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Profiling of Toll-like Receptors and Related Signaling Mediators in the Pathogenesis of Morphea

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ABSTRACT

Introduction: Morphea, also known as localized scleroderma, is a rare fibrosing inflammatory disease of unknown pathogenesis.

Objectives: Although the genetic basis for morphea is important, reports on the evaluation of Toll-like receptors (TLR) in this disease is quite limited. We aimed to evaluate TLR expression levels and serum IL-6, IL-17A, TGF-β1, FGF, and VEGF levels in patients with morphea and compare these results with healthy controls.

Methods: The expression levels of TLRs in the lesional and non-lesional adjacent skin of patients with morphea and in normal skin of healthy controls were evaluated by RT-PCR, whereas serum levels of IL-6, IL-17A, TGF-β1, FGF, and VEGF were evaluated by ELISA.

Results: Based on our findings, TLR1 gene expression increased 34.3-fold in the lesional skin of patients with morphea. In addition, IL-6, IL-17A, TGF-β, FGF, and VEGF were found to be higher in the blood samples of the patient group than in the healthy group.

Conclusion: TLRs are important parts of the pathogenesis of morphea, and a better understanding of them will lead to more directed, effective treatments. We believe that this study will be important for pioneering TLR-targeted therapeutic approaches in the treatment of morphea in the future.

Introduction

Morphea, also known as a localized form of scleroderma, is a rare inflammatory disease characterized by fibrosing of the skin, subcutaneous tissues, and, rarely, underlying organs [1]. It has several subtypes, which are circumscribed (including a superficial and deep variant), linear, generalized, the pansclerotic subtype, and the mixed morphea; the most common type in adults is circumscribed superficial-type morphea [2]. The keystone of the disease is the overproduction of collagen and extracellular matrix deposition; however, the whole pathogenesis has not yet been elucidated. The enlightened part includes multiple factors such as genetic predisposition, immune dysregulation, and environmental factors [3].

Toll-like receptors (TLRs) are one of the pattern recognition receptors required for activation of innate immunity and adaptive immunity [4]. TLRs are located on macrophages and other antigen-presenting cells that discriminate between self and non-self antigens. Upon binding to exogenous or endogenous antigens, TLRs dimerize, and signal transduction is achieved after some conformational changes. Activated TLRs activate two different intracellular pathways, either myeloid differentiation factor 88 (MyD88)-dependent or MyD88-independent [5]. With the induction of immunity by TLRs, dendritic cell maturation and activation of CD4+ and CD8+ T cells are provided [6]. In addition, some TLRs that can be expressed directly in T and B cells can directly stimulate cell differentiation, proliferation, and memory cell and antibody production [7]. In many autoimmune diseases, expressions of TLRs are shown to increase [8-10]. TLR4 expression is elevated in the lesional skin tissue of patients with systemic scleroderma, and induction of TLR4 stimulates fibrotic gene expression and myofibroblast differentiation and also sensitizes fibroblasts to transforming growth factor (TGF)-β [11,12].

In the pathogenesis of morphea, the increased production of endothelin-1 (ET-1), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and decreased prostacyclin activates the dysfunction of endothelial cells and also angiogenesis. In addition, adhesion molecules are upregulated, and reactive oxygen species are generated in morphea [13,14]. Another important step in pathogenesis is the activation of the immune system. CD4+ T lymphocytes are especially predominant and cause the secretion of interleukin (IL)-2, IL-4, IL-6, and GFs such as TGF-β that initiate and/or promote fibrosis and vascular injury. Among these substances, especially IL-4 induces TGF-β production as well as collagen production by fibroblasts. In addition to producing excess collagen, fibroblasts in patients with scleroderma also give an increased response to molecules such as TGF-β, ET-1, and platelet-derived growth factor (PDGF) [15]. According to the information obtained from studies, all T helper 1 (Th1), Th2, and Th17 cytokines may contribute equally to the pathogenesis of morphea [16].

Objectives

A better understanding of the pathophysiology of morphea will lead to more directed and effective treatments. In addition, morphea causes permanent deformity and functional sequelae and has a great impact on patients' life quality of [17]. There is no curative treatment option for morphea yet [16]. The present study aimed to evaluate and compare the expression levels of TLRs in the lesional and non-lesional adjacent skin of patients with morphea with normal skin of healthy controls as well as the serum levels of IL-6, IL-17A, TGF-β1, FGF, and VEGF of both groups. In addition, this study aimed to pioneer TLR-targeted therapeutic approaches for morphea that may emerge in the future.

Methods

This prospective case-control study was conducted according to the Declaration of Helsinki guidelines and was ethically approved by the Ethics Committee (decision 220 on 10.06.2021). All participants in the study provided informed consent.

Study Population

Fifteen patients with morphea aged between 18 and 65 years who were clinically and histopathologically diagnosed with morphea attending the University Hospital Dermatology Outpatient Clinic between July 2021 and February 2022 were included in the study. A detailed anamnesis was taken, a skin examination was performed by the same dermatologist, and the severity of the disease was evaluated according to the Localized Scleroderma Assessment Tool (LoSCAT) [18]. LoSCAT consists of two domains, a modified Localized Skin Severity Index (mLoSSI) and the Localized Scleroderma Damage Index (LoSDI), which measures the severity of morphea. All participants were selected from patients who had not been treated with systemic therapy, including phototherapy, for at least eight weeks before the initiation of the study. Eleven age- and sex-matched healthy volunteers who did not have any systemic inflammatory disease, including cardiovascular and/or neurovascular diseases, or inflammatory skin diseases and who did not take regular medication for any reason were also included as the control group. Patients with a history of cardiovascular or cerebrovascular accident, active infection, and uncontrolled inflammatory systemic disease, including diabetes mellitus (DM) and skin diseases, other autoimmune diseases, and/or malignancy in the last five years were excluded from the study.

Lesions and non-lesional skin samples (both 3 mm in size) were obtained from each patient with morphea and similarly sized skin tissue from healthy controls. In addition, 4 ccs of peripheral blood taken from each of the participants were centrifuged at 2000xg for 10 minutes. All skin tissues and blood samples (after centrifugation) were transferred to 1.5 ml Eppendorf tubes without DNAse RNase and stored in the refrigerator at -20 ˚C until the study day. Then, blood samples were studied by ELISA method and TLR by RT-PCR method.

Serum Levels of Cytokines and GFs

IL-6, FGF, TGF-β1, and VEGF were chosen because they have been proven to play an important role in pathogenesis [19]. Although there are conflicting results about the role of IL-17A in morphea, we included it in our analysis to confirm these results and because of its popularity in recent years [20,21]. The whole blood samples collected using vacuum tubes from healthy individuals and morphea patients were centrifugated at 3000 xg, 10 min for serum separation. The serum samples were transferred into sterile 1.5 ml microcentrifuge tubes and stored at -80 °C until ELISA analysis. The serum levels of IL-6, IL-17A, FGF, TGF-β1, and VEGF were detected by ELISA kit for each parameter (BT Lab, Shanghai, China). Kit sensitivity values were 1.03 ng/L, 2.38 ng/L, 9,85 ng/L, 5.11 ng/L, and 10.42 ng/L for IL6, IL-7A, FGF, TGF-β1, and VEGF, respectively. This assay was based on the biotin double-antibody sandwich technology to quantify specific IL-6, IL-17A, FGF, TGF-β1, and VEGF antigens. Briefly, serum samples were diluted as 1:10 just before analysis, and standards were added to the respective well pre-coated with human-specific primary antibody. Biotinylated, another specific primary antibody, was added to bind to specific antigen in the sample. Followed by first adding streptavidin-HRP and then biotinylated secondary antibody, the samples were incubated with substrate solution for color develops in proportion to the amount of specific antigen. The reaction was terminated by adding acidic stop solution, and absorbance was measured at 450 nm. The serum levels of IL-6, IL-17A, FGF, TGF-β1, and VEGF were calculated according to the specific standard curve for each parameter. The results of three independent experiments for each parameter were triplicated and are expressed as mean ± standard error of mean (SEM).

Real Time-PCR (qPCR)

In this study, we investigated the mRNA expression changes of TLRs in morphea patient samples compared to controls by RT-PCR. Based on this purpose, mRNA expression changes of TLR1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 genes involved in pathways associated with TLRs (MyD88-dependent and independent) and downstream targets were detected. β-actin was used as housekeeping for normalization. The reverse and forward sequences of the genes are given in Table 1. Sequences were designed using OriGene (https://www.origene.com/) online website and BLAST (Basic Local Alignment Search Tool) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) software. Reaction conditions in 96-well plate with 5.5 µl SYBR green master mix (ABT 2x qPCR SYBR Green Master mix), 6.5 µl nuclease-free water, 2 µl (1 Reverse + 1 forward) primer, and 1 µl cDNA per well was set up, and the surface of the plate was sealed with a clear adhesive label (Applied Biosystems™ 96-Well Reaction Plate seal). The plate loaded into the StepOne Plus RT-PCR device was amplified for 40 cycles, 5 min at 95 °C, and 1 min at 60 °C.

Statistical Analyses

SPSS (18.0.0.2; IBM, Armonk, NY, USA) was used for the analyses. The Shapiro Wilk test was used to determine whether the continuous variables were normally distributed. Normally distributed parameters were compared with Student's t test and non-normally distributed parameters were compared with Mann-Whitney U test. The relationship between serum cytokine and factor levels and other variables was evaluated by Spearman correlation analysis. In addition, the relationship between these and disease severity scores was evaluated with the Kruskal-Wallis test. Values with $P < 0.05$ were considered statistically significant.

Results

Fifteen patients with morphea (12 females, 3 males) and 11 healthy controls (9 females, 2 males) were included in our study. While the mean age of the patients was 44.43 years, the mean age in the control group was 43.91 years. No significant difference was seen in terms of age and sex between the two groups ($P>0.05$). Eight of the patients (53.3%) had generalized plaque morphea, 6 (40%) had localized plaque morphea, and 1 (6.7%) had pansclerotic (deep) morphea. The mean disease duration was 4.71 (0.5-12) years. The most common site of involvement was the trunk (73.3%). Among the severity index scores of the patients, the mean mLoSSI score was 15.375, while the mean LoSDI score was 16.875. Itching was the most common symptom in patients (40%). While the disease of 10 patients (66.6%) was stable for the preceding six months, the disease of five patients (33.4%) had progressed. Eleven patients (73.3%) stated that emotional stress could be the cause of the disease. Allergic manifestations (allergic rhinitis, grass allergy, pollen allergy, etc.) were detected in six of the patients. Only two (13.3%)

Table 1. Reverse and Forward Sequences of Genes Used in the Study.

#	Name	Sequence
1	TLR1_F	CAGCGATGTGTTCGGTTTTCCG
2	TLR1_R	GATGGGCAAAGCATGTGGACCA
3	TLR2_F	TTATCCAGCACACGAATACACAG
4	TLR2_R	AGGCATCTGGTAGAGTCATCAA
5	TLR3_F	GGCTAGCAGTCATCCAACAGAA
6	TLR3_R	GCAGTCAGCAACTTCATGGC
7	TLR4_F	CCCTGAGGCATTTAGGCAGCTA
8	TLR4_R	AGGTAGAGAGGTGGCTTAGGCT
9	TLR5_F	CCTTACAGCGAACCTCATCCAC
10	TLR5_R	TCCACTACAGGAGGAGAAGCGA
11	TLR6_F	TTCTCCGACGGAAATGAATTTGC
12	TLR6_R	CAGCGGTAGGTCTTTTGGAAC
13	TLR7_F	CTTTGGACCTCAGCCACAACCA
14	TLR7_R	CGCAACTGGAAGGCATCTTGTAG
15	TLR8_F	ACTCCAGCAGTTTCCTCGTCTC
16	TLR8_R	AAAGCCAGAGGGTAGGTGGGAA
17	TLR9_F	TGAGCCACAACTGCATCTCGCA
18	TLR9_R	CAGTCGTGGTAGCTCCGTGAAT
19	TLR10_F	GGTTAAAAGACGTTCATCTCCACG
20	TLR10_R	CCTAGCATCCTGAGATACCAGG
21	B-AKTIN_F	TCCTCCTGAGCGCAAGTACTC
22	B-AKTIN_R	CTGCTTGCTGATCCACATCTG

of the patients were active smokers. In the family histories of the patients, it was determined that the brother of one patient also had morphea, and the relatives of five patients had various autoimmune diseases (rheumatic diseases, thyroid diseases, etc.). Demographic data of patients and healthy controls are shown in Table 2.

As a result of our study, expressions of TLR genes were investigated in perilesional and lesional morphea tissues and compared to the controls. Expressions of TLR1, 2, 3, 6, 8, 9, and 10 genes were found to be upregulated in the perilesional group, but statistical significance could not be determined ($P>0.05$) (Table 3). TLR 4, 5, and 7 gene expressions were determined to be down-regulated, but statistical significance could not be determined. In the lesional group, a 34.3-fold increase in TLR1 gene expression was found to be statistically significant. The increase in TLR 2, 3, 6, 8, 9, and 10 genes and the decrease in TLR4, 5, and 7 gene expressions were not statistically significant.

To evaluate the changes of key mediators in morphea pathogenesis, IL-6, IL-17A, TGF-β1, FGF, and VEGF levels were measured in serum samples of health individuals and morphea patients. Based on our results, an increase in both levels of cytokine and growth factors were significantly obtained in patients compared to healthy individuals (Table 4).

IL-6 and IL-17A levels, which are the proinflammatory cytokines, were detected as 200.74 ng/L ± 6.96 and 277.71 ± 15.9 ng/L, respectively, in the group with morphea, an approximately 15% and 33% increase, respectively, compared to the 173.54 ± 7.94 ng/L and 209.11 ± 2.25, respectively, in healthy individuals. This increase in IL-17A levels was significantly found as the ratio of 33% (Fig 1), but increased IL-6 levels was not statistically significant (Fig 2). Although not statistically significant, this increase in IL-6 is very important for the morphea group since it has a vital role in the pathogenesis of morphea.

In addition to the pro-inflammatory cytokines, FGF and VEGF levels are known to play a crucial role in dermal fibrosis and are implicated in the pathogenesis of morphea. Based on our results, FGF and VEGF levels were 1096.16 ± 25.73 and 1202.41 ± 37.15 ng/L, respectively, in the healthy group, and 1503.18 ± 83.4 (Fig. 3) and 1568.44 ± 82.16 ng/L, respectively, in the morphea group (Fig. 4). Both growth factor levels were significantly increased in serum samples of morphea patients compared with the healthy group, as the ratio of 37% in FGF and 30% in VEGF ($P<0.05$).

Table 2. Demographic Data of the Participants.

	Patients (n=15)	Healthy Controls (n=11)	P-value*
Age, years	44.43 ± 14.29†	43.91 ± 15.78†	0.623
Female, n (%)	12 (%80)	9 (%81.8)	0.241
Male, n (%)	3 (%20)	2 (%18.2)	
Duration of disease, years	4.71 ± 7.2† (0.5-12)		
mLoSSI score LoSDI score	15.375 ± 17.2† 16.875 ± 27.2†		
Allergic conditions (Allergic rhinitis, pollen allergy, etc.)	6 (%40)	0 (0%)	
Smokers, n (%) Non-smokers, n (%)	2 (%13.3) 13 (%86.7)	1 (%9.1) 10 (%90.9)	0.153
Status of the disease (last 6 months) Stabile Progressive	10 (%66.6) 5 (%33.4)		
Having a family history of morphea, n (%) Having a family history of other autoimmune diseases, n (%)	1 (%6.6) 5 (%33.3)		

p*: Mann Whitney U; †: Standard deviation.

Table 3. Comparison of TLR Expressions in Lesional and Perilesional Skin of the Patient Group With Expressions in the Skin of Healthy Controls.

Genes	Group 1 (Perilesional)		Group 2 (Lesional)	
	Fold Regulation	P-value	Fold Regulation	P-value
TLR1	39.89	0.161145	34.33	0.045850*
TLR2	9.56	0.228178	7.97	0.302169
TLR3	2.68	0.177272	2.41	0.292930
TLR4	-1.03	0.147965	-1.56	0.113440
TLR5	-2.05	0.380609	-3.01	0.173864
TLR6	2.57	0.291077	-1.11	0.399988
TLR7	-1.03	0.308964	-2.62	0.510245
TLR8	6.10	0.242098	19.48	0.076069
TLR9	2.52	0.320951	2.18	0.189571
TLR10	2.31	0.872163	1.26	0.710009

Table 4. Comparison of Serum IL-6, IL-17A, FGF, VEGF, and TGF-β Levels Between Groups.

	Patients (n=15)	Healthy Controls (n=11)	P-value*
IL-6	200.74±7.94†	173.54±6.96†	0.944
IL-17A	277.71±15.9†	209.11±2.25†	0.109
FGF	1503.18±83.4†	1096.16± 25.73†	0.293
VEGF	1568.44± 82.16†	1202.41± 37.15†	0.25
TGF- β	1138.37±62.01†	771.37±24.36†	0.624

Although described as an anti-inflammatory cytokine, TGF-beta is a profibrotic cytokine in the generation of morphea. There are contradictory reports related to serum TGF-beta levels in patients with morphea. However, the important point here is the stage of the disease. In our study, our morphea patients were selected in the inflammatory phase, in which phase lesions appear acutely. The TGF-β1 levels in serum samples of morphea patients at inflammatory

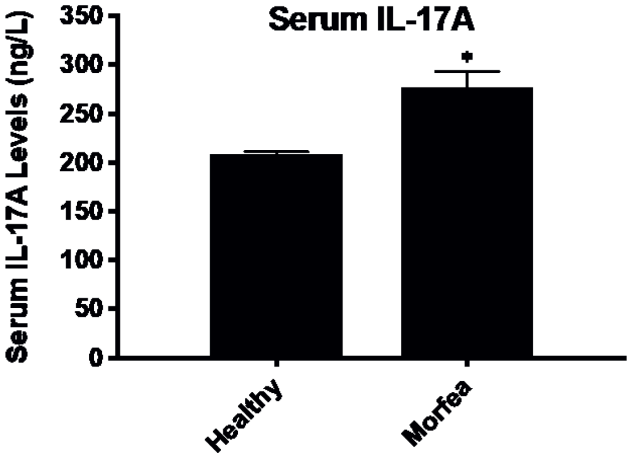

Figure 1. Serum IL-17A levels in healthy individuals and morphea patients. Data are expressed as mean of the percentages of IL-17A levels ± SEM of 3 experiments (*$P < 0.05$ versus healthy group).

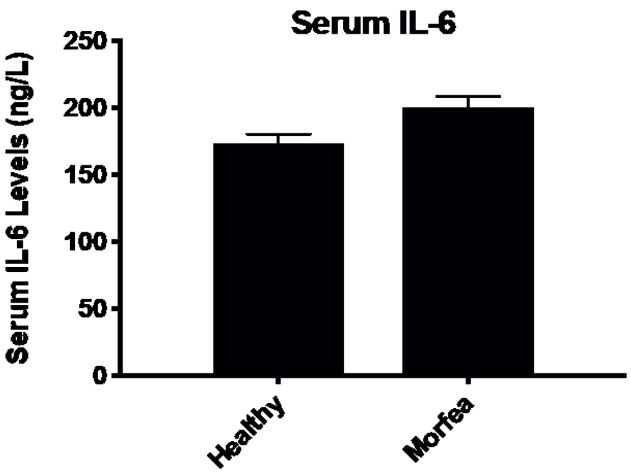

Figure 2. Serum IL-6 levels in healthy individuals and morphea patients. Data are given as mean ± SEM of three experiments.

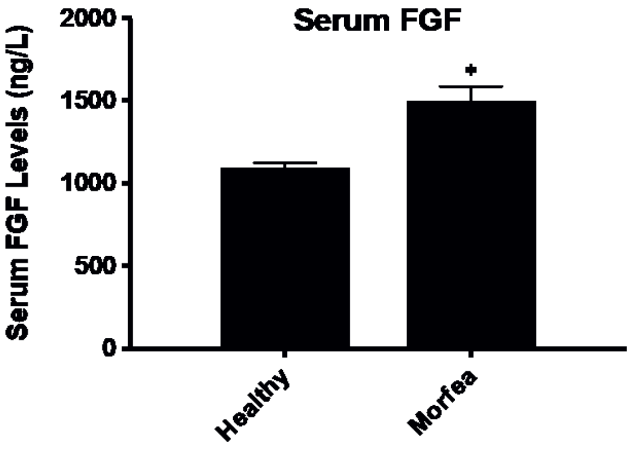

Figure 3. Serum FGF levels in healthy individuals and morphea patients. Data are expressed as mean of the percentages of FGF levels ± SEM of three experiments (*$P < 0.05$ versus healthy group).

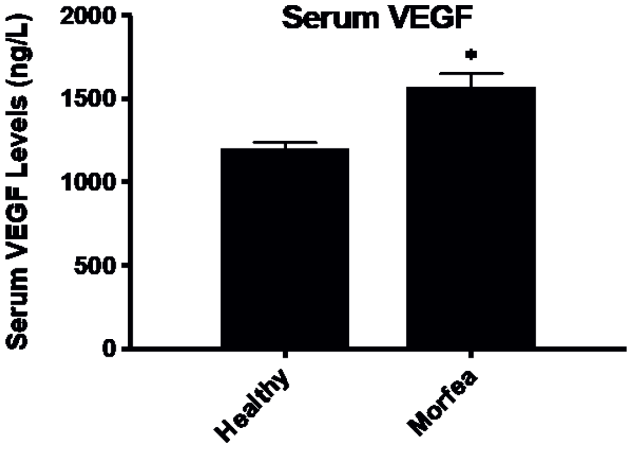

Figure 4. Serum VEGF levels in healthy individuals and morphea patients. Data are expressed as mean of the percentages of VGF levels ± SEM of three experiments (*$P < 0.05$ versus healthy group).

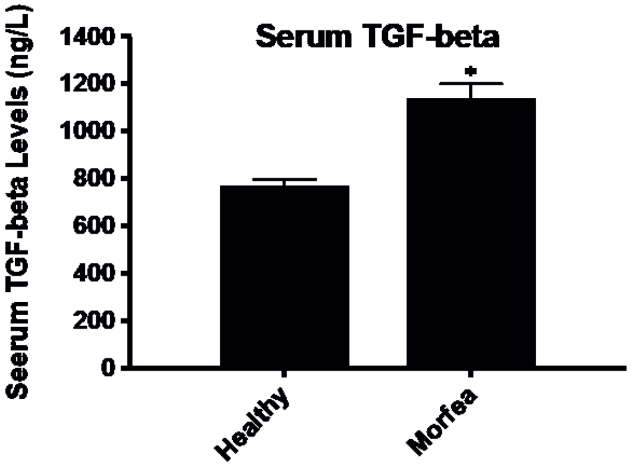

Figure 5. Serum TGF-beta levels in healthy individuals and morphea patients. Data are given as mean ± SEM of three experiments (*$P < 0.05$ versus healthy group).

stage were determined as 1138.37 ± 62.01 ng/L. This value is statistically significant, with an increase of approximately 48% compared to the levels of 771.37 ± 24.36 ng/L measured in healthy individuals ($P<0.01$) (Fig. 5).

Discussion

In this study, we showed that TLR1 gene expression increased 34.3-fold in the lesional skin of patients with morphea. In addition, IL -6, IL -17A, TGF-β, FGF, and VEGF were found to be higher in the blood samples of the patient group than in the healthy group. The role of TLR signaling in fibroblasts is not entirely clear. It has been shown that different fibroblast populations can increase inflammatory infiltration through increased chemokine and cytokine production as a result of

stimulation by TLR. There have been several studies examining TLRs in systemic sclerosis [3,4,7,9,15,16, 22-38], but in localized scleroderma this issue is poorly understood [22]. This may be due to the fact that although morphea and systemic scleroderma have different clinics, they share a common pathological and pathogenetic mechanism [15].

The study by Agarwal and et al. demonstrated that interferon (IFN)-α2 upregulates TLR3 and increases TLR3-induced IL-6 production, and this activation of TLR3 may cause excessive fibroblast activation and collagen production in systemic sclerosis [23]. In our study, we found an increase in TLR3 gene expressions in lesional skin; however, this difference was not significant. This result could be related to the different study designs (in vitro/in vivo) and the number of samples (four samples versus 15 samples).

Many studies have shown that TNFAIP3–a negative regulator of TLR signaling–and TNFAIP3-interacting protein 1 (TNIP1) are both strongly associated with systemic sclerosis [24,25]. In addition, mRNA and protein levels of TNIP1 were significantly reduced in lesional skin and fibroblasts of patients with systemic sclerosis [26].

Some studies have shown increased expression of TLR3 in skin biopsies from patients with systemic sclerosis [21,27]. They also showed that in normal skin fibroblasts, TLR3 stimulation increased IFN-β production and the expression of IFN-regulated genes [27]. In addition, the expression of fibrotic genes was inhibited. Similarly, injection of a TLR3 ligand was found to induce dermal inflammation and the expression of IFN-regulated and fibrotic genes in experimental mice [28]. In our study, we also found higher expression of TLR3 in the patient group, with no significant difference. This difference could again be related to the different study designs (in vitro/in vivo).

Bhattacharyya and et al. showed that the expression of TLR4 was greatly increased in fibroblasts and vascular cells of skin biopsies from patients with systemic sclerosis [11]. Another report by Stifano showed that both TLR4 and its co-receptors–MD2 and CD14–were increased in lesional skin [12]. It was also found that TLR4 levels correlated with skin disease progression. In another study by the same group of authors, tenascin C–another endogenous ligand for TLR-4 and profibrotic molecule–was investigated in systemic sclerosis. Tenascin C plays a role in wound healing and tissue remodeling. The authors demonstrated that tenascin C levels were increased in the skin, serum, and sclerotic fibroblasts of patients with systemic sclerosis and in fibrotic skin tissue of mice. Furthermore, exogenous tenascin-C leads to stimulation of collagen gene expression and transformation of myofibroblasts via TLR4 signaling [29]. In our results, we could not detect an increase in the expression of TLR4. This difference could be related to the type of disease (localized/systemic) or the duration of the disease in our morphea patients.

TLR-2 has been found to be overexpressed in fibroblasts of systemic sclerosis, which produce a large amount of IL-6 and lead to fibrosis [30]. In addition, TLR-2 interacts with TLR1 and TLR6 and forms heterodimers to recognize a variety of pathogen-associated molecular patterns (PAMPs). In our study, we found overexpression of TLR1 and TLR2 in the patient group, with only TLR1 being significant.

The expression of TLR-5 and TLR-10 was also upregulated in fibroblasts of systemic sclerosis [31]. However, the authors claimed that TLR-5 may have a suppressive effect on collagen expression and play a role in regulating fibrosis. In support of this, it has been reported that triggering TLR-5 by PAMP flagellin can prevent collagen deposition in cultured fibroblasts in vitro [31]. One study showed that high mobility group protein 1 (HMGB1), an agonist of TLR-5, interacts with TLR5 and causes the induction of signaling that activates MyD88 and nuclear factor kappa B (NF-kB) [32]. Accordingly, these results may show that damage associated moleculer patterns (DAMPs) other than flagellin or other PAMBs induce the fibrosis process via TLR5 induction in systemic scleroderma. However, in our study, we did not detect any upregulation of TLR5 expression.

TLR-10 has an anti-inflammatory function after forming heterodimers with TLR-2. It has receptors on both B cells and plasmacytoid dendritic cells (pDCs) and activates gene transcription through MyD88 [33]. TLR-10 is thought to play a crucial role in regulating the IFN-I signaling pathway by sequestering dsRNA from TLR-3 [34]. In our study, we found a higher expression of TLR-10 in the patient group, and although this difference was not significant, it may be related to the balance of the inflammatory process in patients with morphea. Fang et al. found a significantly increased expression of the TLR-9 gene in the dermis of patients with systemic sclerosis compared to the dermis of control subjects. In this in vitro study, TLR-9 ligand was shown to stimulate a profibrotic profile and overproduction of TGF-β [35]. Our results were also in good agreement with the literature.

One study showed significantly increased TLR7 gene expression in patients with systemic sclerosis compared to a healthy group [36]. Those authors reported that increased TLR7 expression correlated positively with late-onset disease, active disease, and the presence of digital ulcers. In contrast to the literature, the authors found a lower expression of TLR9 in the patient group. In our study, we could not detect any upregulation of TLR7 expression, while the upregulation of TLR9 was without any statistical significance.

The presence of DAMPs or danger signals and inducing TLRs in association with fibrosis has already been

emphasized [37]. Fibronectin-EDA and tenascin-C are DAMPs that trigger TLR4 on resident cells, leading to stimulation of fibrotic gene expression and myofibroblast differentiation in systemic scleroderma. This TLR4 activation pathway is a potential target for antifibrotic therapeutic approaches in scleroderma and sclerotic diseases [29,38].

The main limitations of our study are that our number of patients was relatively small, and the high number of allergic conditions in the patient group may have affected cytokine levels, since Th2-driven diseases may influence the circulating levels of several cytokines such as IL-6 or TGF-beta, which may be enhanced by IL-4. An additional limitation may be that the analysis of histopathological parameters could not be performed due to budget constraints.

Conclusion

Our findings show that TLR pathways have important roles in the pathogenesis of localized scleroderma, similar to the findings in the systemic scleroderma literature, and we think that these findings will form the basis for the future development of TLR-based agents in the treatment of localized scleroderma.

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