Transforming growth factor beta isoforms and TGF-βR1 and TGF-βR2 expression in systemic sclerosis patients

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Received: 22 January 2022 / Accepted: 9 May 2022 / Published online: 29 May 2022
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Abstract
Systemic sclerosis (SSc) is characterized by chronic inflammation and fibrosis, two processes associated with transforming growth factor β (TGF-β) functions. In the present study, we investigated the expression of TGF-β isoforms in serum and the skin distribution of TGF-β and two receptors (TGF-βR1 and TGF-βR2) and their relationship with some clinical, inflammatory, autoimmune (autoantibodies), and vascular (platelets) biomarkers in SSc patients. A total of 56 SSc patients and 120 control subjects (CS) were included. The serum levels of TGF-β isoforms were quantified by immunoassay with magnetic microspheres, and the skin biopsies were processed by immunohistochemistry. The soluble levels of the three active TGF-β isoforms were lower in SSc patients than in CS \( (p < 0.0001) \). However, sTGF-β1 and sTGF-β3 levels were positively correlated with C-reactive protein levels in SSc patients. Additionally, sTGF-β2 and sTGF-β3 levels were positively correlated with the number of platelets in SSc patients. In skin biopsies, TGF-β1, TGF-βR1, and TGF-βR2 expression levels were higher in SSc patients than in CS. In conclusion, this is the first study showing a joint decrease of the 3 active TGF-β isoforms in SSc patients. However, TGF-β1, TGF-βR1, and TGF-βR2 are possibly increased in clinically involved skin. Therefore, it is likely that a distinct role is played by TGF-β at the local (skin lesions) and systemic levels in SSc patients.

Keywords sTGF-β · TGF-βR · TGF-β isoforms · Systemic sclerosis · Autoantibodies

Introduction
Systemic sclerosis (SSc) is a complex rheumatic disease characterized by microvasculopathy, inflammation, autoimmunity, and fibrosis [1]. The worldwide prevalence and incidence of SSc are very low (~0.05% and ~0.002%, respectively) [2]; however, the clinical characteristics of SSc patients may involve the skin and some internal organs, giving a cutaneous limited (lcSSc) or cutaneous diffuse (dcSSc) affection, with a very high mortality rate [3, 4].

The molecular mechanisms underlying SSc are not entirely understood; however, pathogenicity mechanisms may be influenced by interactions of variants in immunoregulatory genes with environmental factors [5–7]. The pathogenesis leads to inflammation, autoantibodies synthesis [8], vasculopathy, the activation of myofibroblasts, and the accumulation of fibrillar collagens (types I, II, III, IV, and VII) and elastin fibrils in the extracellular matrix (ECM), with the consecutive formation of fibrosis [9, 10]. In these processes, the platelets are important because beyond regulating
hemostasis, they also have a role in inflammation and innate immunity producing molecules such as β-thrombomodulin, platelet-derived growth factor, or transforming growth factor β (TGF-β), that have been observed to elevated in the serum of patients with SSc [11].

The fibrotic tissue is formed by altered endothelial cells, infiltrated autoimmune cells, and collagen-producing myofibroblasts in the ECM [12]; these cells together promote the synthesis of cytokines (e.g., IL-1, IL-4, or TNF-α) and growth factors such as TGF-β, which regulates collagen deposition and the fibrotic process [13].

TGF-β has three soluble isoforms in latent form (sTGF-β1, 2, and 3), which signal through three different receptors (TGF-βR1, TGF-βR2, and TGF-βR3) which are relevant for the stimulation and development of myofibroblasts [14, 15]. Any of the three isoforms may be activated in the cell membrane by proteases or integrins and bind to the TGF-βR2, recruiting TGF-βR1 and activating the SMAD canonical pathway to regulate the transcription of some genes as collagen [16, 17].

TGF-β has been identified as a critical regulator of fibrogenesis in SSc and an attractive therapeutic target because it regulates several processes, such as cell growth, apoptosis, cell differentiation, and ECM synthesis [15]. Some authors have reported high TGF-β isoform levels in SSc [18], but other studies have reported contradictory [19] or not significant findings [20–22]. These discrepancies could be explained by the pleiotropic activities of this cytokine, which can be different in pathogenic or physiological mechanisms; therefore, blocking its signaling is potentially dangerous, because this could even lead to spontaneous immune activation or epithelial hyperplasia due to TGF-β has important physiologic functions for tissue homeostasis [23].

Considering the lack of knowledge and discrepancies on the TGF-β and receptor isoforms in SSc, we undertook this work to evaluate the expression of TGF-β and receptors I and II in the skin, the soluble levels of TGF-β isoforms, and their relationship with some clinical, inflammatory, autoimmune (autoantibodies), and vascular (platelets) biomarkers in SSc patients.

Materials and methods

Study participants

Serum samples of 56 SSc patients and 120 CS were collected from February 2015 to January 2019 in the Department of Internal Medicine/Rheumatology of the General Hospital of Chilpancingo "Dr. Raymundo Abarca Alarcón", in the state of Guerrero, Mexico. A rheumatologist diagnosed all the patients according to the ACR/EULAR 2013 classification criteria [24] and obtained a clinical record, including an evaluation of the modified Rodnan skin score (MRSS), mouth opening, and the health assessment questionnaire disability index (HAQ-DI).

The CS group included subjects without either known medical conditions or treatment, with similar ages, sex, and living in the same geographic regions as the SSc group. They were excluded from the study if they had a history of autoimmune diseases or had the presence of infection, fibrotic, or inflammatory processes.

Blood samples were obtained by trained personnel from all the participants via antecubital venipuncture after an overnight fast. Erythrocyte sedimentation rate (ESR) was determined using the Westergren method; the C-reactive protein (CRP) was quantified using the Immage™ Immunochemistry system (Beckman Coulter System), and the white blood cell count (WBC) and platelet count were also determined by using standard laboratory techniques for hospital use (Abbott CELL-DYN 3700, Illinois, USA).

For the TGF-β and its receptors I and II expression analysis in the skin, the selected individuals were those who had a medical indication for a biopsy for pathological diagnostic analysis. The expression levels of TGF-β, TGF-βR1, and TGF-βR2 were analyzed in the skin biopsies of twelve SSc patients and two CS.

All the procedures performed were adhered to and approved by the ethical standards of the Local Bioethic Committee at the Hospital General de Chilpancingo "Dr. Raymundo Abarca Alarcón" (CI/317/2016) and with the 1964 Helsinki Declaration and its later amendments. Informed consent was obtained from all subjects involved in the study.

Quantification of autoantibodies

Following the manufacturer’s recommendations, the tests for antinuclear antibodies (ANAs) were performed through indirect immunofluorescence microscopy using multisport slides with fixed Hep-2 cells (BioSystems, Barcelona, Spain). The autoantibodies anti-topoisomerase (anti-Scl70, BioSystems Cat. No. COD44863), anti-centromere (CENP-B, BioSystems Cat. No. COD44865), anti-fibrillarin (AFA, CUSABIO Cat. No. COD44863), anti-centromere (CENP-B, BioSystems Cat. No. COD44865), anti-fibrillarin (AFA, CUSABIO Cat. No. CSB-E09697h), and anti-RNA polymerase III (anti-RNA PolIII, CUSABIO Cat. No. CSBEQ027833HU) were measured in all SSc patients with a second-generation enzyme-linked immunosorbent assay (ELISA), following to the manufacturer’s instructions.

Quantification of TGF-β isoforms

The soluble TGF-β (sTGF-β) isoforms were quantified in serum by a multiplex test based on magnetic beads "BioPlex Pro™ TGF-β Assays" (Bio-Rad Laboratories, Hercules, CA, USA). This test recognizes the TGF-β isoforms in their
with 3,3′-diaminobenzidine and stained with hematoxylin.

Immunohistochemical expression of TGF-β, TGF-βR1, and TGF-βR2

Skin biopsies were obtained during serum collection and were fixed in Michel’s solution for later inclusion in paraffin. In SSc patients, the biopsies were taken from a 4 mm puncture with previous anesthesia in the sun-exposed skin around proximal interphalangeal joints (clinically involved skin). For CS, biopsy specimens were obtained from the dorsal surface of the distal forearm.

The samples were embedded in paraffin and cut into 3 μm sections for mounting on charged slides.

Deparaffinization, epitope retrieval, and immunohistochemistry were performed in the BOND MAX automated staining system (Leica Biosystems, Germany), following the manufacturer’s instructions. The deparaffinization process was carried out under heat and with Bond Dewax solution (Leica AR9222). Pretreatment with BOND Epitope Retrieval Solution 1(citrate solution pH = 6) (Leica AR9961) was carried out for 20 min to perform the heat-induced epitope retrieval (HIER). Subsequently, the immunodetection reaction was carried out with the BOND Polymer Refine Detection System (Leica cat. DS9800). The reaction was detected with 3,3′-diaminobenzidine and stained with hematoxylin.

The sections from each biopsy were incubated for 30 min with one of the following primary antibodies: Anti-TGF-β1 antibody [TB21] (ab27969, 1: 300), Anti-TGF beta Receptor I antibody (ab31013, 1: 25), or Anti-TGF-β Receptor II antibody [MM0056-4F14] (ab78419, 1: 50). Positive controls were used for each antibody, and negative controls were conducted without the primary antibody. After staining, the images were qualitatively analyzed by two pathology specialists. Slides were captured with a digital pathology slide scanner (Aperio LV1, Leica Biosystems, Germany). The positive cells for each label were quantified according to their lineage, using a positive control as a reference.

Statistical analysis

All the data were analyzed using the GraphPad Prism v8.0 software and the R version 4.0.0 statistical software program. The Shapiro–Wilks normality test was applied to probe the normal distribution of the data. For descriptive analysis, categorical variables were expressed as frequencies; continuous variables with nonparametric distribution were expressed as medians and percentiles 5th–95th, and parametric variables were expressed as mean ± standard deviation (SD). All data compare between groups showed nonparametric distribution; therefore, the Mann–Whitney U test was used to evaluate the differences between quantitative determinations of two groups, and the Kruskal–Wallis test was for quantitative determinations of three groups. For inferential analysis, the χ2 test was used to compare proportions. To determine the correlations between TGF-β with the clinical variables of SSc patients, we used Spearman correlation tests. To eliminate confounding variables in the correlations, multiple linear regression analysis with the stepwise method was used. The stepwise method created different models. The isoforms were used as the dependent variables, and those variables with a p value of less than 0.10 in the Spearman correlation analysis were used as predictors. The final model for each of the isoforms was the one with the lowest AIC (Akaike information criterion) with a confidence interval of 95 %. A p value < 0.05 was considered significant for all these analyses.

Results

Clinical and demographic characteristics

The clinical and demographic characteristics of both groups are described in Table 1. SSc patients were matched to the CS in age and gender. We observed that limited cutaneous SSc (lcSSc) was more frequent than diffuse cutaneous SSc (dcSSc) (84% vs. 16%, respectively). The average age of disease onset was 40 years old, and the time of disease evolution was five years.

The most prevalent symptoms and signs in SSc patients were sclerodactyly (93%), arthritis (88%), Raynaud’s phenomenon (73%), and puffy fingers (70%). Most patients had an oral opening of 5 cm, MRSS of 6, and an HAQ-DI value of 0.3. Moreover, antinuclear antibodies were present in 77% of patients.

Regarding treatment, methotrexate was the drug more commonly used (43%) by the patients, followed by prednisone (36%) and nonsteroidal anti-inflammatory drugs (NSAIDs) (16%); on the other hand, 39% of patients were receiving no treatment at the time of sampling.

sTGF-β concentrations in SSc patients and CS

Lower concentrations of the three sTGF-β isoforms (sTGF-β1, 2, and 3) were observed in the patients than
CS ($p < 0.0001$, Fig. 1A–C). The comparison of TGF-β1, TGF-β2, and TGF-β3 levels between treated and untreated patients did not exhibit significant differences ($p > 0.05$, Fig. 1D–F).

On the other hand, in SSc patients, sTGF-β1 was negatively correlated with age ($rs = -0.329$, $p = 0.02$) and anti-centromere antibodies (ACAs) levels ($rs = -0.344$, $p = 0.014$), whereas it was positively correlated with C-reactive protein (CRP) levels ($rs = 0.540$, $p = 0.033$) and the number of platelets ($rs = 0.436$, $p = 0.004$), as shown in Table 2. sTGF-β2 levels were negatively correlated with age ($rs = -0.283$, $p = 0.046$) and ACA levels ($rs = -0.291$,
p = 0.040), whereas they were positively correlated with platelets (rs = 0.401, p = 0.008). Additionally, we observed that sTGF-β3 was positively correlated with CRP levels (rs = 0.654, p = 0.008), ACA levels (rs = 0.418, p = 0.0001), anti-RNA pol III levels (rs = 0.291, p = 0.040), and the number of platelets (rs = 0.397, p = 0.008) (Table 2).

In order to identify that the TGF-β isoforms association was not modified by the presence of other confounding variables, multiple linear regression models were constructed (one for each isoform) by the stepwise method.

Table 3 shows the results of multiple linear regression. In model A, the sTGF-β1 levels remained associated with CRP (β coefficient = 142.2; 95% CI = 48.2 to 236.3, p = 0.007) after adjusting for confounding variables. In contrast, in model B, the sTGF-β2 levels after adjustment only remained associated with the number of platelets (β coefficient = 0.124; 95% CI = 0.05 to 0.24, p = 0.04). Finally, model C shows that the association of sTGF-β3 levels with CRP (beta coefficient = 0.30; 95% CI = 0.04 to 0.55, p = 0.003) and the number of platelets (beta coefficient = 0.04; 95% CI = 0.014 to 0.08, p = 0.004).

Expression of TGF-β, TGF-βR1, and TGF-βR2 in the skin

The tissue expression levels of TGF-β, TGF-βR1, and TGF-βR2 were documented in the dermis and epidermis of SSc patients and CS by IHC (Fig. 2). We evaluated by cell line (endothelium, fibroblasts, and keratinocytes) and subcellular location (nucleus, cytoplasm, and membrane). The intensity of the mark was qualitatively scored as weak (+), intermediate (++), and high (+++). The intensity of the mark was qualitatively scored as weak (+), intermediate (++), and high (+++). The intensity of the mark was qualitatively scored as weak (+), intermediate (++), and high (+++). The intensity of the mark was qualitatively scored as weak (+), intermediate (++), and high (+++). The intensity of the mark was qualitatively scored as weak (+), intermediate (++), and high (+++). The intensity of the mark was qualitatively scored as weak (+), intermediate (++), and high (+++). The intensity of the mark was qualitatively scored as weak (+), intermediate (++), and high (+++). The intensity of the mark was qualitatively scored as weak (+), intermediate (++), and high (+++). The intensity of the mark was qualitatively scored as weak (+), intermediate (++), and high (+++). The intensity of the mark was qualitatively scored as weak (+), intermediate (++), and high (+++). The intensity of the mark was qualitatively scored as weak (+), intermediate (++), and high (+++).

Regarding TGF-β1 (Fig. 2a,b), in CS tissues, a weak cytoplasmic signal was only evidenced in endothelial cells of the superficial vascular plexus of the dermis, whereas fibroblasts and keratinocytes were negative. In contrast, in SSc patients, expression was documented in all evaluated cell types. Expression in the endothelium and keratinocytes predominated over fibroblasts. The nuclear signal was more frequent and intense in both cell types (endothelium and keratinocytes) than the cytoplasmic expression. In fibroblasts, the presentation was inverse, with more cytoplasmic intensity than the nuclear mark. Furthermore, the TGF-β1 expression tended to be more frequently positive in dcSSc than lcSSc (Fig. 3a).
Table 2  Correlation of sTGF-β isoforms with clinical variables in SSc patients

| Clinical variable                  | sTGF-β1 (pg/mL) | sTGF-β2 (pg/mL) | sTGF-β3 (pg/mL) |
|-----------------------------------|----------------|----------------|----------------|
|                                   | Rs            | p              | Rs            | p              | Rs            | p              |
| Age (years)                       | −0.329        | 0.020          | −0.283        | 0.046          | −0.263        | 0.065          |
| Age of disease onset (years)      | 0.210         | 0.143          | 0.192         | 0.182          | 0.125         | 0.386          |
| Mouth opening (cm)                | 0.037         | 0.807          | 0.019         | 0.902          | 0.016         | 0.914          |
| ESR (mm/h)                        | 0.030         | 0.848          | 0.031         | 0.841          | 0.045         | 0.774          |
| CRP (mg/dL)                       | 0.540         | 0.033          | 0.476         | 0.064          | 0.654         | 0.008          |
| ACAs                              | −0.344        | 0.014          | −0.291        | 0.040          | 0.418         | 0.0001         |
| ATAs                              | 0.098         | 0.503          | 0.150         | 0.303          | 0.033         | 0.823          |
| Anti-RNA Pol III                  | 0.271         | 0.057          | 0.255         | 0.074          | 0.291         | 0.040          |
| Anti-fibrillarin                  | 0.046         | 0.751          | 0.041         | 0.776          | 0.009         | 0.949          |
| Leukocytes                        | 0.259         | 0.093          | 0.277         | 0.072          | 0.166         | 0.286          |
| Erythrocytes                      | 0.249         | 0.107          | 0.246         | 0.112          | 0.159         | 0.310          |
| Hematocrit                        | 0.254         | 0.101          | 0.177         | 0.256          | 0.099         | 0.526          |
| Hemoglobin                        | 0.267         | 0.084          | 0.216         | 0.164          | 0.114         | 0.469          |
| Platelets                         | 0.436         | 0.004          | 0.401         | 0.008          | 0.397         | 0.008          |
| Glucose                           | 0.032         | 0.854          | 0.018         | 0.916          | 0.165         | 0.345          |
| Cholesterol                       | 0.178         | 0.321          | 0.256         | 0.151          | 0.044         | 0.808          |
| Albumin                           | 0.292         | 0.089          | 0.254         | 0.142          | 0.241         | 0.163          |
| Weight                            | 0.076         | 0.669          | 0.055         | 0.752          | 0.077         | 0.661          |
| Height                            | 0.127         | 0.482          | 0.081         | 0.654          | 0.104         | 0.564          |
| BMI                               | 0.149         | 0.318          | 0.135         | 0.366          | 0.056         | 0.708          |
| HAQ-DI score                      | 0.283         | 0.054          | 0.178         | 0.232          | 0.247         | 0.094          |
| VAS score                         | 0.004         | 0.978          | 0.029         | 0.850          | 0.071         | 0.643          |
| MRSS                              | 0.156         | 0.285          | 0.078         | 0.594          | 0.053         | 0.716          |

ESR erythrocyte sedimentation rate; CRP C-reactive protein; ACAs anti-centromere antibodies; ATAs anti-topoisomerase antibodies; BMI body mass index; HAQ-DI health assessment questionnaire disability index; VAS visual analog scale; MRSS modified Rodnan skin score

Data were analyzed by Spearman correlation (rs). This analysis was carried out for all SSc patients (n = 56). Bold letters indicate significant correlations.

Table 3  Multiple linear regression analysis for the correlation of sTGF-β isoforms with clinical variables in SSc patients

| Variable                  | Model A | Model B | Model C |
|---------------------------|---------|---------|---------|
|                           | sTGF-β1 | sTGF-β2 | sTGF-β3 |
| β coefficient             | IC95%   | p       | IC95%   | p       | IC95%   | p       |
| Age (years)               | Not in the model | – | Not in the model | – | Not in the model | – |
| Age of disease onset (years) | Not in the model | – | Not in the model | – | Not in the model | – |
| CRP (mg/dL)               | 142.2   | 48.2 to 236.3 | 0.007 | Not in the model | – | 0.30 | 0.04 to 0.55 | 0.003 |
| ACAs                      | Not in the model | – | Not in the model | – | Not in the model | – |
| Anti-RNA Pol III          | Not in the model | – | Not in the model | – | Not in the model | – |
| Leukocytes                | Not in the model | – | Not in the model | – | Not in the model | – |
| Platelets                 | Not in the model | – | 0.124 | 0.05 to 0.24 | 0.04 | 0.04 | 0.014 to 0.08 | 0.004 |
| HAQ-DI score              | Not in the model | – | Not in the model | – | Not in the model | – |

For model A, the dependent variable was the sTGF-β1 level. For model B, the dependent variable was the sTGF-β2 level. For model C, sTGF-β3 was selected as the dependent variable.

CRP C-reactive protein; ACAs anti-centromere antibodies; HAQ-DI health assessment questionnaire disability index. CI95%: 95% confidence intervals; β: beta)

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Concerning TGF-βR1 (Fig. 2c,d), in patients and CS, the intensity of the expression range was from low to intermediate. In CS, cytoplasmic expression was observed in the three evaluated cell types, with a nuclear expression of moderate intensity only in keratinocytes. In SSc patients, the cytoplasmic expression was similar to the CS; however, there was no nuclear expression in any cell type in patients, and expression was observed in the membrane of the basal stratum keratinocytes. The membrane expression in basal keratinocytes was observed in all the patients with dcSSc, whereas it was only present in 60% of lcSSc cases (Fig. 3b).

Regarding TGF-βR2 (Fig. 2d,e), in CS, a weak signal was observed in the basal keratinocytes and endothelial cells. In contrast, no expression was observed in the fibroblast or the rest of the epidermis strata. In patients, a weak to intermediate expression was observed in the cytoplasm of the fibroblast and endothelium. The most relevant expression was found in basal keratinocytes whose positivity, although low in intensity, was documented in 92% of cases. In no cases was nuclear or membranous expression observed (Table 4). The overall expression tendency of the TGF-βR2 receptor was more frequent in dcSSc than in lcSSc patients (Fig. 3c).
Finally, TGF-β1 expression in the epidermis of the patients was compared between SSc patients with or without treatment; however, no significant differences were observed ($p > 0.05$, data not shown).

**Discussion**

There are three isoforms of TGF-β, each of them having an important role in fibrosis by activating the canonical SMAD pathway, implicated in different cellular processes such as wound healing and regeneration [17]. TGF-β1 has been associated with the pro-fibrotic process because it is involved in fibroblast activation and collagen production stimulation, whereas TGF-β2 and TGF-β3 presumably have anti-fibrotic roles [17, 25].

Surprisingly, in our study, we observed low levels of all three TGF-β isoforms in SSc patients compared to that observed in CS. This result is in accordance with one study reported in the United Kingdom for sTGF-β1 [19], but in contrast to one reported in Brazil, where sTGF-β1 was higher in SSc patients than in CS [18]. Other studies have reported no significant differences [20–22].

The discrepancies observed in SSc patients regarding TGF-β isoform levels must be clarified with further studies; however, some authors suggest that those could be due to methodological differences in the quantification of TGF-β isoforms because not all of them discriminate between the latent and active forms [19, 20]. Dziadzio et al. evaluated both forms (latent and active) and only found differences for the active TGF-β1 form, which was lower in dcSSc patients than in CS [19]. Similarly to that study, we evaluated the active form of the three TGF-β isoforms. Therefore, the active TGF-β isoforms could have a physiological immunomodulatory role in healthy individuals, and this function could be decreased in SSc patients. Furthermore, we reject that this diminution in active TGF-β isoforms was due to the treatment of the patients because the levels of these isoforms did not vary between individuals with and without treatment.

TGF-β isoforms can be activated by a combination of proteolysis and low pH; however, these forms are also rapidly cleared by mechanisms that include binding to α2-macroglobulin (α2M) or soluble TGF-β receptors and clearance via the liver [19, 26, 27]. In this context, we suggest a possible dysregulation in these processes in SSc at the systemic level. Nevertheless, how these mechanisms are triggered remains unclear, although links between

![Fig. 3 Expression of TGF-β1, TGF-βR1, and TGF-βR2 in the skin of patients with dcSSc and lcSSc: The expression frequencies of the TGF-β1 (a), TGF-βR1 (b), and TGF-βR2 (c) in the skin sections of patients with dcSSc and lcSSc were compared. The results are presented by cell line and subcellular location. C: cytoplasm, N: nucleus, B: basal, S: superficial, M: membrane, dcSSc: diffuse cutaneous systemic sclerosis, lcSSc: limited cutaneous systemic sclerosis. This analysis was carried out on 12 SSc patients (3 patients with dcSSc and 9 with lcSSc). Graphics made with GraphPad Prism v8.0](image-url)
inflammation and TGF-β activation are emerging [13], which provides an increasingly secure framework for understanding TGF-β in SSc pathogenesis.

In other fibrotic diseases such as localized scleroderma (LS), Budzyńska-Włodarczyk et al. reported lower serum TGF-β2 and TGF-β1 levels in patients than in CS [28]. Based on a previous IHC skin biopsy study showing a high binding of TGF-β1 and TGF-β2 proteins in the inflammatory skin of SSc patients [29], Budzyńska-Włodarczyk et al. suggest that the decreases in circulatory TGF-β1 and TGF-β2 could be an indirect effect of their accumulation in inflammatory infiltrations of the skin [28].

On the other hand, in the present study, the sTGF-β2 and sTGF-β-3 levels were positively correlated with the number of platelets of SSc patients. Similarly, sTGF-β1 and sTGF-β-3 levels were positively correlated with CRP levels in SSc patients. Both correlations remained after adjusting for confounding variables (multivariate analysis).

Unlike TGF-β1, the correlation of TGF-β2 and TGF-β3 with platelets has not been well described. However, high TGF-β2 and TGF-β3 expression levels have been reported in megakaryocytes [30]. In addition, after an injury, platelets expressing TGF-β-3 have been reported [31].

Platelets contain 40 to 100 times as much TGF-β1 as other cells, and they rapidly release it on activation [32, 33]. In this sense, it is well known that in the setting of SSc, platelets are detectable in a persistent activated state, which is intimately linked to the concomitant presence of injured endothelium and to the widespread activation of the innate and adaptive immune system [34]. Moreover, platelets can convert latent TGF-β to its active form, which can stimulate nearby fibroblasts and trigger the fibrotic process in SSc. Therefore, TGF-β may enhance platelet activation leading to further TGF-β release and vascular damage, thus creating a vicious cycle with potential pathogenetic implications in SSc [35].

On the other hand, sTGF-β1 and 3 were also positively correlated with CRP levels. This finding is a poorly explored topic; however, TGF-β has been reported as a potential regulator of CRP synthesis by hepatocytes at the post-transcriptional level [36].

In another autoimmune disease such as primary Sjogren’s syndrome, the serum CRP and TGF-β1 concentrations have also been positively correlated \( (r=0.786, p<0.01) \) [37]. The correlation between these two cytokines could be because CRP may induce the synthesis of proinflammatory cytokines such as IL-1β and TNF-α [38], both of which can induce TGF-β expression [39, 40]. Moreover, CRP is an inflammation marker that can promote its effects and regulation through the TGF-β pathway and is related to the severity of the disease [36, 41, 42].

Descriptive analysis of TGF-β expression in skin biopsies showed that SSc patients express more TGF-β in the fibroblasts, keratinocytes, and extracellular matrix than CS. This result agrees with a previous study, where the three TGF-β isoforms were found at higher levels in SSc patients than in controls [29], although this is in contrast to a previous study where only TGF-β2 isoform was reportedly expressed in the dermis, and there was no observed TGF-β1 expression in the dermis [43].

These data also contrast with those observed in the levels of sTGF-β. This could indicate that TGF-β is elevated at a local level (in the skin) but is not at a systemic level; therefore, the role of this cytokine could be tissue-specific in SSc. In this regard, we observed that TGF-β1 expression predominated in the endothelium and keratinocytes in comparison with fibroblasts. This finding is important because the etiology of vascular damage in SSc is uncertain, but immunological dysfunction appears to play a key role [15]. Therefore, TGF-β1 could lead the capillary architecture involvement in patients with SSc and stimulate angiogenesis.

Similarly, the TGF-βR1 and TGF-βR2 receptors were also more expressed in the skin of SSc patients than in CS. These results agree with a previous study in Japan where they found overexpressed TGF-β receptors I and II in the dermal fibroblasts of patients with SSc and remark on their role in the pathogenesis of the disease [44].

Interestingly, nuclear and cytoplasmic expression of TGF-βR1 was observed in CS, whereas in SSc patients it was only found in the membrane and cytoplasm. To the best of our knowledge, the nuclear expression of TGF-βR1 has not been described in open databases such as the Human Protein Atlas [45], and this may be due to a regulation process that has not previously been reported. A limitation of our study was the use of IHC for this observation; therefore, this finding must be corroborated by more sensitive tools such as immunofluorescence. The lack of understanding of these findings can be understood from some of the following studies that have been conducted.

The TGF-βR1 receptor trafficking to the membrane is modulated by some adaptor proteins and enzymes that promote or inhibit the canonical and non-canonical TGF-β pathways by binding to some TGF-βR1-specific domains [46, 47]. TACE is an enzyme that cleaves TGF-βR1 in cancer cells and liberates an intracellular domain (ICD) that binds with p300 in the nucleus and participates in proliferative gene transcription [48]. However, the translocation of TGF-βR1 to the nucleus in healthy tissue cells has not been reported; thus, further studies are necessary to observe the functional effects of the nuclear translocation of TGF-βR1 and 2. Understanding the pleiotropic role of TGF-β in the pathology of SSc could provide new therapeutic strategies and a better understanding of this severe disease.
Conclusions

In conclusion, our findings showed that, in the serum of SSc patients, there is a downregulation of active sTGF-β1, 2, and 3 isoforms; however, the expression of TGF-β, TGF-βR1, and TGF-βR2 is elevated in the skin lesions. This supports a specific role of this multifunctional cytokine at the local and systemic levels in SSc. Moreover, this study highlights the importance of clarifying the possible relationship of TGF-β with platelets and CRP, which could contribute to the explanation of the multiple roles of TGF-β on inflammation, fibrosis, and thrombosis in SSc.

Author Contributions Conceptualization, JHB; Data curation, JALN and JFMY; Formal analysis, CJBH, SGA, MGRD, and IPR; Funding acquisition, JFMY and JHB; Methodology, JALN, JENZ, MGR, SGA, MGRD, IPR, and AVP; Resources, JALN and JENZ; Writing—original draft preparation, JALN and CJBH; Writing—review and editing, JFMY, SGA, and JHB. All authors have read and agreed to the published version of the manuscript.

Funding This research was funded by the PRODEP- SEP Mexico, under Grant (number UDG-PTC-1433) assigned to J.H.-B; by the University of Guadalajara—Strengthening Research and Postgraduate 2018, under Grant (number 244159) assigned to J.F.M.-V, and by the Universidad de Guadalajara through Fortalecimiento de la Investigación y el Posgrado 2021.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the Local Bioethical Committee at the Hospital General de Chilpancingo “Dr. Raymundo Abarca Alarcón” (CI/317/2016) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study. In this consent, the participants accepted to participate in the study and to publish their results.

References

1. Barsotti S, Bruni C, Orlandi M, Della Rossa A, Marasco E, Codullo V, et al. One year in review 2017: systemic sclerosis. Clin Exp Rheumatol. 2017;35(Suppl 106):3–20.
2. Royle JG, Lanyon PC, Grainge MJ, Abhishek A, Pearce FA. The incidence, prevalence, and survival of systemic sclerosis in the UK clinical practice research datalink. Clin Rheumatol. 2018;37:2103–11.
3. Baños-Hernández CJ, Navarro-Zarza JE, Bucala R, Hernández-Bello J, Parra-Rojas I, Ramírez-Dueñas MG, et al. Macrophage migration inhibitory factor polymorphisms are a potential susceptibility marker in systemic sclerosis from southern Mexican population: association with MIF mRNA expression and cytokine profile. Clin Rheumatol. 2019;38:1643–54.
4. Rubio-Rivas M, Corbella X, Pestaña-Fernández M, Tolosa-Vilella C, Guillen-Del Castillo A, Colunga-Argüelles D, et al. First clinical symptom as a prognostic factor in systemic sclerosis: results of a retrospective nationwide cohort study. Clin Rheumatol. 2018;37:999–1009.
5. Lomeli-Nieto JA, Muñoz-Valle JF, Baños-Hernández CJ, Navarro-Zarza JE, Ramírez-Dueñas MG, Sánchez-Hernández PE, et al. TNFA -308G>A and -238G>A polymorphisms and risk to systemic sclerosis: impact on TNF-α serum levels, TNFA mRNA expression, and autoantibodies. Clin Exp Med. 2019;19:439–47.
6. Bossini-Castillo L, Campillo-Daví D, López-Isac E, Carmona FD, Simeon CP, Carreira P, et al. An MIF promoter polymorphism is associated with susceptibility to pulmonary arterial hypertension in diffuse cutaneous systemic sclerosis. J Rheumatol. 2017;44:1453–7.
7. Machado-Sulbaran AC, Ramírez-Dueñas MG, Navarro-Zarza JE, Muñoz-Valle JF, Mendoza-Carrera F, Baños-Hernández CJ, et al. KIR/HLA gene profile implication in systemic sclerosis patients from Mexico. J Immunol Res. 2019;2019:6808601.
8. Yang C, Tang S, Zha D, Ding Y, Qiao J. Classical disease-specific autoantibodies in systemic sclerosis: clinical features, gene susceptibility, and disease stratification. Front Med. 2020. https://doi.org/10.3389/fmed.2020.587773.
9. Bhattacharyya S, Wei J, Varga J. Understanding fibrosis in systemic sclerosis: shifting paradigms, emerging opportunities. Nat Rev Rheumatol. 2011;8:42–54.
10. Wynn TA. Cellular and molecular mechanisms of fibrosis. J Pathol. 2008;214:199–210.
11. Hirigoyen D, Burgos PI, Mezzano V, Duran J, Barrientos M, Saenz CG, et al. Inhibition of angiogenesis by platelets in systemic sclerosis patients. Arthritis Res Ther. 2015;17:332.
12. Jinnin M. Mechanisms of skin fibrosis in systemic sclerosis. J Dermatol. 2010;37:11–25.
13. Lafyatis R. Transforming growth factor β—at the centre of systemic sclerosis. Nat Rev Rheumatol. 2014;10:706–19.
14. Farina G, Lafyatis D, Lemaire R, Lafyatis R. A four-genic biomarker predicts skin disease in patients with diffuse cutaneous systemic sclerosis. Arthritis Rheum. 2010;62:580–8.
15. Ayers NB, Sun C-M, Chen S-Y. Transforming growth factor-β signaling in systemic sclerosis. J Biomed Res. 2018;32:3–12.
16. Vander Ark A, Cao J, Li X. TGF-β receptors: In and beyond TGF-β signaling. Cell Signal. 2018;52:112–20.
17. Lichtman MK, Otero-Vinas M, Falanga V. Transforming growth factor beta (TGF-β) isoforms in wound healing and fibrosis. Wound Repair Regen Off Publ Wound Heal Soc Eur Tissue Repair Soc. 2016;24:215–22.
18. Dantas AT, Gonçalves SMC, de Almeida AR, Gonçalves RSG, Sampaio MCPD, de Vilar KM, et al. Assessing the role of the active TGF-β1 as a biomarker in systemic sclerosis: association of serum levels with clinical manifestations. Dis Markers. 2016. https://doi.org/10.1155/2016/6064830.
19. Dziadzio M, Smith RE, Abraham DJ, Black CM, Denton CP. Circulating levels of active transforming growth factor betal are reduced in diffuse cutaneous systemic sclerosis and correlate inversely with the modified Rodnan skin score. Rheumatol Oxf Engl. 2005;44:1518–24.
20. Majewski D, Majewska KA, Kuznar-Kaminska B, Runowska M, Piorunek T, Batura-Gabryel H, et al. Systemic sclerosis and serum content of transforming growth factor. Adv Exp Med Biol. 2019;1153:63–7.
21. Sato S, Hasegawa M, Takehara K. Serum levels of interleukin-6 and interleukin-10 correlate with total skin thickness score in patients with systemic sclerosis. J Dermatol Sci. 2001;27:140–6.
22. Scala E, Pallotta S, Frezzolini A, Abeni D, Barbieri C, Sampogna F, et al. Cytokine and chemokine levels in systemic sclerosis: relationship with cutaneous and internal organ involvement. Clin Exp Immunol. 2004;138:540–6.
23. Varga J, Pasche B. Transforming growth factor-β as a therapeutic target in systemic sclerosis. Nat Rev Rheumatol. 2009;5:200–6.
24. van den Hoogen F, Khanna D, Fransen J, Johnson SR, Baron M, Tyndall A, et al. 2013 classification criteria for systemic sclerosis: an American College of Rheumatology/European League against Rheumatism collaborative initiative. Arthritis Rheum. 2013;65:2737–47.
25. Sgonc R, Wick G. Pro- and anti-fibrotic effects of TGF-beta in scleroderma. J Dermatol Sci. 2009;47(Suppl 5):v5-7.
26. Blobe GC, Schiemann WP, Lodish HF. Role of transforming growth factor beta in human disease. N Engl J Med. 2000;342:1350–7.
27. Heldin CH, Miyazono K, ten Dijke P. TGF-β1-beta signalling from cell membrane to nucleus through SMAD proteins. Nature. 1997;390:465–71.
28. Budzynska-Wlodarczyk J, Michalska-Jakubus MM, Kowal M, Krasowska D. Evaluation of serum concentrations of the selected cytokines in patients with localized scleroderma. Postepy Dermatol Alergol. 2016;33:47–51.
29. Querfeld C, Eckes B, Huerkamp C, Krieg T, Sollberg S. Expression of TGF-beta isoforms in the skin of patients with systemic sclerosis. J Dermatol Sci. 1999;21:13–22.
30. Yoon S-Y, Tefferi A, Li C-Y. Platelet- and interleukin-10 correlate with total skin thickness score in patients with systemic sclerosis? Arthritis Care Res. 2013;65:1375–80.
31. Kohama K, Nonaka K, Hosokawa R, Shum L, Ohishi M. TGF-beta-3 promotes scarless repair of cleft lip in mouse fetuses. Acta Haematol Karger Publishers. 2000;104:151–7.
32. Assoian RK, Komoriya A, Meyers CA, Miller DM, Sporn MB. Transforming growth factor-beta in human platelets. Identification of a major storage site purification and characterization. J Biol Chem. 1983;258:7155–60.
33. Meyer A, Wang W, Qu J, Croft L, Degen JL, Coller BS, et al. Platelet TGF-β1 contributions to plasma TGF-β1, cardiac fibrosis, and systolic dysfunction in a mouse model of pressure overload. Blood. 2012;119:1064–74.
34. Ramirez GA, Franchini S, Rovere-Querini P, Subbadini MG, Manfredi AA, Mauger N. The role of platelets in the pathogenesis of systemic sclerosis. Front Immunol. 2012;3:160.
35. Netelis K, Bogdanos D, Dimitroulas T, Sakkas L, Daoussis D. Platelets in systemic sclerosis: The missing link connecting vasculopathy, autoimmunity, and fibrosis? Curr Rheumatol Rep. 2019;21:15.
36. Taylor AW, Ku NO, Mortensen RF. Regulation of cytokine-induced human C-reactive protein production by transforming growth factor-beta. J Immunol Baltim Md. 1990;145(10):2507–13.
37. Gao J. Changes and significance of serum C-reactive protein and transforming growth factor-β 1 levels in patients with primary Sjögren’s syndrome. Chin J Rheumatol. 2020;12:125–8.
38. Zhang R, Zhang YY, Huang XR, Wu Y, Chung ACK, Wu EX, et al. C-reactive protein promotes cardiac fibrosis and inflammation in angiotensin II-induced hypertensive cardiac disease. Hypertens Am Heart Assoc. 2010;55:953–60.
39. Sullivan DE, Ferris M, Nguyen H, Abboud E, Brody AR. TNF-alpha induces TGF-beta1/beta1 expression in lung fibroblasts at the transcriptional level via AP-1 activation. J Cell Mol Med. 2009;13:1866–76.
40. Yue TL, Wang XK, Olson B, Feuerstein G. Interleukin-1 beta (IL-1 beta) induces transforming growth factor-beta, (TGF-beta 1) production by rat aortic smooth muscle cells. Biochem Biophys Res Commun. 1994;204:1186–92.
41. Sun W, Yu Y, Gao M, Tian Y, Qi P, Shen Y, et al. C-reactive protein promotes inflammation through TLR4/NF-κB/TGF-β pathway in HL-1 cells. 2019. Biosci Rep. https://DOI.org/10.1042/BSR20190888.
42. Liu X, Mayes MD, Pedroza C, Draeger HT, Gonzalez EB, Harper BE, et al. Does C-reactive protein predict the long-term progression of interstitial lung disease and survival in patients with early systemic sclerosis? Arthritis Care Res. 2013;65:1375–80.
43. Sfikakis PP, McCune BK, Tsokos M, Aroni K, Vayiopoulos G, Tsokos GC. Immunohistological demonstration of transforming growth factor-beta isoforms in the skin of patients with systemic sclerosis. Clin Immunol Immunopathol. 1993;69:199–204.
44. Kubo M, Iin H, Yamane K, Tamaki K. Upregulated expression of transforming growth factor-beta receptors in dermal fibroblasts of skin sections from patients with systemic sclerosis. J Rheumatol. 2002;29:2558–64.
45. Cell atlas - TGFBR1 - The Human Protein Atlas [Internet]. [cited 2021 Aug 7]. Available from: https://www.proteinatlas.org/ENSG00000106799-TGFBR1/cell.
46. Yakymovych I, Yakymovych M, Zang G, Mu Y, Bergh A, Landstrom M, et al. CIN85 modulates TGFβ signaling by promoting the presentation of TGFβ receptors on the cell surface. J Cell Biol. 2015;210:319–32.
47. Yakymovych I, Yakymovych M, Heldin C-H. Intracellular trafficking of transforming growth factor β receptors. Acta Biochim Biophys Sin. 2018;50:3–11.
48. Mu Y, Sundar R, Thakur N, Ekman M, Gudey SK, Yakymovych M, et al. TRAF6 ubiquitinates TGFβ type I receptor to promote its cleavage and nuclear translocation in cancer. Nat Commun. 2011;2:330.

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