been used in animal models in the context of tissue fibrosis. In a bleomycin-induced experimental lung fibrosis mouse model, inhibition of LOX activity by BAPN ameliorated fibrosis progression. In that model, blocking LOX activity at the fibrogenic stage did not efficiently prevent further progression of lung fibrosis, while inhibition of LOX activity at the inflammatory stage impaired inflammatory cell infiltration, TGFβ signaling, and lung fibrosis [21]. In a study of 10 patients with scleroderma, BAPN was not found to be effective, mostly because of severe side effects requiring treatment withdrawal shortly after initiation [22]. Another drug with possible LOX activity is D-penicillamine, once considered a potent anti-fibrotic drug [23]. D-penicillamine is a strong chelator of copper, which is crucial for the activation of LOX. Thus, LOX inactivation may be at least a part of penicillamine's mode of action in SSc. Finally, a humanized antibody, anti-LOX-like 2 (a member of the LOX family), is currently being evaluated as treatment for liver fibrosis [24]. A humanized anti-LOX antibody may be a potential anti-fibrotic drug in SSc.

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