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Transcriptional activity of genes coding tumour necrosis factor α and its receptors in patients with systemic sclerosis and Raynaud's phenomenon

Aktywność transkrypcyjna genów kodujących czynnik martwicy nowotworów α i jego receptorów u chorych z twardziną układową i objawem Raynauda

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Summary

Introduction. The role of TNFα in the pathogenesis of systemic sclerosis (SSc) is still controversial. This factor may play a significant role at the early stage of SSc development, i.e. in vascular changes (Raynaud's phenomenon – RP) and in fibrosis processes.

Aim. The aim of this study was to investigate the changes in the number of mRNA copies of genes coding TNFα and its receptors in peripheral blood leukocytes in patients with SSc and RP.

Material and methods. The research concerned 19 patients with active lSSc, 11 patients with isolated RP and 10 healthy persons. Quantification of TNFα, TNFαRI and TNFαRII genes mRNA was carried out with the use of Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction.

Results. The number of copies of TNFα mRNA in active lSSc patients was significantly lower than in the control group. No statistically significant difference in transcriptional activity of TNFα gene between active lSSc and isolated RP patients as well as isolated RP and the control group was found. Comparing to controls the expressions of TNFαRI and TNFαRII were significantly lower both in active lSSc patients and in isolated RP patients. The TNFα/TNFαRII ratio both in active lSSc and isolated RP patients was significantly higher opposed to the control group. The TNFαRI/TNFαRII mRNA ratio was significantly higher in patients with active lSSc than in the control group.

Conclusions. The decrease in transcriptional activity of TNFα, TNFαRI and TNFαRII genes in SSc may be crucial in fibrosis. Receptors proportion may be important in the regulation of TNFα anti-fibrotic activity.

Key words: real-time QRT-PCR, TNFα, systemic sclerosis, Raynaud phenomenon

Streszczenie

Wprowadzenie. Znaczenie TNFα w patogenezie twardziny układowej (ang. *systemic sclerosis* – SSc) budzi nadal kontrowersje. Czynnik ten może odgrywać znaczącą rolę we wczesnym stadium rozwoju SSc, tzn. w zmianach naczyniowych (objaw Raynauda, ang. *Raynaud phenomenon* – RP) i procesie włóknienia.

Cel pracy. Celem pracy było zbadanie jak się zmienia liczba kopii mRNA genów kodujących TNFα i jego receptory w leukocytach krwi obwodowej chorych z SSc i RP.

Materiał i metody. Badaniem objęto 19 chorych z aktywną limited SSc (lSSc), 11 pacjentek z izolowanym objawem RP i 10 osób zdrowych. Oznaczenie mRNA genów kodujących TNFα, TNFαRI i TNFαRII przeprowadzono techniką ilościowej reakcji amplifikacji z odwrotną transkrypcją w czasie rzeczywistym (ang. *Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction*, real-time QRT-PCR).

Wyniki. Liczba kopii mRNA dla TNFα była znacząco niższa u chorych z aktywną lSSc w stosunku do grupy kontrolnej. Nie stwierdzono znaczących różnic w aktywności transkrypcyjnej dla TNFα, pomiędzy grupą chorych z aktywną lSSc a pacjentami z RP, jak również pomiędzy chorymi z izolowanym RP a grupą kontrolną. W porównaniu z kontrolą, ekspresja TNFαRI i TNFαRII były znacząco niższe, zarówno u chorych z aktywną lSSc, jak i u pacjentów z izolowanym RP. Stosunek TNFα/TNFαRII mRNA w obu grupach, aktywnej lSSc i izolowanym RP, był znacząco wyższy w porównaniu z grupą kontrolna. Stosunek TNFαRI/TNFαRII mRNA był wyższy u chorych z aktywną lSSc niż w grupie kontrolnej.

Wnioski. Obniżenie aktywności transkrypcyjnej genów TNFα, TNFαRI i TNFαRII może być kluczowe w procesie włóknienia związanym z SSc. Proporcje pomiędzy receptorami mogą być ważne w regulacji aktywności antyfibrogennej TNFα.

Słowa kluczowe: real-time QRT-PCR, TNFα, twardzina układowa, objaw Raynauda

INTRODUCTION

Systemic sclerosis (SSc) is a systemic connective tissue disease, in which skin, organs and systems, such as digestive tract, lungs, kidneys and heart are undergoing progressive fibrosis. Etiopathogenesis of this disease is still mostly unknown. Usually, first clinical symptom is Raynaud's phenomenon (RP) – sudden discoloration with subsequent edema of hands – resulting from general vasculopathy of small vessels within the skin and internal organs (1). Destruction and activation of endothelium and fibrosis process are related, among others, with expression of many cytokines. Tumour necrosis factor α (TNFα) belongs to the most important mediators and it stimulates synthesis of many pro-inflammatory cytokines (IL-1, IL-8, IL-6, GMCSF), stimulates proliferation of fibroblasts, decreases matrix metalloproteinase activity, induces expression of ICAM-1, VCAM-1 and E-selectin on endothelial cells, it influences release of: von Willebrand's factor (vWF), vasoconstriction factors – endotheline-1 (ET-1), many cyclooxygenase products (COX), such as thromboksan A2 (1-4). It also influences expression of endothelial nitric oxide synthetase by shortening its half-life time in endothelial cells and increases both proliferation and apoptosis (by activating caspase-3) in vascular smooth muscle cells, therefore regulating the number of these cells (5, 6). However, the role of TNFα for fibrosis in SSc is controversial. Actually there is good evidence that TNFa has anti-fibrotic effects at least *in vitro* (7). Moreover, TNFα is rarely detected in sera of the patients with SSc, and its concentration weakly correlates with the clinical status (8-10). This is explained by a short half-life time of this cytokine and the presence of circulating inhibitors, mostly soluble TNFα receptors (10). Soluble TNFαRI and sTNFαRII receptors are present in the circulation of healthy persons in little amounts, but upon activation of the immune system their concentration may significantly increase exceeding by 100 folds concentration of this cytokine (10). We have reported increased TNFαRI levels in sera of patients with SSc and some of the patients with RP evolving to SSc (11). TNFα protein has not been detected in the examined SSc sera in our studies (8).

Therefore, the aim of this work was to asses the changes in the number of copies of mRNA for TNFα and its receptors in leukocytes from the patients with active lSSc and isolated RP.

AIM OF THE STUDY

The study group consisted of 19 patients (18 women and 1 man) with active lSSc and 11 women with isolated RP. The diagnoses of active lSSc and early SSc were based on clinical, laboratory, capillaroscopic and autoantibody findings. Internal organ involvement, namely lung, kidney, heart, gastroitestinal tract or muscle, was also documented by routine investigation in all patients. Antibodies were marked with the indirect immunofluorescence (IIF) method on Hep-2 cells and double immunodiffusion. SSc pattern on NCM was defined as definitely enlarged capillaries and/or capillary loss of grades and/or capillary telangiectases (12).

Active lSSc was diagnosed based on the American College of Rheumatology (13) criteria and activity index in SSc according to European Scleroderma Study Group (14). Skin changes in these patients corresponded with limited SSc – appeared on the skin of the face, upper limbs up to 1/3 of the forearm. Patients with isolated RP had any of the SSc-specific autoantibodies (anti-Scl 70, anti- centromere, anti-RNA polymerase III) and/or an SSc pattern on NCM. RP was defined as a history of at least 2 of 3 phases of color change (white, blue, red), usually induced by cold exposure, and involving at least 1 finger of each hand (14). These patients did not display clinical manifestations of SSc or another CTD, such as sclerodactyly, digital ulcers, or pitting scars, loss of distal finger pad, clinically visible capillary telangiectases, or calcinosis (15).

Presents a clinical characteristic of patients with isolated RP and active lSSc (tab. 1). Patients qualified for the research were not treated earlier with immunosuppressive agents and (or) steroids. Control samples were obtained from 8 healthy women aged 47.6 ± 8.3 years. The Medical University of Silesia Local Research Ethics Committee approved the study and all subjects provided informed consent to participate.

Table 1. Clinical characteristics of patients with isolated RP and patients with lSSc.

	Isolated RP n = 11	**lSSc n = 19**
Age (years)	44.6 ± 14.1*	49.5 ± 10.8*
Duration of RP	7.1 ± 5.9*	10.7 ± 5.9*
Duration of sclerodactyly	–	3.2 ± 1.9*
SSc pattern on NCM	7	19
Immunologic markers		
Anti-Scl 70	3	12
Anti- RNA polymerase III	–	1
Anti-centromere	4	2
Antibody with homogenous pattern of immunofluorescence	–	3
Visceral involvement		
Oesophagus	–	15
Lungs	–	8
Heart	–	4
Kidney	–	–
Muscle	–	–

*Average ± standard deviation

MATERIAL AND METHODS

Extraction of total RNA. Total RNA was extracted from the 500 μl whole blood samples with the use of acid guanidinium-thiocyanate phenol-chloroform method /20/. Extracts of total RNA were purified with the use of RNeasy Mini Kit (Qiagen Gmbh, Germany), according to the manufacturer's instructions. The quality of RNA extracts was estimated electrophoretically using 1% agarose gel stained with ethidium bromide. The results were analyzed and recorded with the gel documentation system 1D Bas-Sys (Biotech-Fisher, Perth, Australia). The total RNA concentration was determined by spectrophotometric measurement at 260 nm using a Gene Quant II RNA/DNA Calculator (Pharmacia LKB Biochrom Ltd., Cambridge, UK).

mRNA quantification by Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction. The quantitative analysis was carried out with the use of Sequence Detector ABI PRISM™ 7000 (Applied Biosystems, California, USA). The quantity of PCR products was determined after each round of amplification, using fluorescent dye SYBR Green I (Qiagen Gmbh, Germany) that binds double-stranded DNA. The standard curve was appointed for standards of β-actin cDNA (TaqMan® DNA Template Reagents Kit, Applied Biosystems, California, USA). For this assay positive (β-actin mRNA) and negative (no template) controls were carried out. The nucleotide sequences of the PCR primers used to assay TNF, TNFR1, TNFR2 and β-actin (endogenous control) gene expression, chemical and thermal conditions of amplification were as previously (16, 17).

Sequence specificity of amplimers. Sequence specificity of amplimers was proved by analysis with ABI PRISM™ 377 DNA Sequencer (Applied Biosystems, California, USA). Melting temperatures of amplimers were assessed by SYBR Green I Dissociation assay (Dissociation Curve Software – Applied Biosystems, California, USA). The PCR products and molecular weight marker pBR 322/Hae III (Fermentas International Inc., Ontario, Canada) were separated on 8% polyacrylamide gel and visualized using silver staining. The length of amplified fragments was assessed by analysis with GelScan v.1.45 software (Kucharczyk TE, Poland).

Statistical analysis. All calculations were performed with Statistica Version 8.0 software (StatSoft, Tulsa, Oklahoma, USA). The values were expressed as median and range. Quantitative data were compared by a nonparametric Mann-Whitney U test. Correlations were evaluated using the Spearman rank correlation coefficient test. $P < 0.05$ was considered significant. The expression of the TNFα, TNFαRI, TNFαRII, and β-actin genes was expressed as a ratio of the mRNA copy number to the 1 μg of total RNA in samples studied.

RESULTS

β-actin mRNA. In all analyzed samples mRNA of β-actin gene was demonstrated, thus indicating the integrity of the RNA extracts.

TNFα mRNA. The highest number of TNFα mRNA copies/1 μg total RNA was found in the control group (Me = 6143.8). Lower number of TNFα mRNA copies/1 μg total RNA was found in isolated RP patients (Me = 3177.0) and the lowest in active lSSc patients (Me = 2611.0) (fig. 1a). Transcriptional activity of TNFα gene in the group of patients with active lSSc was significantly lower than in the control group ($p = 0.0295$). No statistically significant difference in transcriptional activity of TNFα gene between active lSSc and isolated RP patients as well as isolated RP and the control group was found.

TNFαRI mRNA. Like in case of TNFα no statistically significant difference in transcriptional activity of TNFαRI gene between active lSSc (Me = 11159.9) and isolated RP patients (Me = 4801.9) was found. Comparing to controls (Me = 18283.1) the expression of TNFαRI was significantly lower both in active lSSc patients and in isolated RP patients ($p=0.0294$; $p = 0.0064$, respectively) (fig. 1b).

TNFαRII mRNA. Transcriptional activity of TNFαRII gene both in active lSSc (Me = 16.7) and early SSc patients (Me = 10.8) was significantly lower opposed to the control group (Me=117.3) ($p = 0.0006$ and $p = 0.0105$, respectively) (fig. 1c). Like in case of TNFα and TNFαRI no statistically significant difference in transcriptional activity of TNFαRII gene between active lSSc and isolated RP patients was found.

The TNFα and TNFα receptors ratios. The TNFα/ /TNFαRI ratio did not differ significantly between analyzed groups. The TNFα/TNFαRII ratio both in active lSSc (Me = 151.6) and isolated RP patients (Me = 300.9) was significantly higher opposed to the control group (Me = 26.1) ($p = 0.0257$ and $p = 0.0258$, respectively) (fig. 2a). No statistically significant difference in TNFα/TNFαRII ratio between active lSSc and isolated RP was found. The TNFαRI/TNFαRII mRNA ratio was significantly higher in patients with active lSSc (Me = 449.5) than in the control group (Me = 105.1) ($p = 0.0337$) (fig. 2b). In the group of isolated RP patients the TNFαRI/TNFαRII mRNA ratio (Me = 250.1) did not differ significantly both from active lSSc patients and controls.

Correlations. In the group of patients with active lSSc only a slight trend toward correlation between TNFαRI and TNFαRII mRNA was found ($p = 0.0765$, $R = -0.4159$). This trend was negative, what means that the number of TNFαRI mRNA copies was increasing while the number of TNFαRII mRNA copies was decreasing. No correlation in transcriptional activity between analyzed genes both in isolated RP patients and in the control group was found.

DISCUSSION

TNFα – a protein of molecular weight of 17-kDa is a multi-functional cytokine, involved in pathogenesis of multiply inflammatory and autoimmunological diseases. Monocytes, activated T cells and NK cells may comprise a source of TNFα in blood (18). This factor may

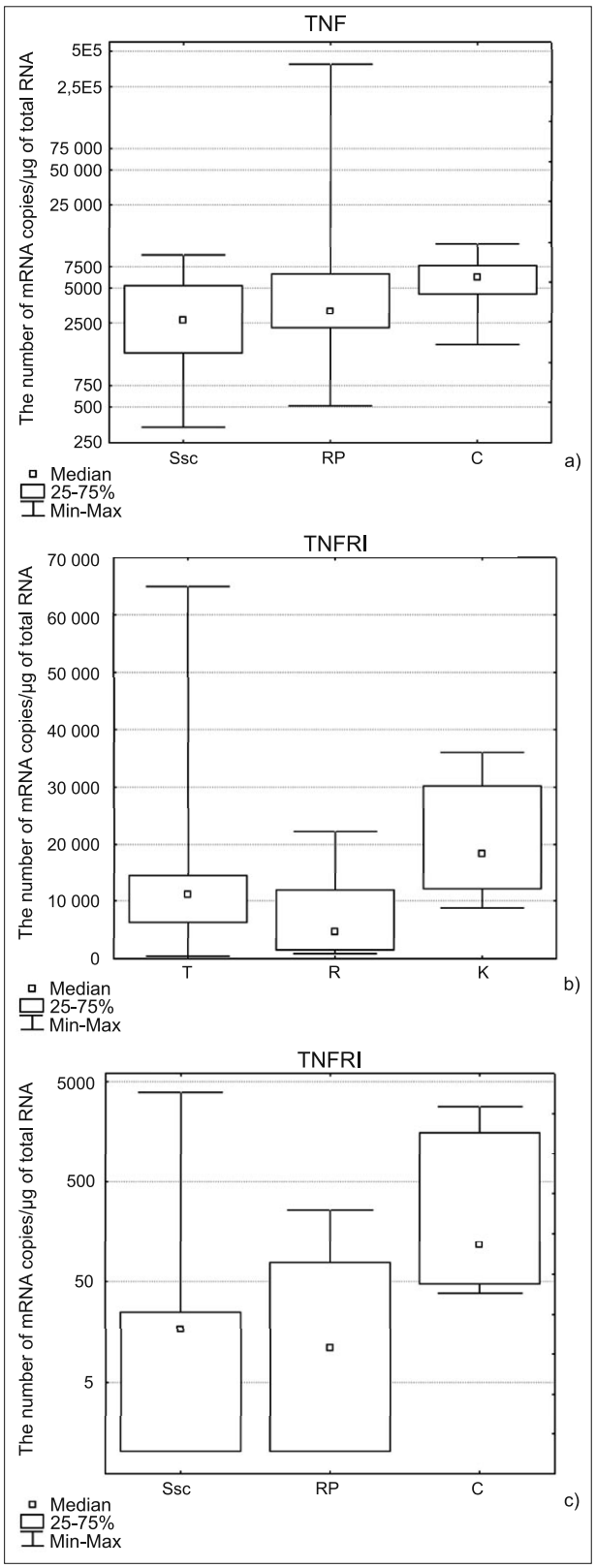

Fig. 1. Comparison of mRNA copy number of TNF (a), TNFRI (b) and TNFRII (c) genes in studied groups. SSc – systemic sclerosis; RP – isolated Raynaud phenomenon; C – control group.

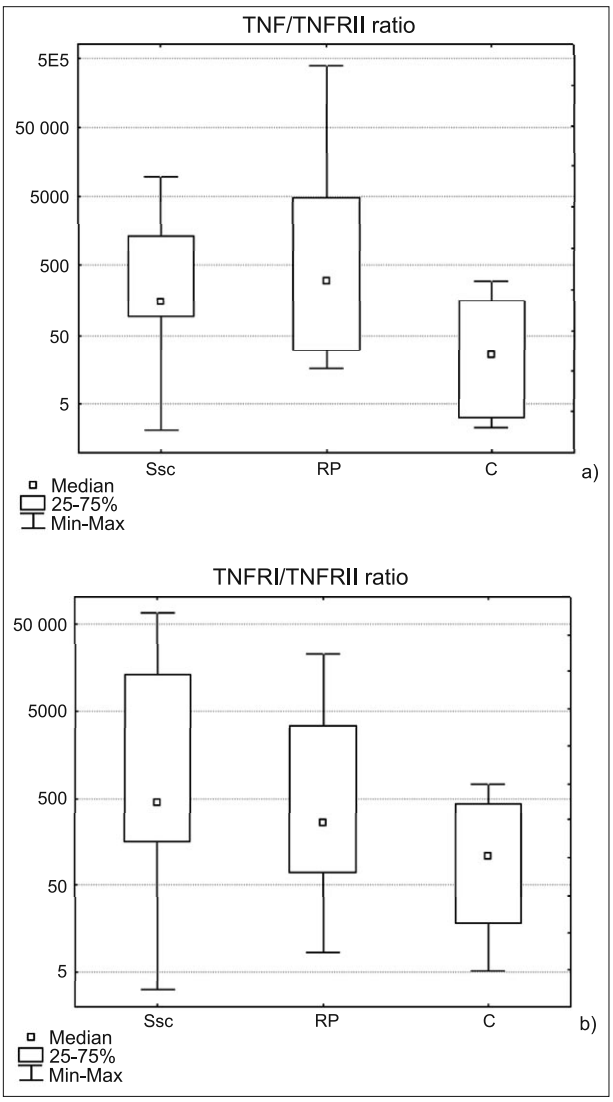

Fig. 2. Comparison of TNF/TNFRII ratio (a) and TNFRI/TNFRII ratio (b) in studied groups. SSc – systemic sclerosis; RP – isolated Raynaud phenomenon; C – control group.

play a significant role at the early stage of SSc development, i.e. in vascular changes. The increased TNFα expression was found in the tissues of the patients with Raynaud's phenomenon and incorrect capillaroscopic image, subsequently followed by SSc development (19). Therefore, this study included also patients with isolated Raynaud's phenomenon, in which capillaroscopy or the presence of immunological markers indicated a risk of SSc development. Due to the influence of circulating inhibitors, mainly sTNFαRI and sTNFαRII, upon the concentration of TNFα in serum, the study of gene transcription (the earlier stage of gene expression) was undertaken. Moreover, the results obtained at transcriptional level may differ from those obtained at protein level. The TNFα protein may be membrane-associated, thus protein detected in serum is only a part of TNFα pool in blood. Other molecular mechanisms such as storage of unprocessed of TNFα pre-mRNAs in immune cells can not be ruled out (20). The number of mRNA copies of the genes encoding TNFα and its receptors was measured using Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (real-time QRT-PCR) (21) the method, which has been used for several years in many medicinal fields. In comparison to the healthy persons, the

patients with SSc showed significantly lower number of TNFα mRNA copies. Our results are in contrast with previous reports. Young et al. (22) observed elevated transcriptional activity of the gene encoding TNFα in leucocytes from patients with SSc. The authors used the same method of mRNA detection as we did. However they did not provide useful data to compare results with phenotypic data (limited or diffuse SSc, early or late disease, internal organs affected, etc.), thus we can not explain difference in our results. Scala et al. (23) found elevated serum level of TNF protein in SSc patients comparing to controls. But, in our and other studies the concentration of TNFα in SSc sera was low or under limit of detection (8-10). As the research of Askew et al (24) performed in mice with systemic sclerosis-like changes in graft versus host disease GVHD – an experimental model of SSc – showed a lack of TNFα production in the early phase of the disease, our study seems to confirm that similar disturbances may take place in the blood of both active lSSc patients and patients with isolated RP evolving to SSc. The decrease in TNFα concentration in circulation and in tissues may play a crucial role in fibrosis process since this is a main cytokine with an anti-fibrotic activity. The SSc patients treated with pamidronate (aminobisphosphonate) showed an increase in TNFα production by PBMCs, which can explain the advantageous therapeutic effect (25). Mice that overexpressed TNFα were protected against both bleomycin and TGF-β-induced pulmonary fibrosis (26). Authors hypothesized that chronic overexpression of TNFα by itself did not produce pulmonary fibrosis but might make lungs more susceptible to fibrotic agents. This may be caused by prolonged immunological reaction observed in chronic inflammation. Pantelidis et al. (27) showed that TNFα is produced at sites of disease in the lung by specific subsets of mononuclear phagocytes. TNFα inhibits TGF-β1-induced collagen synthesis in fibroblasts by inhibiting COLIA2 gene expression (7). Chizzolini at al (18) showed that inhibition of collagen production by dermal fibroblasts is contact-dependent. Th2 cells infiltrating skin lesions in early SSc have capacity to affect both type I collagen and matrix metalloproteinase 1 production by dermal fibroblasts via membrane-bound TNFα. The authors also showed inhibitory effect of soluble TNFαRI on membrane-bound TNFα anti-fibrotic activity. This results provide a new insight into the role of soluble TNFα receptors in the regulation of TNFα anti-fibrotic activity, particularly it's membrane-bound form. It has been showed that sTNFαRs can stabilize a trimeric structure of TNFα, therefore prolonging its activity by a slow release of physiological concentrations (28). sTNFαRs may play a dual role – they protect from negative effects of the increased TNFα concentrations, but they also play a role as a reservoir for a biologically active factor in the case of its down-regulation. Therefore the function of sTNFαRI in the regulation of membrane-anchored TNFα activity seems to be a key in the regulation of fibrotic process in early SSc. In the study of Gruschwitz et al (29) expression of TNFαRI and TNFαRII on PBMC from SSc patients did not differ from controls. The sTNFαRI and sTNFαRII concentrations in sera of SSc patients correlated with their in situ expression in tissues. But, in our previous studies patients with SSc and some of the patients with the isolated Raynaud's phenomenon evolving to SSc. showed an increased sTNFαRI levels in sera (11). In presented study significantly lower number of mRNAs for TNFαRI and TNFαRII receptors was found both in SSc and early SSc patients when comparing to the control group. Like in case of TNFα no significant difference in transcriptional activity of TNFαRI and TNFαRII genes between active lSSc and early SSc patients was found. The TNFαRI/ /TNFαRII mRNA ratio was significantly higher in patients with active lSSc than in the control group. The TNFα/ /TNFαRII ratio both in active lSSc and early SSc patients was significantly higher opposed to the control group.

Like in case of TNFα, TNFαRI and TNFαRII genes expression regulation is complicated and includes regulation both at protein and transcription level. TNFα by itself plays crucial role in the regulation of the number of TNFα receptors molecules on cell surface. Maybe increase of sTNFαRI release to circulation is caused by membrane-anchored TNFα since this form induce proteolytic cleavage of TNFα receptors preventing cells from TNFα activity (30). The increased concentration of sTNFαRI observed in autoimmunological diseases probably reflects an attempt of the host organism to limit the excessive pro-inflammatory and anti-fibrotic TNFα activity (26). Therefore, increase of TNF RI/TNFαRII ratio in SSc patients seems to indicate that TNFαRI may be responsible for fibrotic changes, while TNFαRII may be involved in signalization leading to adverse result.

In conclusion, our studies showed that dysregulation of the genes encoding TNFα and its receptors takes a place in active lSSc patients as well as in patients with isolated RP, preceding skin and organ manifestation. The results suggest that proportion of TNFα, TNFαRI and TNFαRII molecules might be very important in inducing and/or prolonging molecular mechanism leading to disturbances observed in SSc. However, the molecular background of the observed changes in genes' expression requires further studies.

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