Original paper

Transforming growth factor-β1 in plaque morphea

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Postep Derm Alergol 2013; XXX, 6: 337–342
DOI: 10.5114/pdia.2013.39431

Abstract

Introduction: Morphea (localized scleroderma) is a rare cutaneous disease characterized by skin fibrosis of unknown pathogenesis. Transforming growth factor-β (TGF-β) is a potent profibrotic factor. The role of TGF-β in morphea remains unclear.
Aim: The goal of this study was to estimate the expression level of TGF-β1 in skin and peripheral blood mononuclear cells as well as the plasma levels of TGF-β1 in plaque morphea (MEP).
Material and methods: The study involved 20 MEP patients. Three control groups were involved: 1 – plasma: 36 healthy volunteers; 2 – PBMC: 47 healthy volunteers; 3 – skin biopsies: 13 samples collected during mastectomy (breast cancer was not skin involved). The analysis of TGF-β1 plasma levels was performed with the use an adequate ELISA kit, while real-time polymerase chain reaction was employed for the expression of TGF-β1 in peripheral blood mononuclear cells (PBMC) and skin.
Results: In our study we have not detected differences in TGF-β 1 expression in PBMC, skin, nor in plasma levels of TGF-β1 between MEP patients and healthy controls, regardless of disease activity and its duration.
Conclusions: The results of our study contradict the claim of the substantial role of TGF-β1 in the most common morphea subtype – MEP.

Key words: morphea, scleroderma, transforming growth factor-β, transforming growth factor.

Introduction

Morphea (localized scleroderma) is a rare cutaneous disease characterized by two consecutive, inflammatory and sclerotic phases. Additionally, after some time, many patients show spontaneous skin softening, clinically manifested as atrophy with depigmentation [1, 2]. Although skin hardening is the prominent clinical symptom (which is at the same time the reason for disease classification as "scleroderma"), the clinical picture as well as immunologic abnormalities differ from the ones observed in systemic scleroderma [1–8]. Unfortunately, the disorder being rare, clinically diversified and potentially spontaneously reversible leads to the fact that its pathogenesis remains unknown, leaving no undoubtedly proven successful therapy [9, 10].

There are three known isoforms of transforming growth factor-β (TGF-β), i.e. TGF-β1, TGF-β2 and TGF-β3, all synthesized as inactive pro-peptides. The cytokine is multi-functional, yet it is believed to be the leading profibrotic factor in sclerotic disorders [11–13]. It interacts with extracellular matrix components, including fibrillin-1. Interestingly, a defective fibrillin-1 coding gene (FBN1) is the

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Received: 4.07.2013, **accepted:** 17.09.2013.

characteristic of the SSc – Tsk1/+ murine model, while a mutation of this gene has been found in a group of native Americans, in which the risk of developing SSc is higher [14]. Almost 30% of morphea patients show the presence of anti-fibrillin-1 antibodies [15]. The activation of TGF-β is mediated by thrombospondin, plasmin, integrins and THY-1 (CD90), as well as matrix metalloprotease 9. Not only is TGF-β believed to initiate the production of extracellular matrix components (including collagen, fibronectin and proteoglycans) but also it inhibits their degradation due to interactions with matrix metalloproteinases and stimulation of synthesis of their inhibitors [12, 16, 17]. It has been shown that TGF-β increases fibroblast susceptibility for apoptosis [18]. There are other, numerous *in vitro* and *in vivo* studies acknowledging the role of TGF-β in fibrotic diseases. Indirectly, the role of TGF-β in morphea may be underlined by a case study of a patient in whom tyrosine kinase blocking (through which TGF-β mediates a Smad-independent intracellular signal) resulted in an improvement of the clinical condition [19]. Yet, the role of TGF-β in morphea remains unclear, while the results of scientific studies do vary greatly.

Aim

The goal of this study was to estimate the expression level of *TGFB1* in skin and peripheral blood mononuclear cells (PBMC) as well as the plasma levels of TGF-β1 in plaque morphea (MEP).

Material and methods

Material

The study involved 20 MEP patients (10 women and 10 men). The mean age was 43 years, minimum 16, maximum 81, standard deviation (SD) = 19. The mean disease duration was 3.4 years (minimum 1, maximum 20, SD = 4). All patients were diagnosed both clinically and histopathologically. Whole blood samples were acquired from all patients, while 13 of them additionally were the source of skin biopsies. Three control groups were involved: 1 – plasma: 36 healthy volunteers (29 women, 7 men), mean age was 34 years, minimum 20, maximum 57, SD = 9; 2 – PBMC: 47 healthy volunteers (35 women, 12 men), mean age was 33, minimum 20, maximum 57, SD = 9; 3 – skin biopsies: 13 samples collected during mastectomy. The neoplasms were not skin related. Mean age was 52 years, minimum 30, maximum 85, SD = 17. The study was approved by a local bioethical committee. All patients gave written consent. EDTA-collected whole blood samples (5 ml) were spun in ficoll gradient (Ficoll-Histopaque 1.077 g/cm^3, Sigma Diagnostics, Inc. St. Louis, USA). Plasma samples were stored at –80°C. Upon collection, skin samples were immediately frozen in liquid nitrogen and stored at –80°C.

Methods

Evaluation of activity of the disease

A patient was qualified to the active process group, if within recent 6 months there had been an appearance of a new lesion, spread of a previously existing one or a presence of erythematous margins [20, 21].

ELISA

The assessment of TGF-β1 protein plasma level was done with the use of a commercially available kit according to the manufacturer's instructions (R&D System, Minneapolis, USA).

Real-time polymerase chain reaction assessment of transforming growth factor-β1 expression in peripheral blood mononuclear cells

Whole RNA samples were isolated from PBMC according to Chomczynski and Sacchi protocol [22]. Genomic DNA from 1 µg RNA samples was removed with the use of a recombined DNase I (Ambion, USA). Following reverse transcription (Roche Applied Science), cDNA samples were analyzed with the use of real-time PCR in relative analysis mode with standard curves. The analysis was performed with the use of Light Cycler 2.0 thermocycler (Roche Diagnostics GmbH, Germany) and a dedicated commercial SYBR Green kit (Roche Applied Science).

The amplified cDNA fragment was 81 bp long. The sequence covered fragments of exons 4 and 5 of the TGF-β

Table 1. Primers used in this study

Name	5'-3'sequence	Amplicon length [bp]	References
GAPDH-F	CTGCACCACCAACTGCTTAG	105	Ensembl: ENST00000229239 Glyceraldehyde-3-phosphate dehydrogenase [23]
GAPDH-R	TTCTGGGTGGCAGTGATG		
TGFB1-F	GTGACAGCAGGGATAACA-CACTG	81	Ensembl: ENST00000221930 Transforming growth factor, beta1 http://www.rtprimerdb.org/assay_report.php?assay_id=1005 [24]
TGFB1-R	CATGAATGGTGGCCAGGTC		

Table 2. Expression of TGFB1 in PBMC, skin and plasma TGF-β1 level

Variable	MEP			Control groups			Value of *p*
	Median	Minimum	Maximum	Median	Minimum	Maximum	
Expression of TGFβ1 in *PBMC* (per million *GAPDH* copies)	*n* = 20			*n* = 47			0.9
	218674	136538	574074	217453	108746	513292	
Plasma TGF-β1 level [pg/ml]	*n* = 20			*n* = 36			0.4
	159	32	1131	180	40	730	
Expression of *TGFβ1* in skin (per million *GAPDH* copies)	*n* = 13			*n* = 13			0.8
	29503	10067	77760	32090	19469	83284	

Table 3. Expression of TGFB1 in PBMC, skin and plasma TGF-β1 level comparing the active and non-active process groups

Variable	MEP active process			MEP non-active			Value of *p*
	Median	Minimum	Maximum	Median	Minimum	Maximum	
Expression of *TGFβ1* in PBMC (per million *GAPDH* copies)	*n* = 10			*n* = 10			0.3
	231408	139216	574074	218674	136538	276142	
Plasma *TGF-β1* level [pg/ml]	*n* = 10			*n* = 10			0.05
	250	33	1131	70	32	466	
Expression of *TGF-β1* in skin (per million GAPDH copies)	*n* = 8			*n* = 5			0.5
	31906	23431	62500	21504	10067	77760	

1 gene, spanning a 139 bp long intron, which due to short elongation time, minimized the chances of genomic DNA amplification. All RNA samples were subjected to no-RT PCR reactions for genomic DNA contamination analysis. PCR primer sequences, presented in Table 1, were acquired from other studies [23, 24]. The quantitative results are expressed in *TGFB1* copy number for one million reference gene copies. Glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH) was chosen as a reference. The choice of this particular gene had been suggested in literature [25].

Real-time polymerase chain reaction assessment of TGFB1 expression in skin

Skin samples stored at –80°C were rotor-stator homogenized in TriPure reagent (Roche Applied Science). Due to the high level of impurities, phenol-chloroform extraction was doubled and separated with successive isopropanol, 75% ethanol washings and the removal of genomic DNA. The remaining steps of the procedure were the same as for PBMC samples.

Statistical analysis

Arithmetical means and SD values were calculated for age and disease duration. Additionally, the analysis of other variables included median, as well as minimum and maximum values due to lack of normal distribution. The statistical significance of differences was calculated with the use of the two independent Mann-Whitney-Wilcoxon test, while correlation analyses were performed with the Spearman's rank approach. Analyses were considered significant below $p = 0.05$ value.

Results

The median expression level values of *TGFB1* in PBMC and skin as well as plasma TGF-β1 levels are presented in Table 2. No statistically significant differences have been found for these variables between the groups of MEP and controls.

None of the analyzed variables (expression of *TGFB1* in PBMC, plasma TGF-β1 level and expression of *TGFB1* in skin) correlated with the disease duration. Ten MEP patients (50%) were assigned to the group with the active process. There were no statistically significant differences between active and non-active process groups with respect to the three aforementioned variables (Table 3).

Discussion

Although TGF-β is thought to be the main profibrotic cytokine, its possible contribution to the pathogenesis of morphea remains a matter of debate as the results of

scientific studies concerning the subject contradict each other. Higley *et al.* detected elevated TGF-β1 levels in skin of morphea patients compared to healthy controls. Elevated TGF-β1 serum levels were detected in 8 out of 15 morphea patients (53%) compared to 2 out of 31 healthy controls (7%) [26]. Similarly, elevated TGF-β in morphea patients was also detected by Uziel *et al.* and Lipko-Godlewska [21, 27]. On the other hand, Querfeld *et al.* found up-regulated *TGF*β*1*, β*2* and β*3* mRNA production only during the inflammatory phase of morphea, but not in the latter sclerotic one. These results were also supported by immunohistochemical analysis, yet the study involved only 2 patients with morphea in its inflammatory phase and only 1 in the sclerotic phase [28]. Farell *et al.* presented the results of their study where they had found increased intensities of anti-TGF-β1, anti-TGF-β2, but not anti-TGFβ3 antibody staining in the upper and middle layers of the dermis of 2 morphea patients compared to healthy skin of labia [29]. Kawakami *et al*. found that skin of deep morphea is more immunoreactive to anti-TGF-β3 [30]. El-Mofty *et al.* claimed that a group of 21 morphea patients treated with UVA phototherapy showed a statistically significant down-regulation of *TGFB1* expression compared to the levels detected before the treatment. What is more, the change in *TGFB1* expression correlated with the effectiveness of the UVA treatment [31]. This study, however, should be approached with caution as the RNA was isolated from paraffin-embedded skin. Such procedure may cause RNA degradation, resulting in a substantially lowered PCR sensitivity [32, 33]. Additionally, the quantitative assessment technique presented in this study is less precise than relative real-time PCR analysis.

In opposition to the aforementioned results, Restrepo *et al*. did not find differences in anti-TGF-β1 or in anti-TGF-β2 immunohistochemical staining between 10 linear morphea patients and 2 healthy individuals [34]. Concordantly, Antiga *et al.* showed even a decrease in TGF-β+ cells in morphea skin compared to healthy controls and a decreased level of this protein in the studied sera [35]. On the other hand, Kubo *et al.* assessed 5 morphea skin biopsies (2 – MEP, 1 – generalized morphea, 2 – linear morphea) using in situ hybridization and found elevated expression levels of TGF-β type I and II receptors, predominantly in fibroblasts of the dermis, which was further supported by immunohistochemical staining [36]. In our study we have not detected differences in *TGFB1* expression in PBMC, skin, or in plasma levels of TGF-β1 between MEP patients and healthy controls.

The reason for such incomparable results of these studies may be a significant diversity of the disease itself. Some of the authors, interestingly, did not include the relevant subtype of morphea, which might be of some importance regarding their results. On top of that, many of these studies, usually the older ones, involved only a few samples, which significantly decreases their credibility [26, 28, 29]. However, many authors underlined that TGF-β could play a significant role especially during the initial, inflammatory phase [10–12, 26], thus the moment of biopsy acquisition might have had an impact on the results. Yet, we have not observed any correlation between *TGFB1* in skin, PBMC or plasma TGF-β1 levels and disease duration, while it is important to note that 45% of our group suffered from morphea for less than a year, and the following 25% – less than 2 years. Similar conclusions were suggested by Restrepo *et al.* [34]. What is more, regardless of the disease duration, we have not detected relevant differences between active and non-active morphea patients. Antiga *et al.* indicate a potential role of T regulatory lymphocytes in the autoimmunization process in morphea. These lymphocytes are a significant source of TGF-β and their activity results in the phenomenon of tolerance. The decrease in their numbers or their impaired activity may lead to the induction of autoimmunization. During their biopsy studies, Antiga *et al.* found a lowered number of these cells, along with a decreased TGF-β1 levels in sera as well as fewer TGF-β+ cells in skin of morphea patients compared to psoriatic patients and healthy controls [35].

While discussing these results, a failure of a clinical study involving a CAT-125 monoclonal anti-TGF-β1 antibody therapy in SSc is worth noting. The study not only did not confirm the effectiveness of this drug, but also the side effects were more frequent, including 3 deaths (caused by disease complications) compared to the placebo group (no patient died). The most frequent side effect, leading to the elimination of patients from the clinical phase, was a progression of skin involvement. However, there were no statistically significant differences between the studied groups assessed with the use of the modified Rodnan skin thickness score during all study stages. All groups showed improvement that was correlated with disease duration. At the same time, elevated TGF-β1 expression was found in patients before treatment initiation and tended to remain that way during the treatment. Finally, authors postulated the use of TGF-β pathways blocking agents instead [37]. Imatinib is a tyrosine kinase inhibitor, registered as an anti-cancer (preferably as anti-lymphoproliferative neoplasms) drug. The kinases in question are a part of a Smad-independent pathway induced by TGF-β [38]. There were two clinical studies involving Imatinib in SSc. One of them was prematurely ceased due to side effects [39]. The other resulted in no improvement assessed with the modified Rodnan skin thickness score and diffusion capacity for carbon monoxide after 6 months of treatment [40]. To date, the effectiveness of direct TGF-β blocking or any inhibition of its signaling pathways have not been confirmed, yet there has been a report on a morphea patient clinically responding well to Imatinib [19].

Conclusions

Our study was directed to the assessment of the influence of TGF-β1 in the pathogenesis of MEP. The results have not shown significant differences in *TGFB1* gene expression in PBMC and skin, as well as in plasma TGF-β1 levels between MEP patients and healthy controls, disease activity status or significant correlation with the disease duration. We are aware however of certain limitations of our study. It was only directed towards TGF-β1, leaving TGF-β2, TGF-β3 and their receptors without analysis. Additionally, the choice of the *GAPDH* as a reference gene for fibrotic skin assessment remains to be analyzed. The lack of convincing evidence concerning this and other possible reference genes for fibrotic skin is a problem. Generally speaking though, the results of our study contradict the claim of the substantial role of TGF-β1 in the most common morphea subtype – MEP, which was also the conclusion of the study by Restrepo *et al*. [34] and indirectly supported by the results of anti-TGF-β clinical studies in SSc [37, 39, 40].

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