Possible implications of TGF-alpha in oesophageal dysmotility in systemic sclerosis

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ABSTRACT

Objective. *Hypoxia is a characteristic feature of systemic sclerosis (SSc). Transforming growth factor alpha (TGF-α) has an important role in excessive inflammation under hypoxic conditions. Since oesophageal dysmotility is one of the most common signs of SSc, the aim of this study was to explore the relation between TGF-α and oesophageal dysmotility in SSc.*

Methods. *The study included 35 patients with SSc and 32 healthy controls matched for sex and age. Serum concentrations of TGF-α were measured using ELISA. Oesophageal motility was assessed by oesophageal scintigraphy. A multiple-swallow test was performed in the study population with ^{99m}Tc-DTPA. A region of interest over the entire oesophagus was defined and the retention index (RI) was calculated.*

Results. *Statistically significant differences in serum concentration of TGF-α as well as of RI of ^{99m}Tc-DTPA were found between patients with SSc and healthy controls. A statistically significant correlation was found between serum concentrations of TGF-α and RIs of ^{99m}Tc-DTPA. This correlation was inverse, i.e. when serum concentrations of TGF-α increased, the RI of ^{99m}Tc-DTPA decreased (Spearman rho =-0361, p=0.033).*

Conclusion. *These results point to a possible relation between TGF-α and oesophageal dysmotility in SSc. Although the results do not explain the exact role of this cytokine in the pathogenesis of esophageal changes, the finding of inverse correlation between TGF-α and oesophageal dysmotility is intriguing and requires further investigation.*

Introduction

When discussing the pathogenesis of SSc, communication between endothelial cells, epithelial cells and fibroblasts, lymphocyte activation, autoantibody production, inflammation, and tissue fibrosis must be considered (1). Fibroblast activation leads to fibrogenesis and accumulation of extracellular matrix (ECM) which modulates the activity of mediators such as TGFß and TGF-α (2). TGF-α is a member of epidermal growth factor (EGF) family of cytokines. It is a small mitogenic protein that consists of 50 aminoacids linked with 3 disulfide bonds (3). It is excreted by macrophages, brain cells, keratinocytes and fibroblasts (4). In rat cell cultures, TGF-α stimulates the growth of normal fibroblasts (5). For this effect, TGFα requires TGFβ, which augments activation of TGF-α through a separate receptor system (6). Bound to epidermal growth factor receptor (EGFR) TGF-α causes receptor dimerisation, activation of tyrosine kinase, and activation of a signaling cascade (7). EGFR are expressed by many types of cells including skin keratinocytes, fibroblasts, vascular endothelial cells, and epithelial cells of the gastrointestinal tract (GIT) (8). At the cellular level, EGFR stimulates cell proliferation and also can stimulate angiogenesis (9-10). TGF-α has an important role in the stimulation of excessive inflammation in a hypoxic environment (11). Hypoxia is an initial event of ischaemic and lypopolisaccharide stimulated inflammation (12). It is also a strong inductor of TGF-α transcription and translation as well as a crucial mediator of the generalised systemic inflammatory response (13-14). TGF-α has been extensively studied on acute and chronic injury models of the upper gastrointestinal mucosa. During regeneration of upper gastrointestinal mucosal injury TGF-α and EGF serve predominantly to restore the epithelial component (15). TGF-α has been studied also on an interstitial lung disease

model with findings that lung epithelial-specific overexpression of TGF-α leads to progressive and pronounced pulmonary fibrotic lesions in transgenic mice (16-18). On a kidney fibrosis model, interaction of EGF and TGFβ has been documented (19-20). The role of TGFβ in SSc has been studied in detail, but the effects of members of the epidermal growth factor family, such as TGF-α, have not been studied yet (21-22). Clinically, aside from the skin, in SSc patients, the digestive tract is also frequently affected – with the oesophagus being involved in 75–90% of patients with SSc (23). Excessive fibrosis, inflammation, and vascular damage are basic processes of GIT damage with a clinical presentation of dysfunction pertaining to motility, digestion, absorption, or excretion (24). Typical SSc changes include atrophy of smooth oesophageal muscle in the distal 2/3 of oesophagus with consequent reflux oesophagitis and stenosis, strictures (17-29%), and Barrett's oesophagus (0-37%) (25-26). Oesophageal involvement is not necessarily symptomatic, therefore it may be diagnosed late in the course of the disease, when complications have already set in (27). Therefore, we decided to investigate the relation between TGF-α and oesophageal changes. Oesophageal motility was assessed by oesophageal scintigraphy because it is less invasive and is easily performed for patients than manometry or oesophagoscopy (28).

Materials and methods

Study population

The study included 35 consecutive patients with SSc classified according to EUSTAR (European Scleroderma Trial and Research) criteria and 32 control subjects matched for age (± 3 years) and sex. Patient recruitment was performed by three licensed rheumatologists through the Department of Rheumatology, University Hospital Split, Split Croatia in the time period from January 2011 to January 2012. Of the 65 examined SSc patients, 35 patients with diffuse cutaneous SSc (dcSSc) were enrolled in our study. Thirteen of these patients had a limited cutaneous form of disease, 6 patients had active malignant disease, 7 patients had problems with upper gastrointestinal tract other than related to SSc. Four patients did not wish to participate in the study. Due to a low incidence of disease and lack of scientific work on the role of TGF-α in SSc, we decided to enroll only patients with dcSSc (largest homogenous group of patients) and to perform the study on such a homogenous sample. The control group consisted of healthy volunteers and outpatients suffering from cervico-brachial and lumbosacral syndrome having visited our immunology outpatient clinic during the period of recruitment. Controls were free of any problems with upper GIT, having normal endoscopic findings of the distal part of oesophagus within 6 months of the time of recruitment. Disease duration ranged from 18 to 60 months, with a mean of 46 months. SSc was diagnosed according to the revised criteria of the American Rheumatology Association (29). All of 35 patients had diffuse cutaneous SSc, underwent a complete physical examination, and were questioned about possible malignant diseases in their past and any family history of malignant diseases in first-degree relatives before the age of 65. Age, sex and current treatment were recorded. Routine haematological and biochemical measurements were performed. All subjects were tested for ACA-Cenp B, anti-Scl-70 and anti-RNP antibodies from analysis of multiple measurements of autoantibodies (AtheNA Multi – Lyte ANA test system Zeus Scientific, Inc.Raritan, NY, USA) using an adjusted flow cytometer Luminex 100 (Austin, USA). Results were expressed in U/ml. Negative (normal) values ranged up to 40 U/ml, whereas positive (high) values were greater than 40 U/ml. Exclusion criteria were as follows: previous or current malignant disease. All patients (with SSc or controls) with hiatus hernia, oesophageal erosions, ulcers or gastroesophageal reflux disease, *H. pylori* infection were excluded. Written informed consent (according to the Helsinki Declaration) was obtained from all study subjects prior to study participation. The Ethics Committee of the Split University Hospital Center, Split, Croatia approved the study protocol.

Measurement of TGF-α

All subjects were tested for TGF-α. Serum samples were taken via vein puncture in the morning, after an overnight fast, and collected in 2 blood tubes. Serum was extracted and stored in a freezer (temperature: -70°C). TGF-α concentrations were measured in duplicate, using ELISA method on an automatic analyser miniBOS, manufactured by Biomedica (Automatic microplate analyser, Biomedica Gruppe, Wien, Austria). Polyclonal antibody specific for TGFα were pre-coated onto microplates. Standards and samples were pipetted into the wells and any TGFα present was bound by the immobilised antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for TGF-α was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and colour developed in proportion to the amount of TGF-α bound in the initial step. The colour development was stopped and the intensity of the colour was measured. A standard curve was included on each plate using doubling dilutions of pure recombinant TGF-α (R and D systems). Data was processed using GraFit software (Microsoft). Results were expressed in pg/ml (Quantikine, Human TGF-α immunoassay, DTGA00, R&D Systems Inc., Minneapolis, USA) (30).

Measurement of RI of ^{99m}Tc-DTPA

Oesophageal motility was determined by oesophageal scintigraphy with technetium 99 m dietilentriaminopentacetata (Tc-99m-DTPA). The percentage of Tc-99m-DTPA was used as the measure of (RI). The level of 100% was considered as highest oesophageal dysfunction, and levels below 40% were considered normal. The test was performed in the afternoon hours, after subjects fasted for 5 hours. In a supine position, through a 20 ml syringe, a subject was given 300 μCi Tc-99m-DTPA in 3 ml of water per orally (*i.e.* to swallow). Every 15 seconds subject was asked to swallow again, and for 2 minutes dynamics of passage of radioactive bolus through the oesophagus, and oesophageal emptying activities were recorded. RI was

measured at 15, 30, 60 and 90 seconds. RI was shown as a percentage and the most significant results were measured at 60 seconds. These values were used for analysis purposes. Measurements were done using a Siemens Orbiter Gamma Camera and a PDP 11/34 computer, 11 Gamma software package.

Statistical analysis

The level of significance was set at 5% ($p<0.05$), and all confidence intervals were given on the 95% level. Normality of distribution of continuous variables like TGFα was tested by Kolmogorov-Smirnov test for samples greater than 30, or by Shapiro-Wilk test for samples smaller than 30. Median and interquartile ranges were used as measures of central tendency and variability when the distribution statistically significantly deviated from the normal one. Differences in mean values for the continuous, numeric variables between two categories of a nominal variable were analysed by Mann-Whitney U-test. AUC was given as a standardised measure of the effect size for statistically significant results, and is calculated according to the formula: U / (m * n), where U is the result of the Mann-Whitney test (rank sum), m and n are sizes of two samples. Possible impact of covariates on relationship between TGF-α level and systemic sclerosis was controlled by ANCOVA. Differences between two nominal variables were analysed by chi-square test with, and Cramer's V was given as a standardised measure of the effect size for statistically significant results. Relationship between two continuous, numeric variables was analysed by Spearman's rho coefficient. All analyses were carried out using SPSS 17.0 (SPSS Inc., Chicago, IL, USA) statistical software package.

Results

The study population consisted of 35 (52.2%) SSc patients and 32 (47.8%) control subjects. As the distribution of age significantly statistically deviated from normal in the group of SSc patients (Kolmogorov-Smirnov z=0.16; $p=0.040$), the median and interquartile ranges were used as measures of central tendency and variability for both groups. No statistically significant differences regarding gender ($\chi^2=0.013$; $p>0.999$), nor age (Mann-Whitney U=179.5; Z=-0.176; $p=0.871$) were found between the control subjects and the SSc patients. We also measured serum concentration of autoantibodies (anti-Scl-70, ACA Cenp B and anti-RNP). In SSc patients group 12 patients had anti-Scl-70 antibodies (34%), 7 patients had ACA Cenp B antibodies (20%) and 6 patients had anti-RNP antibodies (17%) in serum concentrations of more than 40 U/ml. Sera of 10 SSc patients were negative for these three antibodies (29%). Also, the sera of the control subjects were negative for these three antibodies (100%).

SSc patients had statistically significantly higher average values of TGFα than subjects in the control group (Mann - Whitney U=400.5; Z=-2.003; $p=0.044$; AUC=0.36) (Table I). SSc patients had statistically significantly higher average values of RI of ^{99m}Tc-DTPA than control group (Mann-Whitney U=156; Z=-5.101; $p<0.0001$; AUC=0.14) (Table II). Statistically significant correlation was found between level of TGF-α and value of RI of ^{99m}Tc-DTPA in SSc patients (Spearman rho=-0.361; $p=0.033$). This correlation was inverse, meaning that with an increase in TGF-α, RI of ^{99m}Tc-DTPA decreased (Table III).

There was no statistically significant relation between serum concentration of TGF-α and any of specific antibodies (anti-Scl-70 - Spearman's rho -0.301; $p=0.079$; ACA Cent B - Spearman rho -0.218; $p=0.207$; anti-RNP - Spearman's rho 0.254; $p=0.141$).

There was no statistically significant correlation between serum concentration of TGF-α and Rodnan skin score in SSc patients group (Spearman rho = 0.033; $p=0.851$).

Also, no statistically significant difference was found regarding serum concentration of TGF-α in SSc patients with pulmonary involvement and SSc patients without signs of pulmonary involvement (Z=-0.454, $p=0.650$).

Table I. Difference in TGF-α level between SSc patients and control group.

	SSc group		Control group		
	median	(IQR)	median	(IQR)	p ; effect
TGF-α	16	(8–33.6)	10.3	(7.1–13.6)	0.044; 0.36

IQR: interquartile range; *$p<0.05$

Table II. Difference in RI of ^{99m}Tc-DTPA between SSc patients and control group.

	SSc group		Control group		
	median	(IQR)	median	(IQR)	p ; effect
RI of ^{99m}Tc-DTPA	94	(52–100)	25	(17–37.3)	0.001*; 0.14

IQR: interquartile range; *p<0.05

Table III. Relation of TGF-α and oesophageal dysmotility (RI of ^{99m}Tc-DTPA).

	n	Spearman rho	p-value
Study population	67	0.037	0.740
SSc group	35	-0.361	0.033*
Control group	32	0.344	0.054

*$p<0.05$

Discussion

In our study, we demonstrated that serum concentrations of TGF-α in patients with SSc were statistically significantly higher in comparison to control group. This finding could implicate possible relation between TGF-α and inflammatory processes in SSc, that have an impact on fibroblast activation and fibrogenesis in SSc.

Why did we decide to investigate TGF-α concentrations in the sera of SSc patients? To date, there are neither sensitive nor specific laboratory tests for the diagnosing of patients with SSc. The main diagnostic criteria for SSc are performed almost exclusively by clinical assessment of typical localised skin lesions, but also of fibrotic changes to internal organs (31). Antinuclear antibodies are found in more than 90% of SSc patients, while the more specific anti-Scl-70 antibody (that could serve as serological marker of SSc) is found in only 30–40% of these patients (32). A major pathogenic hallmark of disease is the fibroproliferative process, not only restricted to the skin, but also very prominent in the lungs, heart and gastrointestinal tract (33). Expression of TGFβ receptors is increased in SSc fibroblasts, and increased concentrations of TGFβ is found in the lungs of patients with idiopathic pulmonary fibrosis (34). There is strong evidence that EGF ligands and receptors are critical mediators in the fibrogenic responses to TGFβ. EGFR and ligands are found to be elevated in various interstitial lung diseases (35). Also, inhibition of TGFβ on the animal pulmonary fibrosis model mitigates fibroproliferative changes in the lungs. In addition to the lung, models of renal fibrosis also demonstrate fibrosis progression despite inhibition of the TGFβ pathway (36, 19). These findings point out that both EGFR and TGFβ pathways have a central role in the pathogenesis of fibroproliferative processes (37). To date, TGF-α, the main EGFR ligand, was not studied in SSc patients.

During the recruitment period, 35 dcSSc patients were enrolled in the study group, therefore presenting the largest homogenous group of patients. According to pulmonary involvement, a subgroup of 7 patients with signs of pulmonary involvement was formed. All 7 patients had signs of lung fibrosis on HRCT, five patients had significantly reduced DLCO, and two patients had both lung fibrosis and pulmonary arterial hypertension. Since majority of previous works regarding TGF-α in fibroproliferative processes concentrated on the role of TGF-α on pulmonary fibrotic processes, in our study, serum TGF-α concetrations were analysed in subgroup of SSc patients, comparing those with pulmonary involvement and those without pulmonary involvement. No statistical significant difference was found.

Furthermore, TGF-α and EGF have already been identified in GIT of humans and of rats where they act as potent mitogens for certain types of cells. Besides this mitogenic effect, members of the EGF cytokine family can modulate cell migration, mucus production and secretion, gastric acid secretion and gastrointestinal motility (38). Scheiman *et al*. documented the relation between local TGF-α concentrations in gastric body, antrum and duodenum and medicamentous therapy, namely with PPI and acetylsalicylic acid (39). This study was performed on biopsy specimens measuring local gastric and duodenal levels of TGF-α, while our study measured serum concentrations of TGF-α. Out of 65 patients treated in our facility during the period of recruitment, 7 patients were taking high dosages of PPI due to different pathological conditions in the upper gastrointestinal tract, but due to these pathological endoscopic findings they were excluded from the study during the recruitment process. One enrolled patient was taking acetylsalicylic acid due to comorbid conditions (coronary artery disease). Endoscopic finding of this subject's upper gastrointestinal tract showed no GERD, gastric erosions nor ulcers, and he was therefore enrolled in our study.

In our study, oesophageal scintigraphy was performed and RI of ^{99m}Tc-DTPA was used as a measure of oesophageal dysfunction. Oesophageal scintigraphy was chosen because it is a safe method that causes little discomfort to patients and is better tolerated than manometry. At the same time, it shows great accuracy in diagnosing non-obstructive oesophageal dysphagia (40). It was suprising to find an inverse correlation between serum concentrations of TGF-α and value of RI of ^{99m}Tc-DTPA in SSc patients. Perhaps, this could be due to loss of cellular TGF-α secreting elements during pathologic collagen deposition and fibrous overgrowth in oesophagus in the natural course of SSc. Normally, not only macrophages and keratinocytes, but also epithelial cells of GIT synthesise and secrete TGF-α. Presumably, this secretion would be higher in an inflammatory environment of esophagus at the beginning of oesophageal involvement.

A potential limitation of our study was that the sample size was not large enough due to the low incidence of disease. This small sample was further subdivided into subgroups which related to organ involvement, namely pulmonary involvement. Analysis of such a small subgroup of patients is not statistically meaningful. Also, this study was cross-sectional but, considering the chronic nature of this disease and various rate of progression, it would be interesting to perform a large prospective study of serum TGF-α concentrations at regular time intervals but also related to changes in oesophageal function through the course of the disease in the same patient. It is a promising concept, but further investigation is required.

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