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Research Article
Transforming Growth Factor-β1 in the Skin and Serum of Patients with Non-segmental Vitiligo

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Abstract

Background and Objective: Little is known about cytokine expression in skin of vitiligo patients. Studies of serum cytokine concentrations in vitiligo are few and results are often contradictory. This study aimed to examine the degree of expression of transforming growth factor (TGF-β1) in both the serum and the lesional skin of vitiligo patients. **Materials and Methods:** In this study, 38 patients with non-segmental vitiligo and 40 age and sex-matched controls were recruited. The TGF-β1 1evels were detected in serum using ELISA and in lesional specimens of cases and in controls by immunohistochemistry. The statistical analysis of the data was performed using independent T test, the Mann-Whitney U-test, Kruskal-Wallis test, X^2-test, exact test and Spearman correlation test . **Results:** The levels of serum TGF-β1 in patients with vitiligo showed no significant difference in comparison to controls ($p = 0.523$). Positive epidermal staining for TGF-β1 was detected in 10/24 (41.7%) of patients. The staining was mainly basal and suprabasal with no significant difference from controls. No relation was found between levels of TGF-β1 and disease extent or activity. **Conclusion:** Normal serum levels and lesional expression of TGF-β1 in this investigation do not support a role related to impaired levels of this cytokine in melanocyte cytotoxicity. Further studies about the TGF-β1 mediated signaling pathway in vitiligo is required to establish the role of TGF-β1 in the pathogenesis of vitiligo.

Key words: Transforming growth factor β1, serum TGF-β1, non-segmental vitiligo, immunohistochemistry, ELISA, tissue TGF-β1

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Vitiligo is an acquired condition which occurs secondary to selective destruction of melanocytes. It occurs in about 0.5-1% of the general population[1,2]. It is classified into two major forms: Segmental (dermatomal) and non-segmental types[3]. The destruction of melanocytes may be due to autoimmune mechanism, an intrinsic defect of melanocytes, cytotoxic and neural mechanisms[4]. The most accepted theory is autoimmune destruction of melanocytes by autoreactive CD8+ve T-cells, which have been demonstrated in peri-lesional skin. Also, melanocytes specific T-cells have been found in the peripheral blood of vitiligo patients[5,6].

Transforming growth factor-β (TGF-β) is an important immunoregulatory cytokine. Three members of TFG-β family (TGF-β1, TFG-β2, TFG-β3) have been recognized in mammals, with TGF-β1 being the predominant form expressed in the immune system[7]. The TGF-β has been reported to play a role in the suppressive function of $CD4^+CD25^+$ regulatory T cell (Tregs)[8]. Tregs are specialized subpopulation of $CD4^{++}$ cells that protect against autoimmune reactions to self antigens by several mechanisms. One of these mechanisms involves secretion of inhibitory cytokines mainly TGF-β[9]. This molecule may be an important target for therapy.

Previous studies of TGF-β in vitiligo have concluded different results[10-12] and non performed simultaneous assessment of serum and tissue levels. This study aimed to evaluate the expression of TGF-β1, the main regulatory cytokine of Tregs, both in the lesional skin and the sera of the same patients.

MATERIALS AND METHODS

Participants: In total, 38 patients with non segmental vitiligo (26 women and 12 men) from the outpatient clinic of Dermatology of Mansoura University Hospital and 40 healthy controls matched for age and gender (24 women and 16 men) were included in this study. The mean age of patients and controls was 34.7±16.5 and 32.4±13.1 years, respectively. Recruited patients had not received any treatment for vitiligo in the preceding 4 weeks. Patients who had other autoimmune disorders such as autoimmune thyroid diseases, diabetes mellitus, alopecia areata were not enrolled in this study. A detailed history and physical examination was done for every patient. Age, sex, duration of disease, family history and Koebner phenomenon positivity were noted. Type of vitiligo (focal or generalized) and involvement of body area in quartile percentiles were recorded. The course of vitiligo was defined as active when new lesions had appeared or existing lesions were spreading within the previous 6 months.

Samples collection: Peripheral venous blood samples (2 mL) were obtained from all the patients and control subjects and sera were separated and stored at -70°C until analysis. A 4-mm punch biopsy was obtained from the skin of selected number of the patients and controls who gave consent for taking skin biopsy (24 patients and 11 controls). In patients, biopsies were obtained from the lesional skin.

Quantification of TGF-β1 in the serum: Serum TGF-β1 was measured by solid phase enzyme-linked immunosorbent assay (ELISA) kits based on the sandwich principle (DRG instruments GmbH, Germany). Prior to testing the standards and patient samples were diluted in assay buffer, acidified with HCl then neutralized with NaOH. The neutralized samples were added to the antibody coated microtitre wells. After the first incubation, the unbound sample material was removed by washing and then a monoclonal mouse anti-TGF-β1 antibody, a biotinylated and mouse IgG antibody and streptavidin-HRP enzyme complex were incubated in succession. An immunoenzyme sandwich complex was formed. The unbound conjugate was removed by washing. Subsequently, substrate solution was added. Then colour development was stopped and the absorbance at 450 nm was measured with microtitre plate reader. The standard curve was constructed by plotting the mean absorbance obtained from each standard against its concentration. The concentration of each sample was determined from the standard curve.

Immunohistochemistry for tissue TGF-β1: All specimens were processed by 10% formalin-fixation and paraffin-embedding. Histopathological examination of hematoxylin and eosin sections was carried out to assess epidermal pigmentation. Immunolabelling was performed on tissue sections using broad spectrum histostain-plus kits (invitrogen CA 812251A). The following primary antibodies was used: monoclonal mouse anti-human TGF-β (clone TB21, 1: 50, Cat. # MCA797T, ABC serotec, UK). Briefly, after deparaffinization, Microwave heating in a solution of sodium citrate, pH6 was performed. Slides were quenched in 3% H_2O_2 for 10 min to block endogenous peroxidase and washed in PBS. Sections were then incubated with the primary Abs for 1 h and then with biotinylated secondary Ab followed by ABC reagents. Color development was achieved by incubating diaminobenzidine (DAB) as a substrate. Slides

were counterstained with Mayer's hematoxylin. Substitution of the primary Ab with an irrelevant IgG served as negative controls. In the slides from the control patients, we used a beautiful counterstain for melanocytic lesionsthat involves a 5 min stain in Wright-Giemsa stock solution, followed by a 20 sec hematoxylin counterstain.

The TGF-β1 immunoreactivity was evaluated as negative or positive, the positivity was assigned when positive expression was seen in 5% of cells in fibroblasts, endothelial cells, inflammatory cells and in the epidermis. The intensity of TGF-β1 expression was assessed subjectively as mild, moderate, or strong[13].

Statistical methods: Comparison of quantitative variables between the study groups was performed using independent T test, the Mann-Whitney U-test for independent samples (as appropriate) or Kruskal-Wallis test (if more than two groups were compared). For comparison of categorical data, the X^2-test was performed. The exact test was used when the expected frequency was less than 5. Spearman correlation test was used for the relation between quantitative parameters. The significance level was set at P less than 0.05. Statistical analyses were performed using a software package (SPSS, Version 1 6.0 for Windows, SPSS Inc, Chicago, IL)[14].

RESULTS

The duration of vitiligo ranged from 3 months to 38 years, with a median of 4.5 years. The disease was active in 31 (81.6%) patients. Ten patients (26.3%) had positive family history of vitiligo. Koebner phenomenon was positive in 8 patients (21.1%). Generalized type vitiligo was detected in 33 (86.8%) patients. Involvement of the body area was under 25% in 22 patients (57.9%), between 26-50% in 10 (26.3%) patients, between 51-75% in 4 patients (10%) and more than 75% in 2 patients.

Serum TGF-β1: The levels of serum TGF-β1 in patients with vitiligo showed no significant difference in comparison with controls (Table 1). Likewise, there were no significant difference in the levels of serum TGF-β1 in active vitiligo group when compared with control group or stable vitiligo group ($p>0.05$). In the patient group, no relationship was found between serum TGF-β1 levels and sex, family history, koebner positivity, course, or extent of the disease ($p>0.05$) for each. Serum TGF-β1 was not correlated with duration of the disease or involvement of body area.

TGF-β1 immunohistochemical staining: All cases of vitiligo showed absence of pigmentation by H and E. The TGF-β1 was expressed in dermal fibroblasts and endothelial cells of both normal control skin and vitiligo patients. In the epidermis, positive immunoreactivity for TGF-β1 was detected in 10/24 (41.7%) of patients compared with 5/11 (45.5%) in control skin. The staining was of mild to moderate intensity (Fig. 1) and was mainly focal (basal and suprabasal). There was no significant difference in immunoreactivity or its intensity between vitiligo cases and control (Table 2). Patients with negative epidermal immunoreactivity for TGF-β1 showed significantly lower serum cytokine levels compared to those with positive immunoreactivity (29.45±11.6 vs 39.93±9.08 ng mL^{-1}, $p = 0.027$).

Table 1: Comparison of serum TGF-β1 levels in patients with vitiligo and the control group

Groups	Mean±SD (ng mL^{-1})	Median (ng mL^{-1})	Range (ng mL^{-1})	*p-value
Vitiligo patients (n = 38)	36.341±12.0334	36.35	11.770-57.325	0.523
Control (n = 40)	38.677±15.125	38.66	11.535-66.090	

*Independent sample T test

Table 2: Comparison of TGF-β1 expression in lesional skin of patients with vitiligo and control groups

TGF-β1	Cases (n = 24)	Control (n = 11)	*p
Epidermal expression			
Positive	10 (41.7%)	5 (45.5%)	1.000
Negative	14 (58.3%)	6 (54.5%)	
Intensity			
Mild	5 (50.0%)	3 (60.0%)	1.000
Moderate	5 (50.0%)	2 (40.0%)	
Pattern			
Focal	9 (90.0%)	4 (80.0%)	1.000
Diffuse	1 (10.0%)	1 (20.0%)	
Dermal expression			
Positive	22 (91.7%)	10 (90.9%)	1.000
Negative	2 (8.3%)	1 (9.1%)	

*Fisher's exact test, 10 (41.7%): This means that 10 patients out of 24 (41.7%) showed positive epidermal expression of TGF-β1 in lesional skin

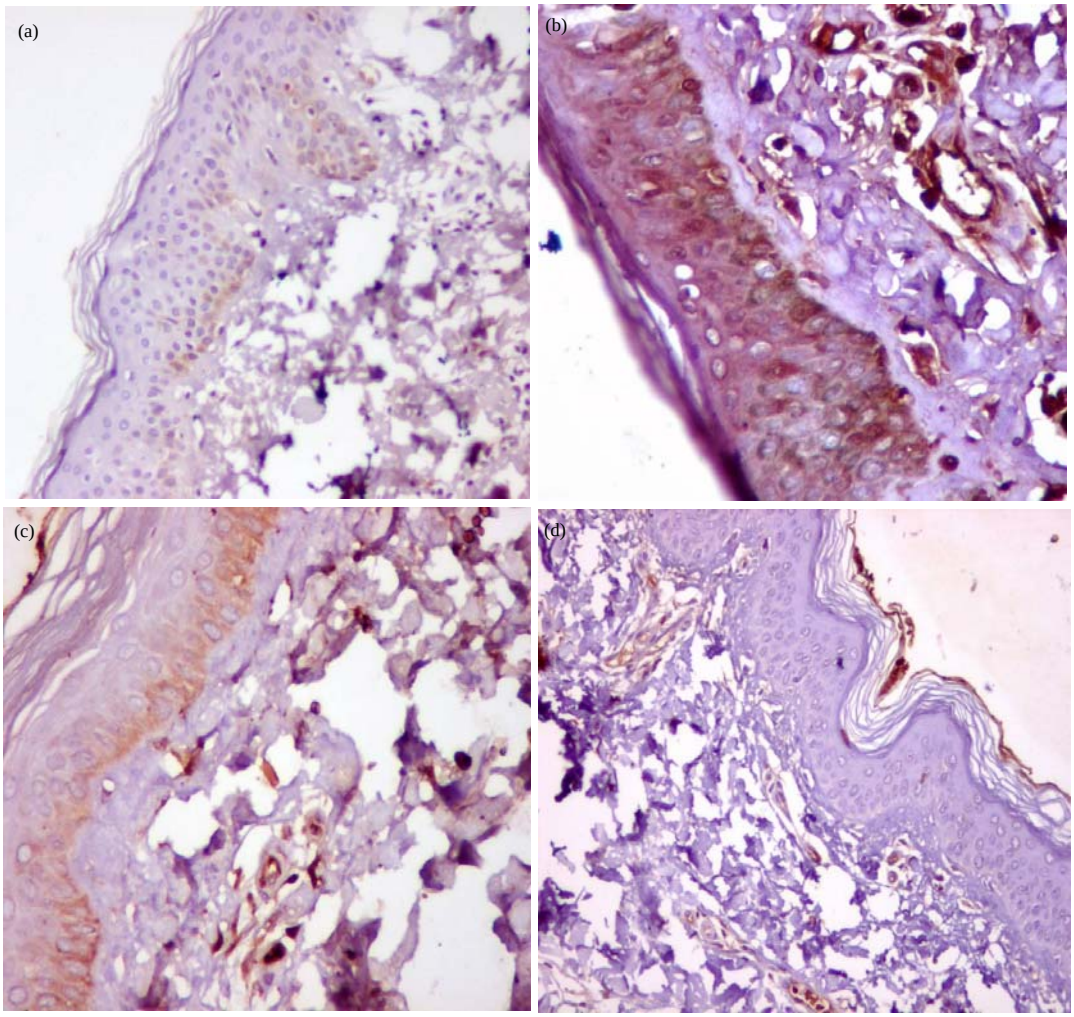

Fig. 1(a-d): (a) Focal mild expression of TGF-β1 in epidermis of control group (DAB×200), (b) Diffuse moderate expression of TGF-β1 in epidermis of control group (DAB×200), (c) Focal moderate expression of TGF-β1 in epidermis of patient group (DAB×200) and (d) Negative stainingTGF-β1of in epidermis of patient group (DAB×200)
Endothelial cells in the dermis show positive staining in all figures (internal positive control)

DISCUSSION

Transforming Growth Factor β (TGF-β) is a potent regulatory cytokine produced by Treg cells. It maintains peripheral tolerance by inhibiting the proliferation and differentiation of self reactive CD4 and CD8 T cells as well as B cells[9]. When TGF-β ,the main product of Tregs is deficient in mice, reduced number of peripheral Tregs followed by deficient production of TGF-β can be demonstrated[15]. Repigmentation in a mouse model of epidermal depigmentation was associated with an increased Treg cells infiltration, supporting their role in preventing immune reaction against melanocytes[16]. As TGF-β has inhibitory role on autoimmunity[17], lower levels of this cytokine is expected in vitiligo. However, TGF-β is known to inhibit melanocyte activity[18] thus its levels may be higher in the sera of patients with vitiligo.

In the present study, there was no significant alteration in serum TGF-B1 in vitiligo patients compared to controls. This is in agreement with Chodorowska *et al.*[10].

Contrasting results however, were obtained by other research groups. In a recent study, Zhou *et al.*[12] demonstrated elevated serum TGF-β1 and increased circulating Th17 cells. Accordingly, they suggested that elevated serum TGF-β1 could contribute to enhanced Th17 cell differentiation in non segmental vitiligo. On the other hand, Basak *et al.*[19] reported reduced serums TGF-β levels in vitiligo patients and suggested that decreased TGF-β levels may contribute to enhanced cellular immunity, reduced maturation of Tregs, thus facilitating occurrence of vitiligo. Subsequently,

Tu *et al.*[11] have found that the TGF-β1 levels both in serum and supernatant of cultured $CD4^+CD25^+$ Tregs were decreased in the active vitiligo group when compared with the control or stable groups. Thus, they suggested that TGF-β1 may play a role in the pathogenesis of non-segmental vitiligo related to the suppressive function of Tregs. Dysregulation of Tregs in patients with vitiligo was reported[20-22]. However, Klarquist *et al.*[23] studied the regulatory T cells in vitiligo and reported a defect in the skin homing of Tregs but circulating Tregs were abundant, functional and capable of inhibiting helper T cell proliferation. The latter data support our finding of intact serum levels of TGF-β1 considering the importance of this cytokine in the development and suppressive function of the peripheral Tregs[8]. Our findings suggest that alteration in serum levels of TGF-β1 is less likely to be involved in melanocyte cytotoxicity in vitiligo. This assumption is supported by the absence of correlation between serum TGF-β1 levels and percentage of body surface area involvement or activity of vitiligo.

The association of TGF-β1 levels with the activity and/or extent of vitiligo is another issue of controversy; some reports revealed no relation between cytokine level and course of the disease [19]. Another study have reported a negative correlation with both the activity and the extent of the disease[11]. While Tembhre *et al.*[24] found that reduced serum TGF-β1 concentrations were positively-correlated with percentage of body surface area involvement and disease duration. On the other hand, Zhou *et al.*[12] found that elevated TGF-β1 was positively correlated with the body surface area of the lesions in non segmental vitiligo. The discrepancies of the results from different studies[11,12,19,24] may be due to difference among the patients in the disease stage or extent or ethnic variations. Also in contrast to this study, Basak *et al.*[19] included patients with other autoimmune diseases which could be responsible for change in cytokine levels.

The TGF-β1 is a multipotent cytokine that regulates cell proliferation and differentiation of many cells[25]. The skin has been shown to be an important target tissue of TGF-β. The expression of TGF-β receptors was detected in epidermal keratinocytes[26]. Within human skin, TGF-β1 production has been reported in keratinocytes, melanocytes, fibroblasts and endothelial cells[13,12,27]. The function of TGF-β varies with its target tissue origin or cell types. In the skin, TGF-β has been demonstrated to inhibit the growth of keratinocytes but stimulate the growth of fibroblasts[25,28]. Nishimura *et al.*[18] showed that the growth of normal human melanocytes in culture was inhibited by exogenous TGF-β1. The regulation of TGF-β in vitiligo skin is not clear. Moretti *et al.*[29] examined the epidermal expression of TGF-β in 15 vitiligo patients and 5 controls, They found no difference between the patients and the controls. The TGF-β was found at minimal levels in all skin specimens including controls. In the present study there was no significant difference in immunoreactivity for TGF-β1 or its intensity in the epidermis or dermis between vitiligo cases and control. Thus, direct inhibitory effect of TGF-β on melanocytes seems unlikely to be involved in depigmentation process in vitiligo. Patients with negative epidermal immunoreactivity for TGF-β1 showed significantly lower serum cytokine levels compared to those with positive staining ($p = 0.027$) suggesting that the mechanisms that control TGF-β1 production and release in both the blood and skin of vitiligo patients may be related.

While TGF-β1 levels were not deficient in our cohort of vitilligo patients, however there may be a possible defect in TGF-β mediated suppression activity either due to defective TGF-β signaling pathway or existence of TGF-β inhibitory effector molecules[24] that may lead to enhanced proliferation of autoreactive T cells and selective destruction of melanocytes from the epidermis.

CONCLUSION

Serum and skin TGF-β1 were analyzed in patients with non-segmental vitiligo. Serum levels and lesional expression of TGF-β1 were not altered. This suggests that the depigmentation process in vitiligo is less likely to be directly mediated by impaired levels of TGF-β1.

SIGNIFICANCE STATEMENTS

This study found normal values of serum and lesional expression of TGF-β1 in patients with non-segmental vitiligo and controls, which does not support a role of TGF-β1 in the pathogenesis of vitiligo. However, larger scale studies and further investigations about TGF-β1 mediated signaling pathway in vitiligo are needed to clarify its role in the pathogenesis of vitiligo.

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