Effectiveness of etanercept in bleomycin-induced experimental scleroderma

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Objectives. To evaluate the effects of etanercept and thalidomide in the mouse model of bleomycin-induced scleroderma (BLM-IS).

Methods. This study involved four groups (n=8 mice in each group). Dermal sclerosis was induced by repeated subcutaneous injections of BLM (10 μg) for 4 weeks in BALB/c mice. Control group received only phosphate-buffered saline. The second group received only BLM; the third and fourth groups were also given an intraperitoneal injection of 100 μg etanercept or 150 mg/kg thalidomide, respectively.

Results. BLM increased serum TGF-β1, tissue hydroxyproline levels and expression of α-smooth muscle actin (α-SMA), and dermal fibrosis was histopathologically prominent. Although thalidomide had no significant effect, etanercept caused decreases in levels of serum TGF-β1, tissue hydroxyproline and number of α-SMA-positive cells.

Conclusion. Inhibition of TNF-α with etanercept in BLM-IS was resulted in a significant reduction of the dermal sclerosis, collagen accumulation and the number of infiltrating myofibroblastic cells. TNF-α may play a key role in the progression of BLM-IS and TNF-α antagonists may be useful in the management of scleroderma.

Key words: Scleroderma, Bleomycin, Etanercept, Animal model.

Introduction

Scleroderma is an autoimmune disease, characterized by progressive fibrosis of the skin and internal organs including lung and gastrointestinal tract. Several growth factors and cytokines that are partly released from inflammatory cells infiltrating affected tissues have been suggested to play a central role in the initiation and the development of fibrosis in scleroderma [1]. Cytokines regulate immune response that is likely to affect fibroblast functions [2, 3]. The infiltration of inflammatory cells into the affected tissues is a process highly regulated by expression of chemokines, and modulated by soluble functional forms of cell adhesion molecules [3]. It is well known that the level of TGF-β, a profibrotic cytokine, increases in scleroderma [1]. TGF-β causes increased production of α-smooth muscle actin (α-SMA) [4, 5]. It has been also shown that TNF-α participates in the activation of vascular endothelium, regulation of immune response and metabolism of the connective tissue by modulation of fibroblastic function. Scleroderma patients exhibit both systemic and local increase of TNF-α levels and consecutively these increases contribute to progression in scleroderma, development of fibrosing alveolitis and skin fibrosis [6]. Increased TNF-α in scleroderma has been reported in clinical and experimental studies [7–11]. Moreover, it has been reported that the level of TNF-α correlates with the disease activity in scleroderma [9]. There are also studies reporting the effective improvement of bleomycin-induced pulmonary fibrosis (BLM-IPF) after anti-TNF-α and soluble receptor of TNF treatments [12, 13].

The exact pathogenesis of scleroderma has not been fully understood yet, despite the known data and ongoing studies about it. Therefore, the therapy of scleroderma is not at a satisfactory level as no highly effective agent is available. The aims of the present study were to evaluate pathogenic roles of TNF-α, and therapeutic effects of etanercept, an anti-TNF-α agent and thalidomide, an immunomodulator agent, in the mouse model of BLM-induced scleroderma (BLM-IS). A mouse model for scleroderma has been established previously by repeated local injections of BLM [4].

Methods

Animal model for scleroderma and administration of etanercept and thalidomide

Specific pathogen-free BALB/c mice (6 weeks old, female), weighing 20–25 g, were used. They were randomly classified into four groups (n=8 in each group). Dorsal skin was shaved for subcutaneous injections. Subcutaneous injections were performed by a 26 gauge needle, every day for 4 weeks. BLM, etanercept, and thalidomide were dissolved or diluted in phosphate-buffered saline (PBS) and sterilized by filtration (0.2 μm filter). The first group was taken as the control one and received only 100 μl PBS. The other three groups received 100 μl (10 μg) BLM, subcutaneously as described by Yamamoto et al. [14]. The third group received additional intraperitoneal 100 μg etanercept three times in a week and the fourth group received additional intraperitoneal 150 mg/kg/day thalidomide every day, as reported by Karrow et al. [15]. Approval of the ethics committee of Firat University was obtained.

Histopathology and histochemistry

Animals were sacrificed by cervical dislocation under anaesthe sia with ketamine hydrochloride on the day following the final applications, and the dorsal skins were harvested for further examinations. The skin specimens were cut into two parts; one part was fixed with 10% formalin solution, and the other was stored immediately at −80°C for tissue hydroxyproline (OH-proline) content assay. The skin specimens embedded in paraffin were sectioned at 4 μm thickness using a microtome. They were then stained with haematoxylin and eosin (H&E) and Van-Gieson. Dermal thickness (measured from the epidermal–dermal junction to dermal–fat junction) was determined from five randomly selected sites of two or more skin sections in each animal by ×100 magnification. They were examined under an Olympus BX-50 light microscope.

The numbers of α-SMA-positive fibroblastic cells were counted using an ocular grid under a light microscope of ×400 high-power...
field (HPF) from five randomly selected fields in each specimen, and the mean number was counted after it was stained with streptavidin biotin technique (Actin Muscle Ab-6 [MSA06] Neomarkers).

Collagen deposition was estimated by determining the total OH-proline content of the skin. The stored skin specimens were washed with normal saline and dried in an oven at 100°C for 72 h, then hydrolyzed with 12 N hydrochloric acid at 130°C for 3 h according to the method of Woessner [16]. After neutralizing with sodium hydroxide, the hydrolysates were diluted with distilled water. OH-proline in the hydrolysates was assessed colorimetrically at 560 nm with p-dimethy lamino benzaldehyde. OH-proline contents were measured and expressed as milligrams per gram of dry tissue.

**Enzyme-linked immunosorbent assay of serum cytokines**

Blood samples were taken by cardiac puncture and sera were obtained after centrifugation at 3000 r.p.m. for 10 min and stored at -20°C until the day of the analyses. Serum TNF-α, IL-6 and TGF-β1 levels were measured using appropriate commercial kits (Biosource International, Camarillo, CA, USA) by the enzyme-linked immunosorbent assay method.

**Statistical analysis**

Data were presented as mean ± s.d. and for comparisons between the groups, Kruskal–Wallis one-way analysis of variance and for dual comparisons Mann–Whitney U-test were used. Statistical evaluations were performed using the SPSS package program, version 11.0. A P-value of < 0.05 was considered to be statistically significant.

**Results**

Injections of PBS did not induce dermal fibrosis (Fig. 1A). However, injections of BLM increased serum TGF-β1 (P < 0.05) and tissue OH-proline levels (P < 0.05) and expression of α-SMA (P < 0.001), compared with the control PBS-treated skin (Table 1). BLM injections increased serum TNF-α and IL-6 levels by approximately 2-fold. However, these increases were not significant (Table 1) and moreover, resulted in histologically prominent approximately 2-fold. However, these increases were not significant (Table 1).

**Discussion**

In the present study, the anti-sclerotic effect of anti-TNF-α treatment on BLM-induced dermal sclerosis was examined in BALB/c mouse model. The mouse model of BLM-IS has been well documented recently [4, 17]. Daily BLM injections induced increased expressions of TGF-β1 and α-SMA, leading to dermal fibrosis in the present study. Moreover, OH-proline contents in the BLM-treated skin were significantly higher than those in the control PBS-treated skin.

The effects of different cytokines have been shown on this mouse model of scleroderma [4, 17, 18]. BLM has been shown to induce synthesis and release of IL-4 and IL-6 [18]. Administration of anti-TGF-β antibody has been reported to be effective in inhibiting dermal sclerosis and BLM-IPF in experimental animal models [18, 19] and TNF-α has been shown to play an important role in the induction of BLM-IPF [12]. In the present study, repeated BLM injections for 4 weeks increased the levels of cytokines and also infiltration of the inflammatory cells.

Scleroderma is an inflammatory disease in which cytokines play pathogenic roles [1, 2, 7–11] as in BLM-induced mouse model [4, 17, 18]. It has been shown that the levels of IL-4, IL-12, IL-13, TNF-α and sIL-6R are high and serum levels of IL-6 and IL-10 correlate with total skin thickness score in patients with scleroderma [10]. Increased IL-6 and TNF-α levels have been reported in another clinical study [11]. It has been shown that TNF-α participates in the activation of vascular endothelium-derived mediators, regulation of immune response and metabolism of connective tissue by the modulation of fibroblastic function. Scleroderma patients exhibit both systemic and local increase in TNF-α level [7–11]. Moreover, it has been reported that the

![Fig. 1. Histopathological evaluation of skin sections in BALB/c mice (H&E stain, ×100).](image)

**Table 1. Effects of etanercept and thalidomide applications on levels of serum cytokines, tissue OH-proline level and histopathological findings in BALB/c mice groups**

<table>
<thead>
<tr>
<th></th>
<th>Control (mean ± s.d.)</th>
<th>BLM (mean ± s.d.)</th>
<th>BLM + etanercept (mean ± s.d.)</th>
<th>BLM + thalidomide (mean ± s.d.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α (pg/ml)</td>
<td>17.36 ± 8.7</td>
<td>27.9 ± 19.6</td>
<td>14.7 ± 8.4</td>
<td>26.7 ± 12.6</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>26.4 ± 20.5</td>
<td>62. ± 16.4</td>
<td>37.5 ± 2.1</td>
<td>39.2 ± 3.4</td>
</tr>
<tr>
<td>TGF-β1 (pg/ml)</td>
<td>587.7 ± 31.5</td>
<td>716.9 ± 49.1¹</td>
<td>514.4 ± 62.6⁶</td>
<td>609.5 ± 161.1</td>
</tr>
<tr>
<td>Tissue OH-proline (mg/g dry tissue)</td>
<td>0.42 ± 0.07</td>
<td>0.82 ± 0.27⁶</td>
<td>0.52 ± 0.39⁶</td>
<td>0.77 ± 0.38</td>
</tr>
<tr>
<td>Dermal thickness (μm)</td>
<td>55.2 ± 10.75</td>
<td>126.4 ± 31.3³⁸</td>
<td>85.83 ± 12.8⁶</td>
<td>105.1 ± 22.7</td>
</tr>
<tr>
<td>α-SMA (cells/HPF)</td>
<td>0.84 ± 0.3</td>
<td>6.87 ± 2.2²⁸</td>
<td>4.5 ± 1.1³</td>
<td>7.4 ± 1.5</td>
</tr>
</tbody>
</table>

HPF, high-power field (×400); vs the control group, *P < 0.05, **P < 0.001; vs the BLM group, *P < 0.05.
level of TNF-α correlates with the disease activity in scleroderma [9]. It has also been shown that treatment with anti-TNF-α agents causes reduction in BLM-IPF [12, 13]. Inhibition of TNF-α with infliximab in patients with collagen vascular disease has been reported to stabilize the progression of pulmonary fibrosis [20].

Anti-TNF-α agents are effective and frequently used in the treatment of a variety of inflammatory diseases [21, 22]. It has been suggested that anti-TNF-α agents might also be efficient in the treatment of scleroderma [22]. Decreased serum cytokines such as IL-1β and IL-6 and reduced expressions of adhesion molecules have been reported in RA patients who have been treated with etanercept [23]. Adhesion molecules are necessary for the migration of inflammatory cells to the extracellular area, and they have prominent roles in the pathogenesis of scleroderma [3]. In the present study, etanercept improved BLM-induced skin fibrosis.

TNF-α increases the release of endothelin-1 and expression of adhesion molecules such as E-selectin, ICAM-1 and VCAM-1 from endothelial cells. Additionally, it stimulates the proliferation of fibroblasts via TGF-β1 and production of matrix metalloproteinases. TNF-α has been suggested to stabilize the progression of pulmonary fibrosis [20].

In conclusion, our results indicate that in the BLM-IS, inhibition of nuclear factor-κB (NF-κB) transcriptional activity induced by TNF-α and has suppressed TGF-β1-induced α-SMA expression and collagen deposition in hepatic stellate cells.

Further studies that evaluate anti-TNF-α agents in treatment of scleroderma are needed.

### References


Disclosure statement: The authors have declared no conflicts of interest.