Pathol. Oncol. Res. (2009) 15:251–256
DOI 10.1007/s12253-008-9119-8

ORIGINAL PAPER

Expression of TGFβ1 and Its Signaling Components by Peripheral Lymphocytes in Systemic Lupus Erythematosus

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Received: 14 April 2008 / Accepted: 20 October 2008 / Published online: 20 November 2008

Abstract Transforming growth factor β1 (TGFβ1) is an important immunosuppressive cytokine. Defects in its production by lymphocytes and the failure of TGFβ1 to regulate immunological functions have been described in SLE. Expression of TGFβ1 and the related signaling pathway was studied in the peripheral lymphocytes of SLE patients. The total plasma TGFβ1 level in active and inactive SLE patients compared to healthy controls was also measured. TGFβ1 and all downstream signaling elements were expressed in normal cells. However, in more than 50% of SLE patients the isolated T cell population showed no TGFβ1 mRNA expression and at least one member of the TGFβ1 pathway was also missing (TGFβ-RI, Smad2 and Smad3) in more than half of the patients. Total plasma TGFβ1 level was increased in both active and inactive SLE groups compared to normal controls ($p< 0.05$). These data raise questions about the availability of TGFβ1 signaling in lymphocytes in SLE patients, however, the elevated total plasma TGFβ1 level suggests that the failure of TGFβ1 effects is not the consequence of low level of this cytokine in SLE.

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Keywords Autoimmune · Signaling · Smad · SLE · TGFβ

Abbreviations

TGFβ1	Transforming growth factor β1
SLE	systemic lupus erythematosus
PBMC	peripheral blood mononuclear cells

Introduction

Systemic lupus erythematosus (SLE) is a generalized autoimmune disorder characterized by T and B cell hyperactivity with autoantibodies against numerous cell components and consequential deposition of immune complexes [1–4]. The disease has a multifactorial pathogenesis with genetic, hormonal and environmental factors.

TGFβ is a family of multifunctional cytokines important in inflammatory and immunological responses [5]. Several epithelial and mesenchymal cell types, including lymphocytes and monocytes, can produce the β1 isoform. It is secreted as an inert precursor molecule and activated in the extracellular space mainly by proteases (e.g. plasmin, matrix metalloproteases, tissue transglutaminases) [6]. TGFβ1 influences differentiation, inhibits proliferation and induces apoptosis of B and T cells [7–10], and it is an important costimulator generating regulatory CD4 +CD25+ and CD8+ T cells [7, 11–13], that help maintain tolerance against self and non-self antigens.

The TGFβ1 signal transduction pathway comprises specific cell surface receptors with serin/threonine kinase activity (TGFβ-RI and TGFβ-RII) and intracellular effector molecules, the Smad proteins [5, 14, 15]. Upon ligand binding the receptors are activated followed by the

phosphorylation of R-Smads (receptor-associated Smads: Smad2, 3) and their complex formation with Co-Smad (common mediator Smad: Smad4). This complex is then translocated to the nucleus where it regulates target genes directly or by recruiting additional transcription factors. I-Smads (inhibitory Smads: Smad6 and 7) have the duty to inhibit R-Smads (e.g. by binding to TGFβ-RI and thereby blocking their activation). As the expression of Smad6 and Smad7 is induced by TGFβ1 they inhibit signaling by a negative feedback loop.

Defects in TGFβ1 production by lymphocytes [16] and the failure of TGFβ1 induced immunoregulation have been described in SLE. Reduced activity of regulatory CD4+CD25+ and CD8+ cells contribute to the generation of autoreactive T helper cells, and to the defects in immunological tolerance [17–19]. Attempts for therapeutic supplement of TGFβ1 were made but led to controversial results [2, 16]. Defective signal transduction may also be a cause of reduced response to TGFβ1, however, no such data are available in the literature. Therefore our aim was to study the expression of TGFβ1 signal transduction elements in lymphoid cells of SLE patients.

Patients and Methods

Patients

Twenty-seven patients with SLE were included in the mRNA expression study (26 females and one male; mean age: 36.6 years). Diagnosis was based on the criteria of the American Rheuma Society [20]. Disease activity was determined by the SLEDAI index [21]—based on clinical and laboratory findings—and the intensity of symptoms. Patients were divided into two groups: patients with inactive disease (group 1; $n = 20$), and with active disease (group 2; $n = 7$). Active SLE patients received no treatment before and during our study. Healthy persons ($n = 11$; mean age: 37.4 years) with no immunologically or hematologically related disease served as normal controls. Samples were obtained with informed consent and all protocols were approved by the Institutional Ethical Review Board (TUKEB 118/1999).

Cell Isolation

Peripheral blood mononuclear cells (PBMC) were separated from 30 ml anticoagulated venous blood samples by Histopaque−1,077 (Sigma) gradient centrifugation. B and T cells were isolated using MACS CD19 and MACS CD3 MicroBeads (Miltenyi Biotec), respectively, according to the manufacturers' instructions. Cells were stored at −70°C until further use.

RT-PCR

Total RNA was extracted with the PureLink Micro-to-Midi RNA isolation kit (Invitrogen, Carlsbad, CA). 100 ng RNA was reverse transcribed with MMLV-RT and random hexamers (Invitrogen). PCR reactions were performed with RedTaq polymerase (Sigma). PCR products were resolved on agarose gels, stained with ethidium-bromide and documented by Eagle Eye video densitometer (Stratagene). Qualitative data evaluation was performed. Cycling parameters were: 95°C 1min, 55°C/57°C/58°C/60°C 1min, 72°C 1min, 30–35 cycles. β-actin was used as an internal control. The following primers were used: Smad2 (746 bp, 57°C) 5′ACA AGA GGC TGT TTT CCT AG3′, 5′GAG AGC CTG TGT CCA T3′; Smad3 (404 bp, 55°C) 5′AAC AAG AAT GCA GCA GTG GA3′, 5′ATG GTG CAC ATT CGG GTC AA3′; Smad4 (205 bp 60°C) 5′GTG GAA TAG CTC CAG CTA TC3′, 5′CGG CAT GGT ATG AAG TAC TCC3′; Smad6 (688 bp 57°C) 5′ACC ACC ATG GAA TCT CCG CCA CCT CCC TAC3′, 5′CCG CCA CTA TCT GGG GTT G3′; Smad7 (501bp 57°C) 5′TCC TTA CTC CAG ATA CCC GAT3′, 5′ACG CCT TCT CGT AGT CGA AAG3′; TGFβ1 (223 bp, 60°C) 5′GCC CTG GGA CAC CAA CTA TTG C3′, 5′ GCT GCA CTT GCA GGA GCG CAC3′; TGFβ-RI (224 bp 58°C), TGFβ-RII (261 bp 58°C), β-actin (538 bp 58°C) 5′GTG GGG CGC CCC AGG CAC CA3′, 5′CTC CTT AAT GTC ACG CAC GAT TTC3′.

ELISA

Blood (5 ml) was collected in pyrogen-free siliconized tubes with EDTA, stored at 4°C and centrifuged for 10 min at 1000 g. Platelet poor plasma was aliquoted and stored at −70°C. (A maximum of 2 h passed between sample acquisition and the freezing procedure during sample processing.) The total (active and inactive) amount of plasma TGFβ1 concentration was measured by Quantikine ELISA sandwich kit (R&D Systems). Absorption was measured at 450 nm wavelength. The calibration curve was determined by standard solutions containing recombinant human TGFβ1 of a given concentration provided by the manufacturer. The sensitivity of the reaction is 7 pg/ml.

Statistical Analysis

For statistical analysis of the data mean arithmetic values (x) and standard deviation (SD) were calculated. Based on Bera-Jarque test the hypothesis of normality of the sample distributions can be clearly rejected. Bootstrap method (10,000 simulations) was performed for comparison of the means, $p < 0.05$ was considered statistically significant.

Results

Gene Expression of TGFβ1 Signaling Components

Normal control cells showed mRNA expression of all examined signaling elements (Smad2, 3, 4, 7, TGFβ1, TGFβ-RI and TGFβ-RII), with the exception of Smad6, which was absent in all — normal and SLE — samples. mRNA expression of Smad2, 3, 7, TGFβ1, TGFβ-R1 genes in T cells from both active and inactive SLE patients was not to be detected in certain cases (Table 1, Fig. 1): in more than 50% of the patients the isolated cells showed no TGFβ1 mRNA expression and the T cell populations (13/20 inactive and 4/7 active SLE patients) failed to express mRNA of at least one element required for the TGFβ1 signal: TGFβ-RI, Smad2 or Smad3. Smad6 expression was found to be absent both in SLE cases and normal controls.

The expression of TGFβ1 signaling elements in B cells can only be partially evaluated due to the low number of isolated cells. Relatively higher number of isolated B cells were obtained from 11 out of 20 SLE patients. In all these cases, B cell populations showed deficient mRNA expression of either Smad2, 3, 7, TGFβ1 or TGFβ-R1 (data not shown).

Plasma Concentration of Total TGFβ1

Blood derived total TGFβ1 plasma levels were 635.02 ± 217.18 pg/ml in controls (n = 10), 900.33 ± 218.03 pg/ml in active SLE patients (n = 7) and 912.11 ± 343.55 pg/ml in inactive SLE patients (n = 18) (Fig. 2). Concentrations were higher in both SLE groups than in the control group ($p<0.05$). No statistically significant difference could be observed between the values of active and inactive SLE patients.

Table 1 Summary of clinical and experimental data of SLE patients. Gene expression of TGFβ1 signal transduction elements in T lymphocytes determined by RT-PCR is shown, qualitative evaluation

Patient[a]	Sex	Age (years)	Disease duration (years)	Disease activity	SLEDAI score	Therapy[b]	Smad2	Smad3	Smad4	Smad7	TGFβ	TGFβ R1	TGFβ R2
I/1	F	30	8	−	2	−	+	+	+	+	−	+	+
I/2	F	45	3	−	4	I	−	+	+	+	−	+	+
I/3	F	39	16	−	4	S	−	+	+	−	+	+	+
I/4	F	24	6	−	4	C+S	−	−	+	−	−	−	+
I/5	F	32	12	−	0	C+S	−	+	+	+	−	+	+
I/6	F	55	16	−	2	S	−	+	+	+	+	+	+
I/7	F	22	2	−	0	I+S	+	+	+	+	+	+	+
I/8	F	50	17	−	4	S	−	+	+	+	+	+	+
I/9	F	27	6	−	6	S	+	+	+	+	−	+	+
I/10	F	61	15	−	4	I	+	+	+	+	−	+	+
I/11	F	30	9	−	0	S	−	+	+	+	−	+	+
I/12	F	27	3	−	2	S	−	+	+	+	−	+	+
I/13	F	39	6	−	0	I	+	+	+	−	+	+	+
I/14	F	40	3	−	2	S	+	+	+	+	+	+	+
I/15	F	26	10	−	6	S+I	−	−	+	−	−	−	+
I/16	F	47	5	−	2	C+I	−	−	+	−	−	+	+
I/17	F	57	36	−	0	−	−	+	+	−	−	+	+
I/18	M	32	12	−	4	S	+	+	+	−	+	+	+
I/19	F	18	4	−	2	S+C+I	−	−	+	−	−	−	+
I/20	F	28	8	−	0	S	−	−	+	−	−	−	+
II/1	F	39	2	+	4	−	+	+	+	+	−	+	+
II/2	F	24	4	+	6	−	+	+	+	+	−	+	+
II/3	F	30	1	+	32	−	−	+	+	+	+	+	+
II/4	F	56	0	+	6	−	+	+	+	+	−	+	+
II/5	F	50	3	+	6	−	−	+	+	−	+	+	+
II/6	F	24	2	+	3	−	−	−	+	−	−	−	+
II/7	F	36	8	+	18	−	−	+	+	+	+	+	+

[a] Group I inactive SLE patients, group II active SLE patients [b] S: steroid (Prednisolon, Medrol), I: immunosuppressive drugs (Imuran, Delagil, Sandimmune), C: cytostatic drugs (Methotrexate)

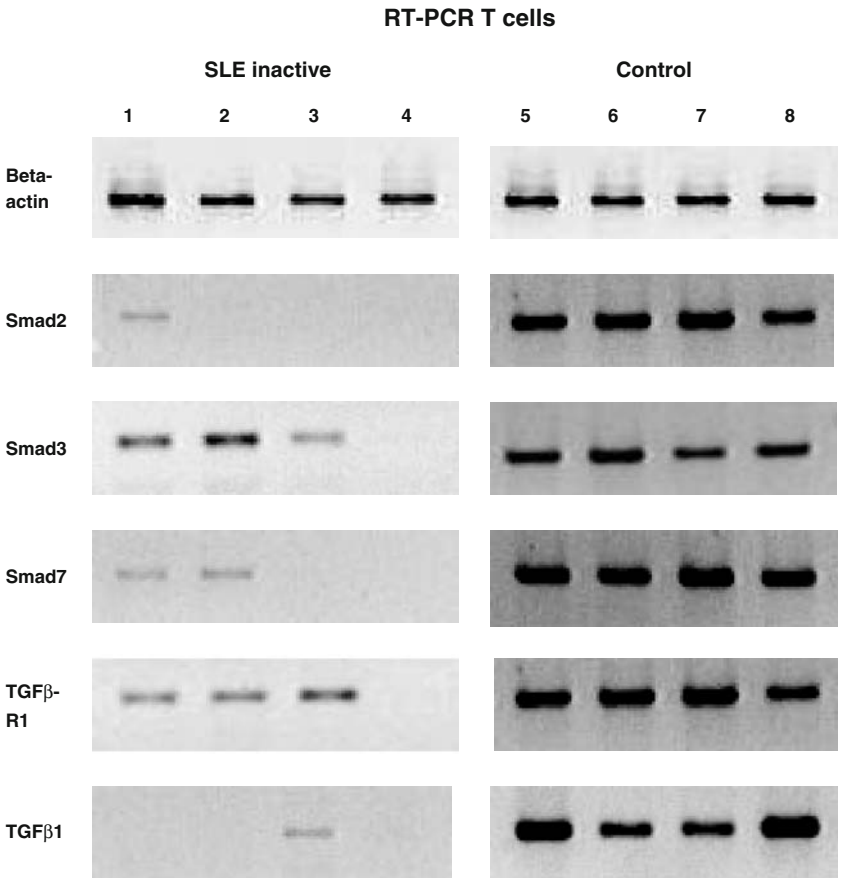

Fig. 1 Expression of TGFβ1 and its signaling pathway elements in T cells of inactive SLE patients (1–4) and normal controls (5–8) (RT-PCR)

Discussion

The role of dysfunctional TGFβ1 in the immunoregulatory defects in SLE are supported by a large amount of data. However, published values on serum and plasma TGFβ1 levels and TGFβ1 production in SLE are rather conflicting. Moreover, expression studies concerning the signaling components of TGFβ are completely lacking. (Serum — in contrast to plasma — lacks coagulation factors, which may bind TGFβ1 directly or indirectly, providing an explanation for differences between plasma and serum TGFβ levels.) The main goal of our study was to characterize the expression of TGFβ1 and its signaling pathway elements in the lymphocytes of SLE patients.

We showed that TGFβ1 mRNA production is defective in T and B cells isolated from SLE patients' peripheral blood. In more than half of SLE patients T cells displayed undetectable levels of TGFβ1 mRNA. Nevertheless, we found slightly increased total plasma TGFβ1 levels both in active and in inactive SLE patients. This may seem paradoxical, but NK cells, monocytes and other non-lymphoid cells are also potential producers of TGFβ1. Ohtsuka et al [16] reported no detectable TGFβ1 production in isolated T cells, but found no difference between TGFβ1 production of monocytes from normal and SLE patients. Their results correlate well with ours, however, they come from in vitro short term culture systems.

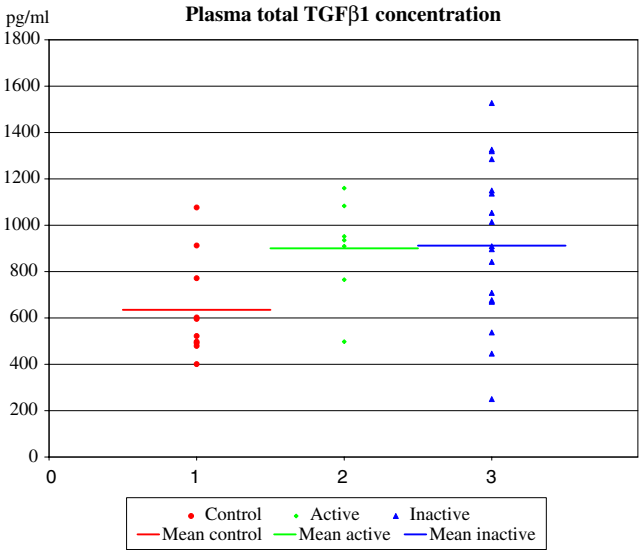

Fig. 2 Plasma total TGFβ1 concentration measured by ELISAl

Alterations of the cytokine context may contribute to the decreased production of TGFβ1 by lymphocytes. Low serum levels of cytokines such as IL-2 and TNF-α which upregulate and elevate the level of IL-10 which down-regulate TGFβ1 were detected in SLE patients [16].

It needs to be clarified whether the dysregulation of TGFβ1 production is under genetic control in SLE lymphocytes. Polymorphisms in the TGFβ1 gene may alter mRNA expression levels and influence plasma TGFβ1 concentration. Having examined the known polymorphisms within the TGFβ1 gene, no significant difference was found in genotype distribution and allele frequencies between SLE patients and healthy controls [22, 23]. Novel single nucleotide polymorphisms have been identified in the promoter region of latent TGFβ1 binding protein-1 L (LTBP-1 L) [24] and in the gene of TGFβ-RI [25]; however, their contribution to autoimmune diseases is still unknown.

The total plasma TGFβ1 level was slightly elevated both in active and inactive SLE patients. Other studies found no difference between the serum TGFβ1 levels of normal controls and SLE patients [26]. Although our results are based on relatively small number of samples, it is suggested that the failure of TGFβ1 function is not the consequence of low blood TGFβ1 level in SLE.

These data raise questions about the availability of TGFβ1 signaling in lymphocytes in SLE patients. Moreover, there is a large amount of data concerning latent TGFβ1 complex conversion defects, for example impaired conversion by decreased plasmin activity was found in SLE [27].

We characterized first the expression of TGFβ1 signaling elements in normal versus SLE lymphocytes. Alterations in the expression of TGFβ1 signal transduction elements in T cells could be observed in certain cases. The expression of TGFβ-RI, Smad2, Smad3 and Smad7 was absent frequently. Factors that regulate R-Smad and TGFβ-R1 expression have not yet been completely identified. Low expression of TGFβRs was found by Espinoza et al [28] in rheumatoid arthritis patients following anti-TNF-α treatment. However, the connection between low TNF-α plasma level and TGFβR expression has not yet been proved [29]. Other reports suggest that TGFβ-R1 expression defects, gene polymorphisms [25] or the effects of other signaling pathways might also underlie these findings. Smad7 (an inhibitory Smad) acts as a negative feedback regulator of TGFβ signaling, and the paradoxical absence of Smad7 expression may be the consequence of defective TGFβ1 function, but may also show the influence of other signaling pathways on the effects of TGFβ1 [15].

Protein expression of Smad2 and 3 in T and B cells measured by flow cytometry showed no difference between healthy and SLE groups (data not shown), however, commercially available antibodies cannot distinguish between Smad2 and 3, and the absence of one R-Smad may be balanced by the presence of the other. More specific antibodies would be required for the distinction in the expression of these proteins.

Additional deficiencies related to TGFβ1 dysregulation have also been described. It is suggested that the failure of TGFβ1-dependent down-regulation of IgG production in SLE might lead to hyperactive plasma cells, and the IgG production correlates well with the amount of IgG - TGFβ1 complexes. Increased amounts of these were found both in the sera of active and inactive SLE patients without statistically significant difference between the two groups [30]. These complexes may have deleterious effects on host defence against microbial infections. These findings are consistent with the impaired capacity of SLE patients to resist infection [8].

It seems that TGFβ1 dysfunction is an important factor in the pathogenesis of SLE. Low TGFβ1 production by lymphocytes, impairment of latent TGFβ1 complex conversion by decreased plasmin activity, genetic factors such as polymorphisms of the TGFβ1 and TGFβ-R1 genes, alterations of the cytokine context and deficient expression of TGFβ1 signal transduction elements in lymphoid cells could be of great importance. Although these factors have not yet been completely described, a thorough knowledge of these mechanisms could help us to better understand the development of SLE.

Acknowledgements This work was supported by grants from the National Science Foundation of Hungary, Ministry of Welfare and Ministry of Education and Culture, Hungary (OTKA (F048380, TS049887) and Békésy Foundation (118/2001).

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