Diminished production of TWEAK by the peripheral blood mononuclear cells is associated with vascular involvement in patients with systemic sclerosis

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Abstract: Widespread vasculopathy and profound fibrosis are key features of the pathogenesis of systemic sclerosis (SSc). We hypothesized that the TNF-like weak inducer of apoptosis (TWEAK), a recently recognized multifunctional cytokine which regulates angiogenesis and tissue remodeling, may play a role in the development of SSc. The production of TWEAK by the peripheral blood mononuclear cells (PBMC) was investigated, by means of ELISA, in 24 SSc patients and 14 healthy subjects. Moreover, production of TWEAK was correlated with clinical features of SSc. PBMC were isolated using density gradient centrifugation on Histopaque and were cultured in FCS supplemented RPMI medium at 37°C under 5% CO₂. Production of TWEAK by PBMC was significantly diminished in patients with more severe microvascular damage, as indicated by the presence of "active" capillaroscopic pattern, compared with SSc patients with less pronounced microangiopathy ("slow" pattern), and healthy subjects. Moreover production of TWEAK correlated inversely with duration of Raynaud's phenomenon. PBMC from patients with scleroderma-related interstitial lung disease tended to produce lower amounts of TWEAK compared with SSc patients without lung involvement but the difference was not significant. The results of our study suggest that diminished production of TWEAK might play a role in the pathogenesis of vascular injury in SSc patients. Whether TWEAK may represent a new therapeutic target in SSc requires further studies.

Key words: systemic sclerosis, scleroderma, TWEAK, pathogenesis, vasculopathy

Introduction

Systemic sclerosis (SSc) is a rare, chronic, autoimmune disease which is characterized by a widespread vasculopathy and a profound fibrosis of the skin and internal organs. The vascular involvement manifests itself in decreased number of capillary vessels, enlargement of the remaining capillaries and/or proliferative angiopathy of small arteries [1]. The excessive fibrosis which is considered a hallmark of SSc results from overproduction of extracellular matrix components by activated fibroblasts [2].

Despite the progress in our understanding of the pathogenesis of SSc, the factors responsible for the development of the disease are still unclear [2]. Moreover, there is still lack of effective treatment strategies for SSc. [3]. The TNF-like weak inducer of apoptosis (TWEAK) is a recently recognized multifunctional cytokine which regulates wound healing and tissue remodeling [4].

TWAER, acting through the polypeptide growth-factor-inducible Fn14 receptor activates several intracellular signaling pathways, including NFKappaB, leading to a wide range of cellular responses crucial to tissue remodeling, including angiogenic, proliferative, and inflammatory responses (reviewed in details in references 4 and 5).

TWEAK contributes to angiogenesis through stimulation of migration, proliferation and survival of endothelial cells [4,5]. Moreover, TWEAK stimulates
smooth muscles cell proliferation [4,5] which might be of importance for new vessel formation, but also may contribute to pathogenesis of proliferative angiopathy seen in SSc-related pulmonary arterial hypertension or scleroderma renal crisis.

TWEAK can also activate human fibroblasts. In human dermal fibroblasts and corneal myofibroblasts TWEAK induced production of proinflammatory cytokines and chemokines: interleukin 6 (IL-6), interleukin 8 (IL-8), RANTES (regulated on activation, normal T expressed and secreted), interferon-γ-inducible protein-10 (IP-10) and/or MCP-1 [6,7]. Human gingival fibroblasts responded to TWEAK stimulation with increased expression of adhesion molecules (VCAM-1 and ICAM-1), IL 8 and vascular endothelial growth factor (VEGF) [8]. Interestingly, TWEAK-mediated induction of ICAM-1, IL-8, MCP-1, RANTES and VEGF were augmented synergistically by transforming growth factor-β (TGF-β) [7,8], a cytokine which is considered of key importance for the pathogenesis of SSc-related fibrosis [9].

Angiogenesis and activation of fibroblasts are key events in both wound healing and pathogenesis of SSc. Deregulated production of TWEAK might therefore contribute to development of SSc. Indeed, Yanaba et al. have recently reported increased TEAWK levels in the sera from SSc patients [10]. The cellular source of these increased TWEAK levels in SSc have not been indicated so far.

Since monocytes/macrophages have been shown to express TWEAK [11] we hypothesized that increased production of TWEAK by the peripheral blood mononuclear cells (PBMC) might be a source of increased levels of TWEAK in the blood of SSc patients. To address this hypothesis, the production of TWEAK was investigated in the PBMC from patients with SSc and healthy controls. Moreover, TWEAK production was correlated with clinical features of SSc.

Materials and methods

Patients. 24 patients with SSc (20 female and 4 men) were investigated. Patients selected for the study fulfilled the ACR classification criteria for SSc. [12] or they had Raynaud's phenomenon and at least one of the following: microangiopathy typical for SSc in capillaroscopy, and/or autoantibodies typical for SSc, and/or the definition of early SSc as proposed by LeRoy et al. [13].

To avoid modification of the activity of leucocytes by immunosuppressive drugs only patients who had not taken any immunosuppressive therapies or in whom immunosuppressive therapies had been stopped at least 6 months before blood collection were considered eligible. SSc patients were classified as having diffuse cutaneous SSc (dSSc) or limited cutaneous SSc (lSSc) based on criteria by LeRoy et al. [14].

Duration of Raynaud’s phenomenon as well as duration of the disease, calculated from the time of the first non-Raynaud’s symptom attributable to SSc, were recorded for every patient. In agreement with generally accepted criteria, early disease was defined as shorter than 3 years in diffuse SSc patients or shorter than 5 years duration in patients with limited SSc measured from first non-Raynaud-symptom.

The presence of scleroderma interstitial lung disease was defined based on the presence of features of interstitial fibrosis and/or "ground glass" opacifications in high resolution computed tomography (HRCT) of the lungs. Lung volumes were evaluated by spirometry. Pulmonary hypertension was defined as pulmonary artery systolic pressure higher than 45 mmHg, as measured by echo-Doppler. Patients without tricuspid regurgitation were considered as having normal pulmonary artery pressure. The presence of autoantibodies and antinuclear antibodies (ACA) antibodies was evaluated by indirect immunofluorescence on Hep-2 cells and the presence of anti-topoisomerase I (anti-topo, anti-Scl70) antibodies – by ELISA technique. ESR and CRP concentrations were used as laboratory parameters of activity of inflammation. Capillaroscopic assessment consisted of evaluation of the four fingers (II-V) of both hands with regard to the presence of megacapillaries, bushy capillaries and/or avascular areas (defined as lack of at least three consecutive capillaries). Patient was considered as having scleroderma-related microangiopathy when at least two megacapillaries per finger in at least two fingers could be found. In addition, capillaroscopic patterns were classified according to the classification by Marique et al. as "slow" pattern characterized by regular distribution of the capillaries, preserved architecture of vascular layout and the presence of megacapillaries, or as 'active" pattern characterized by irregular drop-out of capillaries resulting in disrupted architecture of vascular layout, the presence of megacapillaries and/or bushy capillaries [15].

Control group consisted of 14 healthy subjects (12 female and 2 men). Clinical characteristics of the patients and controls is given in Table 1.

Table 1. Clinical characteristics of the patients with systemic sclerosis and the control group

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SSc patients (n=24)</th>
<th>Controls (n=14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female/Male ratio</td>
<td>20/4</td>
<td>12/2</td>
</tr>
<tr>
<td>Age (range)</td>
<td>47.8±10.8 (28 – 72)</td>
<td>38±9.6 (26 – 50)</td>
</tr>
<tr>
<td>Disease duration in years (range)</td>
<td>3.1±2.8 (0-8)</td>
<td></td>
</tr>
<tr>
<td>Duration of Raynaud’s phenomenon in years (range)</td>
<td>6.5±5.5 (0.5 – 20)</td>
<td></td>
</tr>
<tr>
<td>ARA criteria fulfilled (%)</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>dSSc/lSSc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANA positive (%)</td>
<td>24 (100)</td>
<td></td>
</tr>
<tr>
<td>Anti-Scl70 positive (%)</td>
<td>14 (58)</td>
<td></td>
</tr>
<tr>
<td>ACA positive (%)</td>
<td>6 (25)</td>
<td></td>
</tr>
<tr>
<td>Raynaud’s phenomenon (%)</td>
<td>24 (100)</td>
<td></td>
</tr>
<tr>
<td>SLD by HRCT/Xray (%)</td>
<td>13 (54)</td>
<td></td>
</tr>
<tr>
<td>Restrictive lung disease (%)</td>
<td>3 (12.5)</td>
<td></td>
</tr>
<tr>
<td>Pulmonary hypertension (%)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Digital ulcers (%)</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

*P<0.05; ACA=antinuclear antibodies, ACR=American College of Rheumatology, ANA=antinuclear antibodies, dSSc=diffuse cutaneous systemic sclerosis, HRCT=high resolution computed tomography, lSSc=limited cutaneous systemic sclerosis, SLD=scleroderma lung disease
PBMC cultures and measurements of TWEAK in supernatants. Heparin anticoagulated peripheral blood samples were obtained from cubital vein between 7 and 8 a.m. after patient having fasted for at least 10 hours. Peripheral blood mononuclear cells (PBMC) were isolated from the whole blood using density gradient centrifugation on Histopaque. The isolated PBMC were resuspended in RPMI medium supplemented with 5% fetal calf serum at a density 10^5 cells/ml and cultured at 37°C under 5% CO2 for 24 hours. Subsequently the cells were centrifuged and supernatants were collected and frozen at -80°C until measurements.

Evaluation of TWEAK concentration in the supernatants was performed using commercially available ELSA kits (Bender MedSystems).

Statistical analysis. For the assessment of between-group comparisons the Mann-Whitney U test, and for assessment of correlation the Spearman correlation test were used. The differences were considered significant at p value lower than 0.05. All results are expressed as mean±standard deviation (SD) unless stated otherwise.

Results

Comparison of TWEAK production between SSc patients and healthy controls.

The majority (16 out of 24, 66.7%) of patients included in our study had early SSc. There were no differences in the total number of leukocytes or differential cell counts numbers between SSc patients and the controls (data not shown). TWEAK was detected in the supernatants from PBMC cultures of all SSc patients and healthy controls. There were no significant differences in the production of TWEAK by the PBMC from patients with SSc and those from healthy subjects – Fig. 1.

Although healthy controls were slightly younger than patients with SSc we consider it unlikely that age differences could significantly influence our results since there were no correlations between age and TWEAK production in the whole group (r=-0.004, p=0.98), SSc patients (r=0.049, p=0.82) nor healthy controls (r=0.9, p=0.91).

Associations of the production of TWEAK with clinical features of systemic sclerosis.

To reveal potential role of TWEAK in the pathogenesis of scleroderma-related organ involvement we searched for correlations/associations between TWEAK production and clinical features of SSc.

TWEAK levels showed significant, inverse correlation with the duration of Raynaud's phenomenon (r=-0.54, p=0.007) (Table 2). Moreover, PBMC from patients with "active" pattern of capillary changes produced significantly lower amounts of TWEAK (21.2±4.8 pg/10^5 cells) when compared with PBMC from patients with "slow" pattern (31.9±11.3 pg/10^5 cells, p=0.01) and healthy subjects (27.5±7.1 pg/10^5 cells, p<0.05 vs patients with "active" pattern). Since "active" pattern is associated with greater damage of the capillaries, as indicated by the presences of avascular areas, our findings might indicate that lower production of TWEAK is associated with greater capillary burden and subsequent tissue ischemia.

PBMC from patients with SLD tended to produce lower amounts of TWEAK (24.2±8.7 pg/10^5 cells) as compared with patients without SLD (30.6±9.0 pg/10^5 cells), however the differences were not significant (p=0.088). There was also no significant correlation between TWEAK production and FVC values. However, the majority of patients included in our study had lung volumes within normal limits, this is greater than 80% of predicted, which could diminish the power of this comparisons.

We were not able to demonstrate any association between production of TWEAK by PBMC and any other clinical or laboratory indices in the studied patients (Table 2).

Since only one patient had elevated pulmonary artery systolic pressure and none of them had scleroderma renal...
crisis, we were unable to investigate associations of production of TWEAK and these serious vascular complications of the disease. Also, a low number of patients having active digital ulcers at the time of the study precluded statistical analysis of this complication.

Discussion

Our major finding is that diminished production of TWEAK by PBMC is associated with greater microvascular damage in SSc. In our study, PBMC from patients with "active" capillaroscopic pattern, which is characterized by greater capillary drop-outs, presence of avascular areas and disorganization of capillary layout, produced significantly lower amounts of TWEAK as compared with PBMC from SSc patients with "slow" capillaroscopic pattern which is characterized mainly by the presence of megacapillaries. TWEAK production in SSc patients with "active" capillary pattern was also significantly lower than in healthy subjects. We also found inverse correlation between TWEAK production and duration of Raynaud's phenomenon in our SSc patients. Raynaud's phenomenon is a universal and usually the very first manifestation of SSc which may precede development of other clinical manifestations by up to several years. Raynaud's phenomenon preceding development of definite SSc is usually accompanied by the presence of microangiopathy indicating that microvascular damage takes place from the earliest identifiable stages of the disease. Indeed, Cutolo et al. found significant correlations between duration of Raynaud's phenomenon and capillary damage, in particular capillary loss and disorganization of vascular architecture, which are typical features of "active" scleroderma pattern, in patients with SSc [16]. In a recent prospective study involving almost 600 patients with Raynaud's phenomenon followed-up for 20 years, capillary loss was a late phenomenon associated with development of definite SSc [17]. Taken together, our findings concerning diminished production of TWEAK in patients with greater microvascular damage and longer Raynaud's phenomenon indicate that decreased production of TWEAK is associated with more advanced vascular disease in SSc. Since TWEAK possesses pro-angiogenic activity it is tempting to speculate that decreased TWEAK production may contribute to SSc-associated vascular damage in SSc. This hypothesis is particularly appealing in view of the fact that mononuclear cells are the major constituents of the perivascular infiltrations found in early SSc.

As discussed in the introduction, TWEAK, as a multifunctional cytokine, possesses a broad range of biological activities some of which might be potentially detrimental in the SSc pathogenesis while the others, such as pro-angiogenic activity of TWEAK, might be potentially beneficial. Experimental studies using different models of autoimmune diseases such as rheumatoid arthritis (collagen-induced arthritis), systemic lupus erythematosus (graft versus host disease), or multiple sclerosis (experimental autoimmune encephalomyelitis), indicate that TWEAK contributes to the development of end-organ damage possibly through involvement of maintenance of chronic inflammation (reviewed in ref. 5). None of these disease models is however associated with profound fibrosis which is the major feature and cause of mortality in SSc. In vitro TWEAK activates fibroblasts to release proinflammatory mediators, some of which, such as IL-8 or MCP-1 have been already implicated in the pathogenesis of SSc [18]. However, TWEAK also inhibits differentiation of human keratinocytes into myofibroblasts, cells which possess pro-fibrotic phenotype [7].

So far, only one study concerning TWEAK in patients with SSc has been published recently. Yanaba et al. reported that soluble TWEAK levels are elevated in the sera from patients with SSc compared with healthy individuals and patients with lupus erythematosus [10]. Interestingly, they found inverse association between TWEAK levels and the presence of pulmonary fibrosis which led them to a conclusion that TWEAK has rather protective than deleterious role in SSc. Generally, this is in agreement with our interpretation of the current study suggesting that TWEAK might be protective against vascular damage in SSc.

Elevated serum concentration of TWEAK may result from excessive production or from deficient clearance or receptor binding. The results of our study indicate that, as expected, PBMC might be a potential source of soluble TWEAK in the blood of both SSc patients and healthy subjects. Although we did not find significant differences in in vitro production of TWEAK between PBMC from the whole group of SSc patients and healthy controls, it might be argued that there are numbers of active biological mediators in the circulation of SSc patients which can activate PBMC to release higher amounts of TWEAK in vivo. It could also be speculated that other cell types might contribute to increased TWEAK levels found in the sera of SSc patients. Inflammatory leukocytes are considered as major source of TWEAK, however it cannot be definitely ruled-out that other than PBMC cell types, such as granulocytes, activated endothelial cells or platelets might release TWEAK in vivo.

On the other hand, it has recently been shown that cytokine reach scavenger receptor CD163 binds TWEAK and CD163 positive macrophages can internalize TWEAK decreasing levels of TWEAK [19,20]. Whether CD163-depndent clearance of TWEAK could be responsible for diminished amounts of TWEAK in PBMC cell cultures needs to be determined. Elevated expression of CD163 has been reported in chronic diseases such as atherosclerosis, asthma, and celiac disease [20-22].
Although we did not find significant associations between TWEAK production by PBMC and other than vascular, clinical or laboratory parameters of the disease, PBMC from patients with SLD tended to produce lower amounts of TWEAK compared with those without any evidence of SLD. In general, this is in agreement with observation of Yanaba et al. who found inverse correlations between serum TWEAK levels and development of lung fibrosis [10].

It cannot be ruled out that our study is just underpowered to reveal significant associations between TWEAK production and lung fibrosis or other scleroderma related organ involvement. This seems possible since the majority of patients included in our study had early SSc and, possibly for this reason, infrequently displayed severe organ involvement. Further, longitudinal analysis is required to establish whether production of TWEAK by PBMC is associated with development of any scleroderma-associated organ complication. This hypothesis seems attractive in view of original observations by Maricq et al. revealing that "active" capillaroscopic pattern was associated with greater frequency and severity of organ involvement in SSc which indicates that capillary damage plays a key role in the pathogenesis of SSc [23]. Also, in a recent study by Koenig et al. the occurrence of capillary loss was associated with development of definite SSc in patients presenting with Raynaud's phenomenon [17].

In conclusion, the results of our study indicate that PBMC may be a potential source of soluble TWEAK in the blood. Factors responsible for increased production of TWEAK in the blood of SSc patients need to be determined.

Association between production of TWEAK by PBMC and vascular features of SSc suggest that TWEAK might have protective role against scleroderma-associated vascular injury. Whether TWEAK might appear useful in treating scleroderma associated vascular disease requires further studies.

References


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