Transforming Growth Factor Beta Isoforms and TGF- β R1 and TGF- β R2 Expression in Systemic Sclerosis Patients

José Alvaro Lomelí-Nieto

Instituto de Investigación en Ciencias Biomédicas, Centro Universitario de Ciencias de la Salud, Universidad de Guadalajara

José Francisco Muñoz-Valle

Instituto de Investigación en Ciencias Biomédicas, Centro Universitario de Ciencias de la Salud, Universidad de Guadalajara

Christian Johana Baños-Hernández

Instituto de Investigación en Ciencias Biomédicas, Centro Universitario de Ciencias de la Salud, Universidad de Guadalajara

José Eduardo Navarro-Zarza

Departamento de Medicina Interna-Servicio de Reumatología, Hospital General de Chilpancingo "Dr. Raymundo Abarca Alarcón"

Juliana Marisol Godínez-Rubí

Laboratorio de Patología Diagnóstica e Inmunohistoquímica, Departamento de Microbiología y Patología, Centro Universitario de Ciencias de la Salud, Universidad de Guadalajara

Samuel García-Arellano

Instituto de Investigación en Ciencias Biomédicas, Centro Universitario de Ciencias de la Salud, Universidad de Guadalajara

María Guadalupe Ramírez-Dueñas

Instituto de Investigación en Ciencias Biomédicas, Centro Universitario de Ciencias de la Salud, Universidad de Guadalajara

Isela Parra-Rojas

Facultad de Ciencias Químico-Biológicas, Universidad Autónoma de Guerrero

Arisbeth Villanueva-Pérez

Centro de Diagnóstico e Investigación en Patología y Nefropatología

Jorge Hernández-Bello (✉ jorge89_5@hotmail.com)

Instituto de Investigación en Ciencias Biomédicas, Centro universitario de ciencias de la Salud, universidad de Guadalajara <https://orcid.org/0000-0001-8004-1811>

Research Article

Keywords: sTGF- β , TGF- β R, TGF- β isoforms, systemic sclerosis, autoantibodies

Posted Date: February 7th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1287459/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

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- β R1 and TGF- β R2) 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 (IHC). The soluble levels of the three active TGF- β isoforms were lower in SSc patients than in CS ($p < 0.0001$). However, sTGF- β 1 and sTGF- β 3 levels were positively correlated with C-reactive protein (CRP) levels in SSc patients. Additionally, sTGF- β 2 and sTGF- β 3 levels were positively correlated with the number of platelets in SSc patients. In skin biopsies, TGF- β 1, TGF- β R1, and TGF- β R2 expression levels were higher in SSc patients than CS. In conclusion, this is the first study showing a joint decrease of the 3 active TGF- β isoforms in SSc patients. However, TGF- β 1, TGF- β R1, and TGF- β R2 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.

1. Introduction

Systemic sclerosis (SSc) is a complex rheumatic disease of unknown etiology, characterized by microvasculopathy, autoimmunity, and fibrosis [1]. The worldwide prevalence and incidence of SSc is very low ($\sim 0.05\%$ and $\sim 0.002\%$, respectively), and it is almost five times more common in women than men [2, 3]. SSc patients have heterogeneous clinical characteristics that may involve the skin and some internal organs, giving a cutaneous limited (lcSSc) or cutaneous diffuse (dcSSc) affection with a very high mortality rate [4, 5].

The molecular mechanisms underlying SSc are not entirely understood. However, they may be caused by interactions of genetic predisposition (for example, alterations in immunological regulation genes such as HLA class I and II alleles, *TNFA* or *MIF*) with environmental factors (for example, silica dust or organic solvents) [6–8]. This interaction enables development of the pathogenesis which leads to 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].

The fibrotic tissue is formed by altered endothelial cells, infiltrated autoimmune cells, and collagen-producing myofibroblasts in the ECM [11]; these cells together promote the synthesis of cytokines (e.g., IL-1, IL-4, or TNF- α) and growth factors such as transforming growth factor β (TGF- β) which regulates collagen deposition and the fibrotic process [12].

TGF- β has three soluble isoforms in latent form (sTGF- β 1, 2, and 3), which signal through three different receptors (TGF- β R1, TGF- β R2, and TGF- β R3) which are relevant for the stimulation and development of myofibroblasts [13, 14]. Any of the three isoforms may be activated in the cell membrane by proteases or integrins and bind to the TGF- β R2, recruiting TGF- β R1 and activating the SMAD canonical pathway to regulate the transcription of some genes as collagen [15, 16].

TGF- β has been identified as a critical regulator of pathological fibrogenesis in SSc and an attractive therapeutic target because it regulates several processes, such as cell growth, apoptosis, cell differentiation,

and ECM synthesis [14]. However, these pleiotropic activities make non-selective blocking of its signaling potentially dangerous; this could even lead to spontaneous immune activation or epithelial hyperplasia, because TGF- β has important physiologic functions for tissue homeostasis [17].

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, and the soluble levels of TGF- β isoforms in SSc patients and CS.

2. Materials And Methods

2.1. 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 [18] and obtained a medical record, an evaluation of the modified Rodnan skin score (MRSS), and performed the health assessment questionnaire disability index (HAQ-DI) for all the patients.

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.

All the procedures performed were adhered to and approved by 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. Informed consent was obtained from all subjects involved in the study.

2.2. 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. 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.

2.3. Quantification of TGF- β isoforms

The soluble TGF- β (sTGF- β) isoforms were quantified in serum by a multiplex test based on magnetic beads "Bio-Plex Pro™ TGF- β Assays" (Bio-Rad Laboratories, Hercules,

CA, USA), following the manufacturer's instructions. All samples and controls were analyzed in duplicate; the coefficients of variation were within the acceptable range (<20%). The assay's sensitivity was 6.35 pg/mL for

sTGF- β 1, 4.57 pg/mL for sTGF- β 2, and 4.12 pg/mL for sTGF- β 3 (data were obtained from the results issued by the Bio-Plex Magpix instrument). Data were fitted to a five-parameter logistic function.

2.4. Immunohistochemical expression of TGF- β , TGF- β R1, and TGF- β R2

The expression levels of TGF- β , TGF- β R1, and TGF- β R2 were analyzed in the skin biopsies of twelve SSc patients and two CS. The selected individuals were those who had a medical indication for a biopsy for pathological diagnostic analysis. 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 μ m 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 minutes 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 minutes with one of the following primary antibodies: Anti-TGF- β 1 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.

2.5. Statistical analysis

All the data were analyzed using the GraphPad Prism v6.0 software and the R version 4.0.0 statistical software program. The Shapiro–Wilk normality test was applied to probe the normal distribution of the data; parametric or non-parametric tests were used depending on the results. The Mann–Whitney U test was used to evaluate the differences between two groups, and the Kruskal–Wallis test was utilized for three or more non-normally distributed groups. A p-value < 0.05 was considered significant for all the analyses.

3. Results

3.1. 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.

Table 1
Clinical characteristics of SSc patients and CS.

	SSc (n = 56)	CS (n = 120)	p-value
Demographic data			
Age (years) ^a	50 (18-75)	47 (29-64)	0.448
Male/ Female ^b	12 (7) / 88 (49)	8 (10) / 92 (110)	0.383
Type of SSc and clinical record			
IcSSc ^b	84 (47)	-	
dcSSc ^b	16 (9)	-	
Age of disease onset (years) ^c	40 ±17	-	
Disease evolution (years) ^a	5.5 (0.6-20.3)	-	
Signs and symptoms % (n)			
Sclerodactyly ^b	93 (52)	-	
Arthritis ^b	88 (49)	-	
Active Raynaud's phenomenon ^b	73 (41)	-	
Puffy fingers ^b	70 (39)	-	
Telangiectasia ^b	59 (33)	-	
Digital ulcers ^b	43 (24)	-	
Pulmonary Arterial Hypertension ^b	9 (5)	-	
Interstitial lung disease ^b	7 (4)	-	
Clinical evaluation			
Mouth opening (cm) ^a	5 (3-7)	-	
MRSS score ^a	6 (0-25)	-	
HAQ-DI, 0-3 score ^a	0.3 (0.0-1.3)	-	
Laboratory findings			

^a Data are provided as medians (p5-p95); ^b Data are provided as percentages and (n); ^c Data are provided as the mean ± standard deviation. ESR: erythrocyte sedimentation rate. CRP: C-reactive protein. ANAs: antinuclear antibodies. NSAIDs: nonsteroidal anti-inflammatory drugs; DMARDs: disease-modifying antirheumatic drugs. MRSS: modified Rodnan skin score. IQR: interquartile range.

	SSc (n = 56)	CS (n = 120)	p-value
ESR (mm/h) ^a	28 (11-51)	27 (6-47)	0.110
CRP (mg/dL) ^a	11 (0-64)	12 (3-45)	0.730
ANAs ^b	77 (43)	0 (0)	
Treatment % (n)			
NSAIDs ^b	16 (9)	-	
Prednisone ^b Median 5 mg/day (IQR: 5-7.5 mg/day)	36 (20)	-	
DMARDs ^b		-	
Methotrexate ^b Median 25 mg/week (IQR: 15-25 mg/day)	46 (26)	-	
Chloroquine ^b 150 mg/day	13 (7)	-	
No treatment ^b	39 (22)	-	
^a Data are provided as medians (p5-p95); ^b Data are provided as percentages and (n); ^c Data are provided as the mean ± standard deviation. ESR: erythrocyte sedimentation rate. CRP: C-reactive protein. ANAs: antinuclear antibodies. NSAIDs: nonsteroidal anti-inflammatory drugs; DMARDs: disease-modifying antirheumatic drugs. MRSS: modified Rodnan skin score. IQR: interquartile range.			

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.

3.2. 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 ($p < 0.0001$, Figure 1A-C). The comparison of TGF-β1, TGF-β2, and TGF-β3 levels between treated and untreated patients did not exhibit significant differences ($p > 0.05$, Figure 1D-F).

On the other hand, in SSc patients, sTGF- β 1 was negatively correlated with age ($r_s = -0.329$, $p = 0.02$) and anti-centromere antibodies (ACAs) levels ($r_s = -0.344$, $p = 0.014$), whereas it was positively correlated with C-reactive protein (CRP) levels ($r_s = 0.540$, $p = 0.033$) and the number of platelets ($r_s = 0.436$, $p = 0.004$), as shown in Table 2.

Table 2
Correlation of sTGF- β isoforms with clinical variables in SSc patients.

	sTGF- β 1 (pg/mL)		sTGF- β 2 (pg/mL)		sTGF- β 3 (pg/mL)	
Clinical Variable	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.						

sTGF- β 2 levels were negatively correlated with age ($r_s = -0.283$, $p = 0.046$) and ACA levels ($r_s = -0.291$, $p = 0.040$), whereas they were positively correlated with platelets ($r_s = 0.401$, $p = 0.008$). Additionally, we observed that sTGF- β 3 was positively correlated with CRP levels ($r_s = 0.654$, $p = 0.008$), ACA levels ($r_s = 0.418$, $p = 0.0001$), anti-RNA pol III levels ($r_s = 0.291$, $p = 0.040$), and the number of platelets ($r_s = 0.397$, $p = 0.008$) (Table 2).

In order to identify that the TGF- β isoforms association were not modified by the presence of other confounding variables, multiple linear regression models were constructed (one for each isoform). In these models, the isoforms were used as the dependent variables, and those variables which had a p -value of less than 0.10 in the correlation analysis were used as predictors. Construction of the models was carried out using the stepwise method. The final model for each of the isoforms was the one with the lowest AIC (Akaike information criterion).

Table 3 shows the results of multiple linear regression. In model A, the sTGF- β 1 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- β 2 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- β 3 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, $p = 0.004$).

Table 3

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

Model A				Model B			Model C		
sTGF- β 1				sTGF- β 2			sTGF- β 3		
Variable	β coefficient	CI95%	<i>p</i>	β coefficient	CI95%	<i>p</i>	β coefficient	CI95%	<i>p</i>
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- β 1 level. For model B, the dependent variable was the sTGF- β 2 level. For model C, sTGF- β 3 was selected as the dependent variable. CI95%: 95% confidence intervals; β : beta).									

3.3. Expression of TGF- β , TGF- β R1, and TGF- β R2 in the skin

The tissue expression levels of TGF- β , TGF- β R1, and TGF- β R2 were documented in the dermis and epidermis of SSc patients and CS by IHC (Figure 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 (+++) (Table 3).

Regarding TGF- β 1 (Figure 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, the 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 cytoplasmatic intensity than the nuclear mark. Furthermore, the TGF- β 1 expression tended to be more frequently positive in dcSSc than lcSSc (Figure 3a).

Concerning TGF- β R1 (Figure 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 (Figure 3b).

Regarding TGF- β R2 (Figure 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- β R2 receptor was more frequent in dcSSc than in lcSSc patients (Figure 3c).

Table 4
Cell count of positive/negative cases for TGF- β 1, TGF- β R1, and TGF- β R2 in SSc.

TGF- β 1 (n = 12) IHC intensity score				
Cell	Positive, n (%)	+	++	+++
Fibroblasts				
Nucleus	5 (42)	1	1	3
Cytoplasm	7 (58)	4	1	2
Endothelium				
Nucleus	10 (83)	0	0	10
Cytoplasm	9 (75)	3	5	1
Basal keratinocytes				
Nucleus	11 (92)	0	2	9
Cytoplasm	6 (50)	1	3	2
Superficial keratinocytes				
Nucleus	9 (75)	0	3	6
Cytoplasm	4 (33)	1	3	0
TGF- β R1 (n = 11) IHC intensity score				
Cell	Positive n (%)	+	++	+++
Fibroblasts				
Cytoplasm	4 (36)	4	0	0
Endothelium				
Cytoplasm	11 (100)	10	1	0
Basal keratinocytes				
Cytoplasm	10 (91)	4	6	0
Membrane	9 (82)	4	4	1
Superficial keratinocytes				
Cytoplasm	4 (36)	4	0	0
Membrane	4 (36)	4	0	0
TGF- β R2 (n = 12) IHC intensity score				
Cell	Positive n (%)	+	++	+++

n: number of analyzed cases. The intensity of the mark was qualitatively scored as weak (+), intermediate (++), and high (+++).

TGF-β1 (n = 12) IHC intensity score				
Fibroblasts				
Cytoplasm	2 (17)	1	1	0
Endothelium				
Cytoplasm	7 (58)	4	3	0
Basal keratinocytes				
Cytoplasm	11 (92)	7	4	0
Membrane	5 (42)	1	4	0
Superficial keratinocytes				
Cytoplasm	4 (33)	3	1	0
Membrane	1 (8)	1	0	0
n: number of analyzed cases. The intensity of the mark was qualitatively scored as weak (+), intermediate (++), and high (+++).				

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).

4. 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 [16]. 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 [16, 19].

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 [20], but in contrast to one reported in Brazil, where sTGF-β1 was higher in SSc patients than in CS [21]. Other studies have reported no significant differences [22–24].

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 [20, 22]. 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 [20]. 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 α 2-macroglobulin (α 2M) or soluble TGF- β receptors and clearance via the liver [20, 25, 26]. 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 inflammation and TGF- β activation are emerging [12], 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- β 2 and TGF- β 1 levels in patients than in CS [27]. Based on a previous IHC skin biopsy study showing a high binding of TGF- β 1 and TGF- β 2 proteins in the inflammatory skin of SSc patients [28], Budzyńska-Włodarczyk et al. suggest that the decreases in circulatory TGF- β 1 and TGF- β 2 could be an indirect effect of their accumulation in inflammatory infiltrations of the skin [27].

On the other hand, in the present study, the sTGF- β 2 and sTGF- β 3 levels were positively correlated with the number of platelets of SSc patients. Similarly, sTGF- β 1 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- β 1, the correlation of TGF- β 2 and TGF- β 3 with platelets has not been well described. However, high TGF- β 2 and TGF- β 3 expression levels have been reported in megakaryocytes [29]. In addition, after an injury, platelets expressing TGF- β 3 have been reported [30].

Platelets contain 40 to 100 times as much TGF- β 1 as other cells, and they rapidly release it on activation [31, 32]. 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 [33]. 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 [34].

On the other hand, sTGF- β 1 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 [35].

In another autoimmune disease such as primary Sjogren's syndrome, the serum CRP and TGF- β 1 concentrations have also been positively correlated ($r = 0.786$, $p < 0.01$) [36]. The correlation of these two cytokines could be because CRP may induce the synthesis of proinflammatory cytokines such as IL-1 β and TNF- α [37], both of which can induce TGF- β expression [38, 39]. 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 [35, 40, 41].

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 [28], although this is in

contrast to a previous study where only TGF- β 2 isoform was reportedly expressed in the dermis, and there was no observed TGF- β 1 expression in the dermis [42].

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- β 1 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 [14]. Therefore, TGF- β 1 could lead the capillary architecture involvement in patients with SSc and stimulate angiogenesis.

Similarly, the TGF- β R1 and TGF- β R2 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 [43].

Interestingly, nuclear and cytoplasmic expression of TGF- β R1 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- β R1 has not been described in open databases such as the Human Protein Atlas [44], 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 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- β R1 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- β R1-specific domains [45, 46]. TACE is an enzyme that cleaves TGF- β R1 in cancer cells and liberates an intracellular domain (ICD) that binds with p300 in the nucleus and participates in proliferative gene transcription [47]. However, the translocation of TGF- β R1 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- β R1 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.

5. Conclusions

In conclusion, our findings showed that, in the serum of SSc patients, there is a downregulation of active sTGF- β 1, 2, and 3 isoforms; however, the expression of TGF- β , TGF- β R1 and TGF- β R2 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.

Declarations

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.

Author Contributions: Conceptualization, Jorge Hernández-Bello; Data curation, José Alvaro Lomelí-Nieto, and José Francisco Muñoz-Valle; Formal analysis, Christian Johana Baños-Hernández, Samuel García-Arellano, Maria Guadalupe Ramírez-Dueñas, and Isela Parra-Rojas; Funding acquisition, José Francisco Muñoz-Valle and Jorge Hernández-Bello; Methodology, José Alvaro Lomelí-Nieto, José Eduardo Navarro-Zarza, Marisol Godínez-Rubí, Samuel García-Arellano, Maria Guadalupe Ramírez-Dueñas, Isela Parra-Rojas, and Arisbeth Villanueva-Pérez ; Resources, José Alvaro Lomelí-Nieto and José Eduardo Navarro-Zarza; Writing—original draft preparation, José Alvaro Lomelí-Nieto and Christian Johana Baños-Hernández; Writing—review and editing, José Francisco Muñoz-Valle, Marisol Godínez-Rubí, and Jorge Hernández-Bello. All authors have read and agreed to the published version of the manuscript.

Ethics 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.

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

References

1. Barsotti S, Bruni C, Orlandi M, et al. One year in review 2017: systemic sclerosis. *Clin Exp Rheumatol*. 2017;35 Suppl 106:3–20.
2. Allanore Y, Simms R, Distler O, et al. Systemic sclerosis. *Nat Rev Dis Primer*. 2015;1:15002.
3. Royle JG, Lanyon PC, Grainge MJ, et al. The incidence, prevalence, and survival of systemic sclerosis in the UK Clinical Practice Research Datalink. *Clin Rheumatol*. 2018;37:2103–11.
4. Baños-Hernández CJ, Navarro-Zarza JE, Bucala R, 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.
5. Rubio-Rivas M, Corbella X, Pestaña-Fernández M, 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.
6. Lomelí-Nieto JA, Muñoz-Valle JF, Baños-Hernández CJ, 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.
7. Bossini-Castillo L, Campillo-Davó D, López-Isac E, 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.

8. Machado-Sulbaran AC, Ramírez-Dueñas MG, Navarro-Zarza JE, et al. KIR/HLA Gene Profile Implication in Systemic Sclerosis Patients from Mexico. *J Immunol Res*. 2019;2019:6808061.
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. Jinnin M. Mechanisms of skin fibrosis in systemic sclerosis. *J Dermatol*. 2010;37:11–25.
12. Lafyatis R. Transforming growth factor β —at the centre of systemic sclerosis. *Nat Rev Rheumatol*. 2014;10:706–19.
13. Farina G, Lafyatis D, Lemaire R, Lafyatis R. A four-gene biomarker predicts skin disease in patients with diffuse cutaneous systemic sclerosis. *Arthritis Rheum*. 2010;62:580–8.
14. Ayers NB, Sun C-M, Chen S-Y. Transforming growth factor- β signaling in systemic sclerosis. *J Biomed Res*. 2018;32:3–12.
15. Vander Ark A, Cao J, Li X. TGF- β receptors: In and beyond TGF- β signaling. *Cell Signal*. 2018;52:112–20.
16. 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.
17. Varga J, Pasche B. Transforming growth factor- β as a therapeutic target in systemic sclerosis. *Nat Rev Rheumatol*. 2009;5:200–6.
18. van den Hoogen F, Khanna D, Fransen J, 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.
19. Sgonc R, Wick G. Pro- and anti-fibrotic effects of TGF-beta in scleroderma. *Rheumatol Oxf Engl*. 2008;47 Suppl 5:v5-7.
20. Dziadzio M, Smith RE, Abraham DJ, et al. Circulating levels of active transforming growth factor beta1 are reduced in diffuse cutaneous systemic sclerosis and correlate inversely with the modified Rodnan skin score. *Rheumatol Oxf Engl*. 2005;44:1518–24.
21. Dantas AT, Gonçalves SMC, de Almeida AR, et al. Reassessing the Role of the Active TGF- β 1 as a Biomarker in Systemic Sclerosis: Association of Serum Levels with Clinical Manifestations. *Dis Markers*. 2016;2016:6064830.
22. Majewski D, Majewska KA, Kuznar-Kaminska B, et al. Systemic Sclerosis and Serum Content of Transforming Growth Factor. *Adv Exp Med Biol*. 2019;1153:63–7.

23. 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.
24. Scala E, Pallotta S, Frezzolini A, et al. Cytokine and chemokine levels in systemic sclerosis: relationship with cutaneous and internal organ involvement. *Clin Exp Immunol*. 2004;138:540–6.
25. Blobel GC, Schiemann WP, Lodish HF. Role of transforming growth factor beta in human disease. *N Engl J Med*. 2000;342:1350–8.
26. Heldin CH, Miyazono K, ten Dijke P. TGF-beta signalling from cell membrane to nucleus through SMAD proteins. *Nature*. 1997;390:465–71.
27. Budzyńska-Włodarczyk 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.
28. Querfeld C, Eckes B, Huerkamp C, et al. Expression of TGF-beta 1, -beta 2 and -beta 3 in localized and systemic scleroderma. *J Dermatol Sci*. 1999;21:13–22.
29. Yoon S-Y, Tefferi A, Li C-Y. Cellular Distribution of Platelet-Derived Growth Factor, Transforming Growth Factor- β , Basic Fibroblast Growth Factor, and Their Receptors in Normal Bone Marrow. *Acta Haematol*. Karger Publishers; 2000;104:151–7.
30. Kohama K, Nonaka K, Hosokawa R, et al. TGF-beta-3 Promotes Scarless Repair of Cleft Lip in Mouse Fetuses. *J Dent Res*. SAGE Publications Inc; 2002;81:688–94.
31. 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.
32. Meyer A, Wang W, Qu J, et al. Platelet TGF- β 1 contributions to plasma TGF- β 1, cardiac fibrosis, and systolic dysfunction in a mouse model of pressure overload. *Blood*. 2012;119:1064–74.
33. Ramirez GA, Franchini S, Rovere-Querini P, et al. The role of platelets in the pathogenesis of systemic sclerosis. *Front Immunol*. 2012;3:160.
34. Ntelis K, Bogdanos D, Dimitroulas T, et al. Platelets in Systemic Sclerosis: the Missing Link Connecting Vasculopathy, Autoimmunity, and Fibrosis? *Curr Rheumatol Rep*. 2019;21:15.
35. Taylor AW, Ku NO, Mortensen RF. Regulation of cytokine-induced human C-reactive protein production by transforming growth factor-beta. *J Immunol Baltim Md 1950*. 1990;145:2507–13.
36. 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;125–8.
37. Zhang R, Zhang YY, Huang XR, et al. C-Reactive Protein Promotes Cardiac Fibrosis and Inflammation in Angiotensin II-Induced Hypertensive Cardiac Disease. *Hypertension*. American Heart Association;

2010;55:953–60.

38. Sullivan DE, Ferris M, Nguyen H, et al. TNF-alpha induces TGF-beta1 expression in lung fibroblasts at the transcriptional level via AP-1 activation. *J Cell Mol Med*. 2009;13:1866–76.
39. 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.
40. Sun W, Wu Y, Gao M, et al. C-reactive protein promotes inflammation through TLR4/NF-κB/TGF-β pathway in HL-1 cells. *Biosci Rep*. 2019;39:BSR20190888.
41. Liu X, Mayes MD, Pedroza C, 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.
42. Sfrikakis PP, McCune BK, Tsokos M, et al. Immunohistological demonstration of transforming growth factor-beta isoforms in the skin of patients with systemic sclerosis. *Clin Immunol Immunopathol*. 1993;69:199–204.
43. Kubo M, Ihn 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.
44. Cell atlas - TGFBR1 - The Human Protein Atlas [Internet]. [cited 2021 Aug 7]. Available from: <https://www.proteinatlas.org/ENSG00000106799-TGFBR1/cell>
45. Yakymovych I, Yakymovych M, Zang G, et al. CIN85 modulates TGFβ signaling by promoting the presentation of TGFβ receptors on the cell surface. *J Cell Biol*. 2015;210:319–32.
46. Yakymovych I, Yakymovych M, Heldin C-H. Intracellular trafficking of transforming growth factor β receptors. *Acta Biochim Biophys Sin*. 2018;50:3–11.
47. Mu Y, Sundar R, Thakur N, et al. TRAF6 ubiquitinates TGFβ type I receptor to promote its cleavage and nuclear translocation in cancer. *Nat Commun*. 2011;2:330.

Figures

Figure 1

Comparison of the serum isoforms for sTGF- β 1 by study groups and treatment use: Figures (a), (b), and (c) show the comparison of sTGF- β 1, 2, and 3 in SSc patients and CS, respectively. Figures (d), (e), and (f) compare these isoforms in patients with and without treatment. Data are provided as medians and interquartile range (IQR). The p-value was calculated using the Mann–Whitney U test. sTGF- β : soluble TGF- β ; SSc: systemic sclerosis; CS: control subject. Graphics made with GraphPad Prism v8.0

Figure 2

Tissue expression of TGF- β , TGF- β R1, and TGF- β R2 in skin biopsies: Figures show representative tissue sections revealed with 3,3'-diaminobenzidine (brown color) and hematoxylin counterstain. **(a)** and **(b)** show TGF- β 1 staining: nuclear and cytoplasmic markings of moderate to high intensity are seen in basal and superficial keratinocytes in these cases, whereas in CS, the expression is weak in the cytoplasm of a few endothelial cells and negative in the rest of the cells. **(c)** and **(d)** TGF- β R1 staining: a weak expression is observed in the keratinocyte's cytoplasm and membrane and endothelial cells in these cases, which contrasts with the moderate-intensity nuclear marking in the keratinocytes and endothelium of CS. **(e)** and **(f)** TGF- β R2 staining: in SSc, a mark of moderate intensity is identified in the cytoplasm of fibroblasts, endothelium, and keratinocytes. In the latter, membrane expression is also observed. All images are at the same scale (50 μ m). Black arrow, keratinocytes; red arrow, endothelium; green arrow, fibroblasts. Slides were captured with a digital pathology slide scanner (Aperio LV1, Leica Biosystems, Germany).

Figure 3

Expression of TGF- β 1, TGF- β R1, and TGF- β R2 in the skin of patients with dcSSc and lcSSc: The expression frequencies of the TGF- β 1 **(a)**, TGF- β R1 **(b)**, and TGF- β R2 **(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 in 12 SSc patients (3 patients with dcSSc and 9 with lcSSc). Graphics made with GraphPad Prism v8.0