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Joanna Busquets
Thomas Jefferson University

Francesco Del Galdo
Thomas Jefferson University, Francesco.DelGaldo@jefferson.edu

Eugene Y. Kissin
Boston University School of Medicine

Sergio A. Jimenez
Thomas Jefferson University, Sergio.Jimenez@jefferson.edu

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Assessment of tissue fibrosis in skin biopsies from patients with systemic sclerosis employing Confocal Laser Scanning Microscopy: An objective outcome measure for clinical trials?

Joanna Busquets, M.D.[1]; Francesco Del Galdo, M.D., Ph.D. [1]; Eugene Y. Kissin, M.D. [2]; and Sergio A. Jimenez, M.D. [1]

[1]Jefferson Institute of Molecular Medicine, Department of Dermatology and Cutaneous Biology

Thomas Jefferson University, Philadelphia, Pennsylvania, USA and

[2]Section of Rheumatology, Boston University School of Medicine, Boston, MA, USA

Running head: CLSM for SSc

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Address all correspondence to:

Sergio A. Jimenez, M.D.

Jefferson Institute of Molecular Medicine

Thomas Jefferson University

233 S. 10th Street, Room 509 BLSB

Philadelphia, PA 19107-5541

Phone: 215-503-5042

Fax: 215-923-4649

E-mail: sergio.jimenez@jefferson.edu

ABSTRACT

Objectives. To obtain an objective, unbiased assessment of skin fibrosis in patients with systemic sclerosis (SSc) for use in clinical trials of SSc disease-modifying therapeutics.

Methods. Skin biopsies from the dorsal forearm of six patients with diffuse SSc and six healthy controls, and skin biopsies from the forearem of one patient with diffuse SSc before and following 1 year treatment with mycophenolate mofetil were analyzed by confocal laser scanning microscopy (CLSM) with specific antibodies against collagens type I and III or fibronectin. The integrated density of fluorescence (IDF) was calculated employing NIH-ImageJ® software in at least four different fields per biopsy spanning the full dermal thickness.

Results. The intensities of types I and III collagen and fibronectin immunofluorescence were 174%, 147%, and 139% higher in SSc skin than in normal skin, respectively. All differences were statistically significant. The sum of the IDF values obtained for the three proteins yielded a comprehensive fibrosis score. The average fibrosis score for the six SSc samples was 28.3×10^6 compared to 18.6×10^6 for the six normal skin samples ($p < 0.0001$). Comparison of skin biopsies obtained from the same SSc patient before treatment and following 12 months mycophenolate mofetil treatment showed a reduction of 39% in total fibrosis score after treatment.

Conclusion. CLSM followed by quantitative image analysis provides an objective and unbiased assessment of skin fibrosis in SSc and could be a useful endpoint for clinical trials with disease modifying agents to monitor the response or progression of the disease.

KEYWORDS

Systemic Sclerosis

Scleroderma

Confocal Laser Scanning Microscopy

Skin Collagen

Clinical Trials Outcome Measure

Fibrosis

Systemic sclerosis (SSc) is a heterogeneous autoimmune disorder characterized by excessive deposition of collagen and other extracellular matrix proteins in skin and multiple internal organs, microvascular dysfunction and humoral and cellular immune dysregulation (1). Uncontrolled production of collagens and other extracellular matrix (ECM) proteins by fibroblasts leads to excessive accumulation of connective tissue in various organs (1,2). The most apparent and almost universal clinical features of systemic sclerosis are related to the progressive thickening and fibrosis of the skin (3). The histopathologic findings in the skin include marked thickening of the dermis with massive accumulation of dense collagen causing epidermal atrophy, and replacement of sebaceous and sweat glands as well as hair follicles. A prominent inflammatory infiltrate is often present, especially in early lesions (4,5). Although the pathogenesis of SSc skin changes is not completely understood, the hallmark of skin involvement is the increased content of collagen and other extracellular matrix components such as fibronectin in the dermis (1,2). The extent and rate of progression of tissue fibrosis is critically important in determining the clinical features and prognosis of SSc. Skin thickening correlates with both survival[6] and functional limitations in the disease (7), and extent or severity of skin involvement correlates with an overall assessment of clinical disease severity (8).

The primary criterion used to classify patients as either SSc with diffuse or limited cutaneous involvement is the extent and severity of skin thickening (9). Skin involvement is the most widely used outcome measure in SSc interventional trials (10). Currently, there is no widely accepted objective and quantitative measure of the amount of skin fibrosis in SSc for use in clinical trials to assess treatment success or disease progression and the gold standard outcome measure of skin involvement is the modified Rodnan skin score (mRSS), a semiquantitative assessment of cutaneous thickening performed by palpation and scoring of the degree of local

skin thickness in 17 body surface areas (11,12). Studies have shown that higher skin scores correlate with worsening of internal organ involvement and higher morbidity and mortality in SSc (6-8) and, therefore, it is generally accepted that the skin score can be used as a clinical surrogate of the overall disease process (10). Although the mRSS is accessible, noninvasive and cost-effective, it is a subjective assessment with potential inter- and intra-observer variability, which has been calculated to be 25% and 12%, respectively (13). This variability requires the recruitment of a large number of patients to appropriately power clinical studies, a difficult task given the low incidence of the disease. Also, since the scale employed to grade the severity of skin thickening is limited to four integer values, it is difficult to quantify subtle skin changes. Furthermore, it is not possible to differentiate fibrotic skin thickening from that resulting from edema, inflammation, vascular bed engorgement, or skin tethering (14).

To reduce subjectivity and increase reliability, investigators have employed device-assisted measurements such as durometry and ultrasonography (14,15). Although durometers provide a continuous scale and correlate with the mRSS, there is a similar inter-observer variability and a non-homogeneous sensitivity in uneven body regions (*i.e.* fingers, face) (15). As a result, none of the devices have been accepted as valid outcome measures for clinical studies.

Increased expression of the genes encoding interstitial collagens types I and III accompanied by marked elevations of the production of the corresponding proteins are the hallmark of SSc and are responsible for the pronounced abnormalities in skin and various internal organs. Given the marked increase in the production and biosynthesis of interstitial collagens in SSc, numerous studies have investigated circulating or urinary levels of collagen molecules or collagen fragments as biomarkers that may reflect the activity of the ongoing

fibrotic process (16-19). Although the measurements of metabolites derived from the biosynthesis and degradation of collagen are very likely a reflection of the fibrotic process, owing to the fact that most of the type I collagen in the body is present in bone, metabolites derived from this molecule would reflect, to a large extent, the remodeling and degradation of type I collagen in the skeletal system.

Another protein that has been suggested as a potential biomarker to reflect the fibrotic process in SSc is the cartilage oligomeric matrix protein (COMP). This large molecular weight pentameric protein was initially discovered as a product of articular cartilage chondrocytes and was considered to be specific for this tissue. However, more recently it has been shown that COMP is also an important fibroblast and synoviocyte product (20) and therefore it has been suggested that serum COMP may reflect fibroblast biosynthesis of extracellular matrix components. Indeed, some studies have measured serum levels of COMP and described significant correlations with the extent of skin involvement and with the severity of SSc (21). Increased expression of COMP was also demonstrated in skin samples from SSc patients as well as in fibroblasts cultured from these samples (22,23). However, the possibility that COMP originated from articular cartilage may be a component of the serum COMP being detected or, more importantly, that it may represent a product of inflamed synovium needs to be further evaluated. Thus, more extensive studies would be required to confirm the validity of measurements of collagen derived peptides and of COMP as markers of tissue fibrosis in SSc.

Histopathological quantitation of fibroblastic cells expressing α-smooth muscle actin (myofibroblasts) has been suggested as another potential biomarker of SSc skin fibrosis (24). In this study myofibroblast content in skin correlated with clinical measures of skin disease as well as with mRSS and showed a significant reduction following treatment with cyclophosphamide.

Thus, myofibroblasts may represent an early marker of the fibrotic process capable of being modulated by effective therapeutic interventions.

The availability of an objective, reliable and reproducible quantitative method to assess the amount of skin fibrosis in SSc would be extremely valuable in determining the efficacy of disease-modifying interventions in clinical trials. Reproducible observer-independent measures of skin disease would allow for smaller sample sizes and enhanced detection of effective therapies in clinical trials. Here, we aimed to identify and quantify histopathologic markers of fibrotic activity in SSc skin biopsies that can be used as reliable biological endpoints for clinical trials of SSc disease modifying therapeutic agents. For this purpose, we employed confocal laser scanning microscopy (CLSM) based quantitative analysis to assess the abundance of pertinent ECM proteins such as collagens types I and III, and fibronectin.

PATIENTS AND METHODS

Patient selection. Systemic sclerosis patients were recruited from the Scleroderma Center at Thomas Jefferson University. All patients fulfilled the SSc classification criteria of the American College of Rheumatology (25), and had diffuse cutaneous involvement based on the criteria of LeRoy et al. (9). All patients studied had SSc of recent onset (less than 18 months from first evidence of skin induration) and clinically evidenced rapid progression. Skin biopsies were obtained within the first six months for four of the six patients studied.

Skin biopsies. The Scleroderma Tissue Bank is a repository of tissue from patients with SSc which was established in 1987. The skin samples maintained in the Tissue Bank are tissues remaining from biopsies obtained for diagnostic purposes from clinically affected skin. For this study skin samples obtained from the dorsal forearm of six untreated patients with rapidly

progressive diffuse SSc of recent onset (less than 18 months from clinically detectable skin induration) were employed following an IRB approved protocol. Normal skin samples were obtained from patients undergoing unrelated surgical procedures. To examine the effects of a therapeutic intervention, skin biopsies were obtained from one patient with diffuse SSc of recent onset (less than 10 months from clinically detectable skin induration) before treatment and following 12 months treatment with 2.0 g/day mycophenolate mofetil (MMF) orally. The second biopsy was obtained from an area in close topographic proximity to the initial biopsy. The samples were fixed with 10% buffered formalin and embedded in paraffin.

Skin Immunofluorescence. After deparaffinizing the slides, tissue sections were incubated with a 1:100 dilution of specific primary rabbit polyclonal antibodies (Rockland Immunochemicals, Gilbertsville, PA) against collagens type I and type III, fibronectin or α-SMA for 1 h at room temperature in a moist, dark chamber. Sections were washed twice with PBS. To minimize non-specific staining, slides were incubated with FAB'-sheep anti-rabbit Cy3 antibody (Sigma, St. Louis, MO) for 1 h at room temperature. The slides were then washed again in PBS and counterstained with DAPI-1500. No antigen retrieval was performed since commonly used antigen retrieval procedures may cause variable extraction of tissue collagens or cell membrane alterations and cellular disruption resulting in increased background staining.

Analysis of Fluorescence. The entire skin section was scanned and four different low power magnification (20X) fields consecutively spanning the full thickness of the dermis were analyzed for each biopsy employing a Zeiss LSM 510 META confocal laser scanning microscope. The analyses were carried out in a blinded manner to disease/non-disease source of sample. A computer generated 2.5D image which plots the intensity of fluorescence of a specific microscopic field was employed for further analyses. Each skin section was analyzed with

Image J® software, which calculated the sum of the intensity of each pixel in a given microscopic field as the Integrated Density of Fluorescence (IDF). The IDF value is a measure of the overall amount of fluorescence intensity in a given microscopic field. The averages of the four IDF values per specimen were calculated. Statistical analysis of the average IDF values was performed using a 2-tailed unpaired t-test. *P* values < 0.05 were considered significant.

RESULTS

Six patients with diffuse SSc (4 women, 2 men; median ± SD disease duration 8 ± 5 months, age 39 ± 11 years) were recruited for the study. The disease duration was less than six months for 4 of the 6 patients. The SSc biopsy samples were obtained from patients with early disease since this is the target population most commonly included in SSc disease modifying clinical trial studies.   Skin samples from 6 healthy subjects (4 women, 2 men; age 43 ± 17 years) were used as normal controls. The relevant clinical and serological characteristics of the patients are shown in **Table 1**.

Figure 1 shows representative images of the analysis of fluorescence intensity in normal and SSc skin samples. The left panels show representative images of the visual intensity of fluorescence, the center panels show the computer generated 2.5D analyses of the fluorescent images and the right panels show the Image J® software generated IDF values in arbitrary units. The Image J® generated IDF value for collagen I (**Figure 1A**) shows a high correlation with the visual intensity of fluorescence and, most importantly, it is an accurate assessment of total collagen I expression in the tissue section. The average collagen I IDF in the normal skin was $5.82 \pm 0.87 \times 10^6$ vs. $10.13 \pm 0.92 \times 10^6$ in the SSc skin (p = 0.0018). **Figures 1B** and **1C** show a similar analysis for collagen type III and fibronectin. The average collagen III in normal skin

was 5.53 ± 0.69 x 10^6 vs. 8.10 ± 1.57 x 10^6 in the SSc skin (p = 0.0197). The average fibronectin in the normal skin was 7.28 ± 0.51 x 10^6 vs. 10.10 ± 0.94 x 10^6 in the SSc skin (p = 0.0086). The calculated amounts of type I collagen, type III collagen, and fibronectin were 174%, 147%, and 139% higher in SSc skin than in normal skin, respectively. All these differences were highly significant statistically. Analysis of α-SMA was of little value as the calculated IDF included, in addition to myofibroblast or activated fibroblast signals, the intense signal originating from smooth muscle cells in small arterioles or surrounding hair follicles.

The average IDF values of the 4 microscopic fields from the dermis of each of the 6 SSc skin samples and 6 normal skin samples stained for collagen I, collagen III, and fibronectin were analyzed by scatter plot (**Figure 2A**). In order to obtain a comprehensive value of the amount of collagen I, collagen III, and fibronectin, we calculated and plotted the sum of the IDF values for all 3 ECM proteins as the total fibrosis score for each of the 6 SSc skin samples and 6 normal skin samples (**Figure 2B**). The average total IDF score was 28.3 x 10^6 for the SSc skin samples compared to 18.6 x 10^6 for the normal skin samples. Statistical analysis performed using a 2-tailed unpaired t-test indicated that the differences in expression were all statistically significant (p < 0.0001).

To examine whether the fibrosis score obtained as described above is static over time or sensitive to change we compared the fibrosis score in skin biopsies from one patient with diffuse SSc before and following 12 months of treatment with MMF. The second biopsy was taken in close topographic proximity to the initial biopsy site and the ECM protein content and fibrosis score were obtained following staining for collagen I, collagen III, and fibronectin. Two representative CLSM fields of the type I collagen staining in the dermis are shown in **Figure 3A** and the plotted IDF values of 7 separate microscopic fields spanning the dermis are shown in

Figure 3B. A profound decrease in fluorescent intensity is observed in the sample obtained following treatment compared to the initial biopsy obtained prior to treatment. The IDF values obtained for all 7 microscopic fields were highly statistically significant , demonstrating the reliability of the method. The sum of the average IDF values for collagen I (COL 1), collagen III (COL 3), and fibronectin (FBN) of the SSc skin biopsies taken before and following 1 year of treatment with MMF was analyzed as the total IDF fibrosis score. The results showed that there was a 39% reduction in the total fibrosis score following 1 year of oral MMF treatment (**Figure 3C**).

DISCUSSION

The goal of this study was to obtain an assessment of the extent of skin fibrosis in SSc patients that can be used as an objective and unbiased end point in clinical trials for SSc disease-modifying therapeutics. We determined the amounts of three ECM proteins known to be upregulated in SSc skin employing CLSM in skin biopsies from six patients with diffuse SSc and six healthy controls. The amounts of type I collagen, type III collagen, and fibronectin were 174%, 147%, and 139% higher in SSc skin than those in normal skin, respectively. All differences were statistically significant. In order to obtain an overall assessment of the amount of dermal fibrosis and of the total amount of collagen I, collagen III, and fibronectin in the tissue, the sum of the IDF values for all 3 ECM components was calculated as a total fibrosis score for each of the samples. The total fibrosis score demonstrated a more pronounced difference between SSc and normal skin compared to the individual IDF values obtained separately for each ECM protein. This observation may be explained by differences in stage of the disease which may result in increased abundance of a particular ECM protein at certain stages of disease

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progression, and, therefore, a global or total fibrosis score may be more representative of the overall level of fibrosis in the tissue.

Our results also suggest that the measurements obtained might be sensitive to change over time and are not static as they displayed a highly statistically significant reduction following 12 months of oral treatment with MMF, a drug that has been recently suggested as a potentially effective antifibrotic agent (26,27) and has been used as a disease-modifying therapy for SSc-associated lung disease (28-31) and diffuse cutaneous SSc of recent onset (32,33). Our analyses showed a statistically significant 39% reduction in total fibrosis score following treatment which demonstrates that the assessment described here is capable of detecting a difference in skin fibrosis. However, it was not possible to demonstrate a direct correlation between the fibrosis score values obtained with this procedure and the mRSS or the assessment of skin induration obtained with a durometer in a previous study (14) (data not shown). The failure to establish these correlations suggests that the parameters that are evaluated by the mRSS and durometer measurements are not the same as those being evaluated by the procedure described here. It is likely that our method is a direct measurement of the accumulation of extracellular matrix macromolecules in the tissue and indeed reflects the level of tissue fibrosis, whereas the mRSS and durometer measurements may reflect in addition to tissue fibrosis other parameters such as, for example, tissue edema, severity and extent of the inflammatory process, tissue tethering, vascular engorgement, etc.

The assessment described here is a substantial improvement compared to the qualitative histopathologic assessment performed routinely in skin biopsies. Although the demonstration of increased collagen content in SSc skin biopsies is a rather obvious finding, the relevance and importance of the results reported here is twofold. First, the values were obtained with an

objective and unbiased ("investigator interpretation free") method, and second, the assessment generated absolute numbers that can be used as an objective outcome measure of histopathological changes in SSc. The values obtained by analyzing and quantifying the immunofluorescence intensity are sensitive enough to clearly distinguish between fibrotic and non-fibrotic skin.

Although clinical trials are a valuable means of evaluating the effectiveness of new treatments in SSc, the lack of an objective outcome measure with appropriate end-points for measuring treatment success in this disease makes interpretation and comparison of the findings difficult. The development of an objective and reliable method of assessing the severity of skin fibrosis, therefore, would be invaluable in determining the efficacy of a given treatment in clinical trials, both by providing a method independent of the potential bias of study investigators and by allowing a reduction of the number of patients needed to achieve statistical power for the study.

The modified Rodnan skin score (mRSS) is a clinical semi-quantitative assessment of skin thickness that is currently the only outcome measure widely used in clinical trials of disease modifying agents for SSc. Although accessible and non-invasive, the mRSS has several limitations, ranging from the subjective assessment of skin palpation to the difficulty of scoring borderline skin changes and the confounding effects of the presence of edema, inflammation, or tethering and variability in skin adipose tissue. Although hydroxyproline assays would yield a precise quantification of the amount of collagen in skin, there are drawbacks that limit its applicability, including the variability in wet weight of the samples caused by the difficulty of controlling loss of water content, the confounding and variable contribution of elastin

hydroxyproline to the overall results, and more importantly, the inability of hydroxyproline assays to differentiate between collagen types and to evaluate fibronectin content.

We do not foresee that CLSM followed by quantitative image analysis would be widely applicable in the clinical practice setting since skin biopsies are not typically necessary to establish the diagnosis. However, this objective method may be used in clinical trials to compare the amount of tissue fibrosis before and following administration of a disease-modifying drug since two biopsies are often required as part of a clinical trial. Thus, CLSM followed by quantitative image analysis may be a sensitive tool to assess the amount of skin fibrosis in SSc. Such analysis could be a useful endpoint for clinical trials to accurately monitor the response or progression of the disease. However, further assessment of a larger number of patients will be required to validate this procedure.

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Table 1. Relevant clinical and serological characteristics of systemic sclerosis (SSc) patients.

	Sex	Age	Duration*	mRSS†	Lungs±	Heart	Kidneys	Gastro-intestinal	ANA Titer	ANA Pattern	Scl-70	ACA
SSc-1	F	50	12	14	+	+	−	+	1:640 1:160	Speckled Homogeneous	−	−
SSc-2	F	31	18	13	+	−	−	+	+	Not done	+	−
SSc-3	M	57	4	23	+	+	−	+	1:610 1:160	Homogeneous Nucleolar	+	−
SSc-4	F	33	4	42	+	+	−	+	1:1280 1:80	Homogeneous Nucleolar	+	−
SSc-5	M	35	6	24	−	+	−	+	Direct = 266	Not done	−	−
SSc-6	F	30	4	13	−	−	+	−	> 1:640	Nucleolar	−	−

* Disease duration in months from earliest clinically detectable skin involvement.

† mRSS at time of skin biopsy

± Lung involvement classified as interstitial lung disease

LEGENDS FOR ILLUSTRATIONS

Figure 1. Analysis of the fluorescence intensity for collagen I **(A)**, collagen III **(B)** and fibronectin **(C)** in normal skin (N) and SSc skin (SSc). The left panels show the immunofluorescence images, the center panels show the computer generated 2.5D image analysis plots of the corresponding microscopic fields, and the right panels show the IDF of the two microscopic fields calculated by Image J® software.

Figure 2. **(A)** The average IDF for collagen I (COL-1), collagen III (COL-3), and fibronectin (FBN) from 4 different microscopic fields for each of the 6 SSc skin samples and 6 normal skin samples (N) analyzed by scatter plot. * = $p < 0.05$; ** = $p < 0.01$; ***$p < 0.001$. **(B)** The sum of the IDF values for collagen I, collagen III, and fibronectin of each of the 6 SSc skin samples and 6 normal skin samples was analyzed as the total fibrosis score by scatter plot. The red dotted line demonstrates that there is no overlap between the total fibrosis scores calculated for SSc skin and normal skin samples. ***$p < 0.001$

Figure 3. **(A)** CLSM images of 2 representative fields of the dermis stained for collagen I (COL I) of a patient with diffuse SSc before treatment and after receiving oral treatment with 2.0 g/day MMF for 1 year. **(B)** The IDF values of 7 microscopic fields stained for collagen I of the same SSc patient before treatment and after treatment were plotted. ***$p < 0.001$. **(C)** The sum of the average IDF values for collagen I (COL I), collagen III (COL III), and fibronectin (FBN) of the SSc skin biopsy taken before and of the skin biopsy taken after treatment was analyzed as the total IDF score. There is a reduction of 39% in fibrosis score after treatment with MMF for 1 year.

KEY MESSAGES

- CLSM fibrosis score is an objective SSc skin fibrosis measurement independent from edema, inflammation, vascular engorgement, and other factors.

- CLSM fibrosis score reflects clinically relevant therapeutic effects.