Expression of NLRP3 Inflammasome, Cytokines and Vascular Mediators in the Skin of Systemic Sclerosis Patients

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ABSTRACT: Background: The activated NLRP3 inflammasome is associated with the etiology of fibrotic diseases. The role of inflammasomes in SSc is still poorly understood.

Objectives: To determine the expression of NLRP3 (nucleotide-binding domain, leucine-rich-repeat-containing family, pyrin domain-containing 3) in the skin of patients with systemic sclerosis (SSc) and its relationship with pro-inflammatory cytokines and vascular mediators expression.

Methods: Skin biopsies were taken from 42 patients with either limited or diffuse SSc (21 lcSSc and 21 dcSSc), and from 13 healthy individuals. Using real-time polymerase chain reaction (PCR), the relative expression of caspase-1, IL-1β, IL-18, IL-33, TGF-β, ET-1, iNOS and eNOS genes, were measured. The location of NLRP3 and IL-1β were also determined by immunohistochemistry. Clinical characteristics were evaluated.

Results: The mean age of the patients was 49.3 ± 12.9 (lcSSc), 44.6 ± 13.8 (dcSSc), and 45 ± 14.1 (healthy individuals). Compared to healthy individuals, the skin of both subsets of SSc showed a significant increase (P < 0.05) in NLRP3, caspase-1, IL-1β, IL-18 and ET-1. Samples of lcSSc also showed a significant increase of eNOS (P < 0.029), iNOS (P < 0.04) and TGF-β (P < 0.05). Dermal fibrosis evaluated by modified Rodnan skin score (MRSS) had significant correlation with NLRP3, IL-1β, IL-18, and ET-1. Immunohistochemical analysis showed stronger staining of NLRP3 and IL-1β cytoplasmic expression in the keratinizing squamous epithelium of skin from SSc patients compared to controls.

Conclusions: This study identified NLRP3 over-expression in skin of patients with SSc. Skin thickness correlates positively with the NLRP3 inflammasome gene expression and with the vascular mediator and profibrotic ET-1, suggesting that NLRP3 inflammasome plays a role in the pathophysiology of skin fibrosis in human SSc.

KEY WORDS: inflammasome, NLRP3, systemic sclerosis (SSc), caspase-1, skin

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Systemic sclerosis (SSc) is an autoimmune disease that manifests as skin fibrosis, systemic vascular alterations and collagen accumulation. The cutaneous involvement, which develops slowly, is termed limited cutaneous SSc (lcSSc); when appearing rapidly, with extensive skin thickening and affecting organs early it is termed diffuse cutaneous SSc (dcSSc) [1]. The skin fibrosis is measured by the modified Rodnan skin score [2].

In SSc, there is a simultaneous occurrence of chronic inflammation, tissue remodeling and repair processes, which are driven by abnormal production of growth factors [3], proteolytic enzymes of vascular mediators (ET-1, NOS) [4], and profibrotic cytokines [2,5]. Evidence indicates that SSc begins with vascular dysfunction, followed by inflammatory/immunologic changes and fibrosis [1]. Inflammasome gene transcripts of fibroblasts obtained from patients with SSc has demonstrated that fibrosis is dependent upon caspase-1 activation and interleukin IL-1β secretion resulting from innate immunity activation [6].

The innate immune system is activated when the key receptors are stimulated in macrophages, neutrophils, monocytes, lymphocytes, epithelial cells and dendritic cells. Among such receptors are NLRs (previously termed NALPs), including nucleotide-binding domain and leucine-rich repeat receptors [7]. These NLRs are critical factors in the activation of caspase-1 [8], which is essential for the processing and secretion of IL-1β/IL-18 [9]. This molecular platform has been termed the inflammasome. The activated NLRP3 inflammasome is associated with the etiology of fibrotic diseases [6,10]. Since the role of inflammasomes is still poorly understood in SSc, we undertook this study to analyze the over-expression of NLRP3 inflammasome, vascular mediators and profibrotic factors in the skin of SSc patients.

PATIENTS AND METHODS

Forty-two consecutive patients with SSc were enrolled in the study after they gave their permission and signed a written informed consent form. All patients were attended in the
internal medicine outpatient department of La Raza National Medical Center, Mexico City. The study was conducted under a protocol approved by the hospital’s Ethics Committee.

Of the 42 SSc patients, 21 were classified in the lcSSc subset and 21 in the dcSSc subset according to the criteria described by LeRoy and Medsger [11]. We included 13 unrelated control subjects. Patients with lcSSc or dcSSc were further classified according to disease duration and clinical features.

**CLINICAL ASSESSMENT**

Skin thickness was evaluated using the Rodnan skin score by clinical palpation of 17 body areas on a scale from 0 to 3 [2]. Organ evaluations were performed according to the disease severity scale for SSc developed by Medsger et al. [12]. Active Raynaud’s phenomenon (RP) was identified by sudden pallor of digits followed by reactive hyperemia or cyanosis after cooling. Digital ulcers or gangrene were registered. For tendon involvement we measured the distance from the fingertips in maximum flexion to the center of the palms. Skeletal muscle disease was defined as proximal muscle weakness or atrophy recorded at clinical evaluation. Lung interstitial fibrosis was confirmed by high resolution computed tomography scan and forced vital capacity (VCV) < 80% on pulmonary function tests. Pulmonary arterial hypertension was defined as ≥ 35 mmHg by echocardiography. Cardiac disease was considered when diastolic dysfunctions on an echocardiogram or conduction disturbance on an electrocardiogram were present. Gastrointestinal involvement was considered when abnormal manometry or persistent abdominal distension and biochemical markers of malnutrition were present. Weight loss ≥ 5 kg during the previous 6 months was registered.

Antinuclear antibodies and anti-centromere antibodies were determined by indirect immunofluorescence. Anti-Scl-70 (anti-topoisomerase I) was determined by enzyme-linked immunoassay.

**BIOPSY SAMPLES**

We obtained 0.8 cm punch biopsy samples of the affected skin over the dorsal mid-forearm from each individual. Affect ed skin areas were defined as those with a thickness value ≥ 2 points, according to the modified Rodnan skin score (MRSS) [2]. Control skin samples were obtained from 13 healthy subjects. From each biopsy sample, 4 mm were immediately placed in RNAlater® (Ambion, USA) and stored at -20°C until preparation of RNA, and 3 mm were frozen at -60°C and stored until they were cut on the cryostat.

**REVERSE TRANSCRIPTION AND QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION**

In this study we investigated the gene expression of NLRP3, caspase-1, related pro-inflammatory cytokines, transforming growth factor-beta (TGFβ), Endothelin-1 (ET-1) and inducible and endothelial nitric oxide synthase (iNOS and eNOS) in the skin of SSc patients.

Total RNA was isolated (Pure Link™ Mini RNA Kit, Invitrogen, Carlsbad, CA, USA), and reverse transcription was carried out using 0.5 µg of total RNA with random hexamers in a 20 µl volume of reaction mixture (SuperScript® II™ cDNA Synthesis Kit, Invitrogen) according to the manufacturer’s instructions. Reactions were performed in a Mastercycler thermocycler (Eppendorf, Hamburg, Germany). Amplified cDNA was quantified on a photometer at 260 nm. Real-time polymerase chain reactions (PCR) were carried out using the Human Universal ProbeLibrary (Roche Diagnostics, GmbH, Germany). Specific oligonucleotide primers were generated by using online assay design software (ProbeFinder http://www.universal-probelibrary.com). The genes are shown in Table 1.

The 20 µl reaction mixture contained 1X LightCycler TaqMan Master reaction mixture (Roche Diagnostics, GmbH), 200 nM of each primer, 100 nM of Universal ProbeLibrary probe, 0.5 U LightCycler Uracil-DNA Glycosylase and 2 µl of standard DNA in the appropriate dilution. The amplification was performed in borosilicate glass capillaries (Roche). The specificity of the reaction products was confirmed by visualization of a single band of the correct size using 2% agarose gel electrophoresis. Each sample was tested in triplicate, and the fold change in mRNA levels was calculated using the standard curve method against 18S rRNA to correct for differences in both RNA quality and quantity.

Quantification of gene expression was calculated using the ΔΔCt method as described by Livak and Schmittgen [13]. This method uses a single sample, the calibrator sample for comparison of every unknown sample’s gene expression. This method of analysis and quantification has been shown to give similar results as the standard curve method [14]. Briefly, \( \Delta \Delta C_{t} = C_{t}(\text{sample}) - C_{t}(\text{calibrator}) \) was calculated for each sample and calibrator. \( \Delta \Delta C_{t} \) was then calculated for each sample, and relative quantification was calculated as 2ΔΔCt.

**Table 1. Nucleotide sequences**

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Primer sets used for real-time PCR designs are based on the ensemble transcript ID of the Human ProbeLibrary.
IMMUNOHISTOCHEMICAL ANALYSIS

Sections of 7 μm were cut into a cryostat and were fixed with acetone for 20 minutes. The slides were then hydrated with phosphate-buffered saline (PBS), and the activity of endogenous peroxidase was blocked by incubation with 0.3% H2O2 and 0.1% of NaN3 in PBS for 10 min. After washing, the samples were incubated with 5% bovine serum albumin for 30 min. A blocker of the endogenous biotin system (BioGenex, Fremont, CA, USA) was used before the application of primary antibodies, following the instructions of the manufacturer. The sections were then washed with light agitation in 0.05% Tween-20 and PBS (PBS-T), incubated with 5% bovine serum albumin for 30 min. A blocker of the endogenous biotin system (BioGenex, Fremont, CA, USA) was then used. The incubation time for the primary antibodies was 12 hours at 4°C, and for the DAKO LSAB+System (Cat. K0679) was then used. The incubation time for the primary antibodies was 12 hours at 4°C, and for the DAKO LSAB+System 60 min in a humidified chamber at room temperature. After incubation the slides were washed with PBS-T for 10 min. The peroxidase reaction was revealed by the Karnovsky method with 3,3′-diaminobenzidine (DAB) (Pierce, Rockford, IL, USA). Control stains were performed following the same protocol for each antibody, with the exception of the primary antibody for which PBS was used. The intensity of the reaction to both molecules was analyzed using the program Image-Pro Plus 5.1, assigning average density arbitrary units.

STATISTICAL ANALYSIS

The data are presented as the mean ± SD of multiple determinations. The significance of differences between experimental and control groups was determined by one-way ANOVA or Kruskal-Wallis according to the data distribution, using GraphPad (PRISM software, version 4, La Jolla, CA, USA). To analyze categorical data we used the chi-square test. P < 0.05 was considered statistically significant, and P < 0.01 highly significant. Correlation analyses between the expression values of genes were performed using Spearman’s correlation coefficient, using IBM SPSS Statistics 20.0.

RESULTS

The age of patients in each of the two SSc subtypes was similar (49.3 ± 12.9 vs. 44.7 ± 13.8 years, for limited vs. diffuse SSc, respectively), as was the time of evolution of SSc. We included 13 healthy controls (9 females, 4 males) whose mean age was 45 ± 14.1 years. Systemic alterations were most severe in the dcSSc group. The patients’ characteristics are summarized in Table 2.

NLRP3 INFLAMMOSOME IN SSC

The relative expression of the NLRP3 inflammasome was elevated in the skin of patients with SSc compared to the control group (6.2 and 1.6-fold increase in lcSSc and dcSSc, respectively, P < 0.01) [Figure 1]. Dermal fibrosis evaluated by MRSS had a significant correlation with NLRP3, IL-1β, IL-18, and ET-1 [Figure 1]. The caspase-1 gene, which is necessary for the activation of IL-1β and IL-18, showed higher expression in the lcSSc group than in the dcSSc or control group (P = 0.04) [Figure 1]. The mRNA for IL-1β and IL-18 showed higher expression in both subtypes of SSc than the controls. As with the NLRP3 and caspase-1 genes, the expression of IL-1β was higher in the lcSSc group than in the dcSSc or control group (P = 0.026) [Figure 1]. The expression of IL-33 was minimal and with no difference in expression between the three groups [Figure 1].

NLRP3 INFLAMMOSOME, CYTOKINES, AND VASCULAR MEDIATOR

The NLRP3 expression was in direct correlation with MRSS and skin expression of caspase-1, IL-1β, IL-18, IL-33, eNOS and ET-1 [Figure 1]. There were no differences in gene expressions when we compared the severities of organ involvement (data not shown).

The relative expression of the ET-1 vascular mediator was also significantly higher in both SSc groups compared to the control group, but ET-2 showed no significant changes among the three groups [Figure 1]. The relative expression of ET-1 correlated positively with MRSS, NLRP3, IL-1β, IL-18 and iNOS enzymes (eNOS and iNOS) [Figure 1].

The expression of eNOS and iNOS increased significantly in the lcSSc group in relation to the dcSSc and control groups [Figure 1]. The gene expression of eNOS was in significant and direct correlation with NLRP3, caspase-1, IL-1β, IL-18 and IL-33, but not with MRSS [Figure 1]. The gene expression of ET and NOS in skin was similar among patients with or without digital ulcers.

TGF-β was approximately four times greater in injured skin of the lcSSc group than in the dcSSc group [Figure 2]. Analyzing all groups of SSc patients, gene expression of TGF-β correlated
Figure 1. Quantitative real-time PCR assays of the NLRP3 inflammasome cytokines and vascular mediators. Total RNA was prepared from human skin of diffuse subtype (dcSSc) and limited subtype (lcSSc) systemic sclerosis patients versus healthy controls. Data represent the mean ± SD. (lcSSc n=21, dcSSc n=21 and control n=13). ANOVA* P < 0.05. Correlations between modified Rodnan skin score (MRSS), NLRP3 inflammasome, pro-inflammatory cytokines, and vascular mediators gene expression in skin of systemic sclerosis patients are shown. They were further analyzed by Spearman’s correlation analysis. Regression values (r) and P values are shown.

<table>
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<th>NLRP3</th>
<th>Casp-1</th>
<th>TGFβ</th>
<th>IL-33</th>
<th>IL-1β</th>
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**P < 0.0001, *P < 0.05
directly with mRNA expression of NLRP3 ($r = 0.667, P < 0.001$) but did not correlate with MRSS, cytokines or vascular mediators [Figure 1].

**IMMUNOHISTOCHEMICAL ANALYSIS**

The expression of the NLRP3 inflammasome and IL-$\beta$ was compared among the three groups by qRT-PCR and immunohistochemical analysis. We found that NLRP3 and IL-$\beta$ expression in affected skin showed different cytoplasmic expression pattern compared to the control group. The strongest staining of keratinizing squamous epithelium was observed in lcSSc and dcSSc skin. Staining was scarcely detectable in healthy skin [Figure 2].

Staining for NLRP3 and IL-$\beta$ in the skin of healthy patients was negative in both epidermis and dermis, showing melanic pigmentation in some germinal layer cells. Staining for NLRP3 and IL-$\beta$ in the skin of lcSSc and dcSSc patients showed a positive reaction in the cytoplasm of cells in every epidermis layer. NLRP3 staining was also positive in the stratum basal/germinatal layer and in some spinous layer/stratum spinosum keratinocytes. As for IL-$\beta$ staining, a more intense reaction was found in the granular layer and the stratum corneum. Melanin was found in some germinal layer cells [Figure 2].

Densitometric analysis of the immunohistochemical assay for NLRP3 and IL-$\beta$ protein expression was assigned a value using arbitrary units. When comparing NLRP3 protein expression in SSC samples to control samples ($5.02 \pm 0.31$), protein levels were significantly higher in both subtypes ($6.42 \pm 0.34$ in lcSSc, $P < 0.001$; $5.74 \pm 0.23$ in dcSSc, $P < 0.01$). Regarding IL-$\beta$, compared to the control group ($6.18 \pm 0.78$), protein levels were also significantly higher in both SSC subtypes ($10.67 \pm 1.17$ in lcSSc, $P < 0.001$; $8.53 \pm 1.06$ in dcSSc, $P < 0.01$) [Figure 2].

**DISCUSSION**

In this study, we demonstrated a significant increase of the NLRP3 inflammasome gene expression in the skin of SSC patients. Severity of dermal fibrosis evaluated by MRSS correlated positively with the overexpression of NLRP3, pro-inflammatory cytokines and ET-1. The potent vasoconstrictor and pro-fibrotic ET-1 was significantly elevated in both groups of SSC compared to the control group. The relative expression of ET-1 correlated positively with MRSS, and expression of NLRP3, IL-$\beta$, IL-$\beta$-18 and enzymes that produce NO (eNOS and iNOS). Of interest, pro-fibrotic TGFβ showed a higher expression only in the lcSSc group compared to controls. TGFβ was in direct correlation with MRSS expression. The skin immunohistochemical analysis showed a significant increase of NLRP3 and IL-$\beta$ in comparison to controls.

We included patients with both lcSSc and dcSSc with the objective of evaluating the presence of inflammasome molecular platform and its relationship to the severity of dermal fibrosis, and comparing the organ damage in SSC subtypes. There were no differences in gene expression when we compared the severities of organ involvement.

The inflammasome is an essential regulator of the processing and secretion of the pro-inflammatory cytokines IL-$\beta$ and IL-18. NLRP3 activation is triggered by a wide range of substances such as various microcrystals, nanoparticles, oxidized DNA, reactive oxygen species, and the list is increasing [15]. In SSC, skin fibrosis is an early characteristic hallmark as a consequence of collagen overproduction and collagen-modifying enzyme imbalance from unknown stimuli. These changes have been linked mainly to fibroblast activation, abnormal production of epithelial/endothelial factors and TGFβ induction [16,17]. Artlett et al. [6] showed that the innate immune response contributes to fibrosis. The expression of genes associated with the inflammasome signaling molecules is increased in SSC fibroblasts. In vitro inhibition of innate immunity via caspase-1 leads to a decrease in collagen secretion from myofibroblasts. In our study, expression of caspase-1 was significantly higher in SSC patients than controls, in direct correlation with all related NLRP3 cytokines and vascular mediators, supporting the participation of an innate immune response in SSC skin.

From TGFβ stimulation, human skin fibroblasts produce connective tissue growth factor (CTGF). The expression of CTGF in the skin and plasma correlates well with the degree of fibrosis [18,19]. Interestingly, in patients with dcSSc, circulating levels of TGFβ are reduced in comparison to both healthy people and patients with lcSSc [3,20]. We also found that in dcSSc skin, TGFβ gene expression was reduced with respect to lcSSc. These paradoxical findings suggest an autocrine regulation in the circulation and in the skin, or the presence of an inhibitor of gene expression of TGFβ.

There is accumulating evidence suggesting that ET-1 is an important mediator in the pathogenesis of SSC, acting not only...
as a potent vasoconstrictor but also as a fibrogenic mediator. In skin from dcSSc patients, staining reveals up-regulation of ET-1 [21]. We observed a significant increase in ET-1 expression in skin from dcSSc and lcSSc patients compared to controls. Beyond the over-expression of ET-1, this correlated positively with MRSS, NLRP3, IL-1β, IL-18, eNOS and iNOS. Previous studies have found up-regulation of ET-1 by IL-1β stimulation, and our results confirm and extend the information about molecular changes in the pathophysiology of SSc [4].

There are controversial reports about nitric oxide (NO) production in SSc patients. Nitric oxide regulates vascular tone and has anti- and pro-inflammatory actions. Its over-production has been implied in endothelial dysfunction in SSc [22]. Additionally, the over-production of reactive oxygen species (ROS), such as superoxide anions (O2-) and peroxynitrite (ONOO-), have also been associated with endothelial dysfunction in SSc [23,24]. In our study, the expression of eNOS and iNOS was significantly higher in lcSSc compared to dcSSc and controls. The eNOS expression correlated positively with NLRP3 and caspase-1, but not with the severity of dermal fibrosis according to MRSS or Raynaud’s phenomenon severity according to Medsger’s scale. The iNOS expression had no correlation with dermal fibrosis and had weak correlation with caspase-1, IL-18 and IL-33. Also, eNOS correlated positively with iNOS expression. These observations indicate that NOS expression could occur from endothelial and inflammatory cells principally in lcSSc and are involved in vascular changes more than dermal fibrosis.

With regard to treatment of skin fibrosis, our findings could have therapeutic implications. Our findings indicate that ET-1 is over-expressed in the skin of SSc patients in direct correlation with MRSS. The receptor antagonist of ET-1, bosentan, competes with ET-1 receptors and has been shown to be effective in treating skin fibrosis [25]. Therefore, the presence of the NLRP3 platform opens the door for new therapeutic options.

In conclusion, this study demonstrates that the inflammasome platform in the skin of patients with SSc is an important sensor of fibrosis and vascular damage. NLRP3 and related cytokine agonists/antagonists could be therapeutic targets for limiting fibrosis in SSc.

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