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The Protective Effects of Bevacizumab in Bleomycin-Induced Experimental Scleroderma*

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Abstract

Background. The capillary networks are less dense and have irregular structures in scleroderma. These abnormalities result in lower capillary blood flow causing severe tissue hypoxia, which is a major stimulus for angiogenesis. However, current knowledge about compensatory angiogenesis is ambiguous in scleroderma. Bevacizumab is an inhibitor of vascular endothelial growth factor (VEGF).

Objectives. The aim of the present study is to evaluate the protective effects of bevacizumab in bleomycin (BLM)-induced dermal fibrosis.

Material and Methods. This study involved 4 groups of Balb/c mice (n = 10 per group). Mice in the control group received 100 µL/day of phosphate-buffered saline (PBS) subcutaneously, while the other 3 groups were given 100 µg/day of BLM (dissolved in 100 µL PBS) subcutaneously, for 4 weeks. Mice in BLM-treated 3rd and 4th groups also received bevacizumab (1 or 5 mg/kg twice a week, intraperitoneally). At the end of the fourth week, all mice were sacrificed and blood and tissue samples were obtained.

Results. The BLM applications increased the dermal thicknesses, tissue hydroxyproline contents, and α-smooth muscle actin-positive (α-SMA+) cell counts, and led to histopathologically prominent dermal fibrosis. The bevacizumab treatments decreased the tissue hydroxyproline contents and dermal thicknesses, and these improvements were more prominent at doses by which α-SMA+ cell counts were markedly decreased, in the BLM-injected mice.

Conclusions. In our study, inhibition of VEGF with bevacizumab treatments prevented the BLM-induced dermal fibrosis suggesting that VEGF expression contributes to the pathogenesis of scleroderma (*Adv Clin Exp Med* 2016, 25, 2, 249–253).

Key words: bevacizumab, scleroderma, dermal fibrosis, VEGF.

Scleroderma is a chronic inflammatory disease characterized by widespread fibrosis of the skin and internal organs [1–3]. Although its pathogenesis is not fully understood, vasculopathy, characterized by intimal fibro-proliferation and episodic vasospasms, leads to endothelial injury and subsequently increases the adhesions and migrations of inflammatory cells [1–4]. Moreover, intimal fibro-proliferation leads to severe and chronic hypoxia [5]. Hypoxia is a major stimulus of angiogenesis, leading to the expression of pro-angiogenic

molecules including vascular endothelial growth factor (VEGF) which is a well-characterized regulator of physiological and pathological angiogenesis [6, 7]. Despite markedly increased VEGF [7], there is no evidence of compensatory angiogenesis [8], in scleroderma. This defective angiogenesis is reported to be associated with largely alteration in the balance between pro- and anti-angiogenic factors [9].

Activated endothelial and inflammatory cells activate the fibroblastic cells through cell-cell interactions or by cytokines and growth factors includ-

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ing interleukin (IL)-4 and transforming growth factor (TGF)- β 1 [1–3]. Activated fibroblasts (myofibroblasts), which are the key effectors of extracellular matrix (ECM) production, also express pro-fibrotic cytokines and growth factors, including IL-6, TGF- β 1, platelet-derived growth factor, and connective tissue growth factor [1–3]. Therefore, activated fibroblasts show autocrine behavior and require no exogenous stimuli for persistence in their activation [1–3]. In addition to the autonomy of fibroblasts, transformation of the non-fibroblastic cells to fibroblastic cells is the other possible pathogenic way leading to scleroderma. Endothelial cells cultured with fibroblast growth factor [10], and exposed to tumor necrosis factor alpha (TNF- α) or IL-1 β [11] are observed to transform into myofibroblastic cells. Endothelial-myofibroblastic cell transformation is also reported by the application of homocysteine, a potent oxidant [12].

The aim of the present study was to evaluate the possible protective effectiveness of bevacizumab, an antibody against VEGF, in the bleomycin (BLM)-induced dermal fibrosis in an experimental scleroderma model.

Material and Methods

Animals and Experimental Protocols

Forty female Balb/c mice, 6 weeks old and weighing 20 to 25 g, were used for the experimental procedures. They were classified into 4 groups (n = 10 in each group). Mice were maintained in the animal facility of Firat University Experimental Research Center, housed in a climate-controlled environment with a 12 h light/dark cycle, in polystyrene cages containing wood shavings, and were fed standard rodent chow and water *ad libitum*. The Animal Care and Ethics committee of Firat University approved the care of mice and the experimental procedures.

Mice in the control group received 100 μ L/day of phosphate-buffered saline (PBS) subcutaneously to the shaved upper back skin. To induce dermal fibrosis, the remaining 3 groups received 100 μ g BLM (Nippon Kayaku Co., Ud., Tokyo, Japan) dissolved in 100 μ L PBS to the shaved upper back skin. Two groups of these BLM-treated mice were also intraperitoneally administered either 1 or 5 mg/kg bevacizumab (purchased from Roche, Istanbul, Turkey) twice in a week [13].

At the end of the 4th week, animals were sacrificed under anesthesia with ketamine hydrochloride on the day following the final applications. The blood samples and upper back skin samples were harvested.

Enzyme-Linked Immunosorbant Assay (ELISA)

Blood samples were taken by cardiac puncture. After centrifugation at 3000 rpm for 10 min the sera were obtained and stored at -20°C until the analysis. Serum levels of IL-2, IL-4 and TGF- β 1 were measured using the appropriate commercial kits (Biosource International, Camarillo, California USA) by the ELISA method.

Histopathology and Immunohistochemistry

The skin specimens were cut into two parts. The one part was fixed with 10% formalin solution for histopathological analysis. The other part was stored immediately at -80°C for tissue hydroxyproline (OH-proline) content analysis. The skin specimens that were embedded in paraffin were cut into 4 μ m thick sections. Afterwards, they were stained with hematoxylin and eosin (H & E) and Masson's trichrome (MT). Dermal thickness was measured from the epidermal-dermal junction to dermal-fat junction and was determined from five randomly selected sites of at least three skin sections in each animal by $\times 100$ magnification using an Olympus BX-50 light microscope.

The number of alpha-smooth muscle actin-positive (α -SMA+) fibroblastic cells (myofibroblasts) staining with streptavidin biotin (Actin Muscle Ab-6 [MSA06] Neomarkers) was counted using an ocular grid under a light microscope at $\times 400$ high-power field (HPF) from five different fields in each specimen and the mean number was calculated.

Measurement of Hydroxyproline

Collagen deposition was estimated by determining the total OH-proline content of the skin. The stored skin specimens were washed with saline and were dried in an oven (100°C for 72 h). And then they were hydrolyzed with 12 N hydrochloric acid at 130°C for 3 h [14]. The hydrolysates were diluted with distilled water after neutralizing with sodium hydroxide. OH-proline was assessed colorimetrically at 560 nm with *p*-dimethylaminobenzaldehyde and expressed as mg/g dry tissue.

Statistical Analysis

Statistical evaluations were performed using the SPSS v. 11.0. Data was presented as mean $\pm$ standard deviation. Kruskal-Wallis one-way analysis of variance and Bonferroni adjusted Mann-Whitney U test for dual-comparisons were used for statistical analysis. $P < 0.05$ was considered to be significant.

Results

Daily injections of BLM caused the increases in invaded inflammatory cell counts, in the number of α -SMA+ fibroblastic cells, and in dermal thick-

ness compared with the PBS-treated control group (Table 1, Fig. 1A, B). In addition, serum TGF- β 1 level was relatively higher in the BLM-treated group. The examinations with H & E and MT staining revealed an increased deposition of colla-

Table 1. Cytokines levels, tissue OH-proline contents, and histopathological findings in the study groups

	PBS	BLM	BEV (1 mg/kg)	BEV (5 mg/kg)
IL-2 (pg/mL)	7.53 $\pm$ 0.45	8.44 $\pm$ 2.82	7.07 $\pm$ 0.71 [†]	7.03 $\pm$ 0.87 [†]
IL-4 (pg/mL)	7.04 $\pm$ 0.13	8.43 $\pm$ 2.90	7.11 $\pm$ 0.24	7.29 $\pm$ 0.70
TGF- β 1 (pg/mL)	1098 $\pm$ 486	1363 $\pm$ 487	661 $\pm$ 585 [†]	939 $\pm$ 498
OH-proline (mg/g dry tissue)	0.52 $\pm$ 0.19	1.71 $\pm$ 0.58 [§]	1.01 $\pm$ 0.25 [‡]	0.96 $\pm$ 0.45 [‡]
Dermal thickness (μ m)	192.8 $\pm$ 53.5	435.5 $\pm$ 77.8 [§]	346.6 $\pm$ 92.8 [†]	281.6 $\pm$ 47.3 [‡]
Inflammatory cells (/HPF)	7.9 $\pm$ 3.1	34.4 $\pm$ 27.5 [§]	20.4 $\pm$ 8.1	14.9 $\pm$ 5.3 [†]
α -SMA+ cells (/HPF)	1.02 $\pm$ 0.4	2.4 $\pm$ 1.1 [§]	2.3 $\pm$ 0.7	1.6 $\pm$ 0.6 [†]

Data are expressed as mean $\pm$ standard deviation; PBS – phosphate-buffered saline; BLM – bleomycin; BEV – bevacizumab; IL – interleukin; TGF – transforming growth factor; OH-proline – hydroxyproline; α -SMA+ – alpha-smooth muscle actin-positive; HPF – high power field; vs. PBS group: [§]p < 0.001; vs. BLM group: [†]p < 0.05, [‡]p < 0.01, [§]p < 0.001.

Fig. 1. Representative photomicrographs of hematoxylin and eosin (H & E) staining. (A) Normal histopathological view in the PBS-injected control group. (B) Increased dermal thickness and infiltration of inflammatory cell in the BLM-injected sham group. (C, D) Bevacizumab treatments decreased the dermal thickness in the BLM injected mice, dose dependently. PBS – phosphate-buffered saline; BLM – bleomycin; BEV – bevacizumab

gen and histologically prominent dermal fibrosis, characterized by thickened collagen bundles with cellular infiltrates, which mimicked scleroderma in the BLM-treated group (Fig. 1B). The decrease in the amount of subcutaneous fat tissue was also observed in BLM-treated mice (Fig. 1B).

The decreases in inflammatory cell infiltration and α -SMA expressions were prominent in the bevacizumab treated group with 5 mg/kg dose. Bevacizumab applications with all doses decreased dermal thickness (Fig. 1C, D) and OH-proline content compared to the BLM-treated sham group (Table 1). Improvements of the dermal thickness and OH-proline content were more prominent in the treatment groups those are reached greater reduction in the inflammatory cell infiltration and the expression of α -SMA (Table 1).

Discussion

In this study, it was observed that the applications of bevacizumab decreased the BLM-induced inflammatory cell infiltration, fibroblastic activation, and deposition of collagen in dermal tissue, and thus prevented the development of fibrosis and thickening of the skin.

Hypoxia and cytokines are the major stimulators of VEGF expression and neovascularisation [9, 15]. It is known that appropriate vessel formation does not occur in patients with scleroderma, despite the presence of several stimuli which induce the formation of new vessels such as tissue hypoxia [9] and increased VEGF levels [8, 16]. The overexpression of VEGF in serum [8, 16] and in skin samples [6, 7] of patients with scleroderma is demonstrated. Therefore, it may be anticipated that the cause of insufficient effect of VEGF could be the decreased expression of VEGFR; however, VEGFR expression has also been reported to be increased [17]. On the other hand, Distler et al. have reported that in scleroderma VEGF does not increase as VEGFR is increased, and this situation may suggest that insufficient angiogenesis may be caused by the insufficiently increased VEGFR despite increased VEGF [17].

VEGF and other angiogenics stimulate the migrations of local- and bone marrow originated-endothelial cells [18]. Del Papa et al. [19] documented that circulating endothelial progenitor cells were not decreased in scleroderma. If so, migrated local and bone marrow derived endothelial cells may transform into the different cells for instance fibroblastic cells and thus may abrogate the fibrotic process instead of neovascularization. Endothelial cells handled by oxidative stress [12] and cytokines [11] have been reported to transform into fibroblastic cells.

VEGF enhances the production of ECM which should be used for tube formation, subsequently [20]. One other possible cause of insufficient angiogenesis in scleroderma may be insufficient activity of proteolytic enzymes required for tube formation. uPA/uPAR is required for ECM degradation at the tube formation. The level of MMP-12 which deactivates uPAR was reported to be decreased in scleroderma [21]. In that case, VEGF leads to increased production of ECM and higher interstitial pressure, and thus it also aggravates contrarily tissue hypoxia via microvascular collapse.

In the present study, bevacizumab an anti-VEGF agent ameliorated the BLM-induced dermal fibrosis. This result suggests that VEGF level increases and it plays a harmful role instead of providing a beneficial effect, at least in the early stage of dermal fibrosis. In addition to the topics mentioned above, VEGF increases vascular permeability for angiogenesis. However, inflammatory cells, the main determinants of the scleroderma pathogenesis, may infiltrate the affected tissues easily. In the present study, bevacizumab decreased inflammatory cell infiltration in addition to dermal thickness.

This study has some limitations. Firstly, it would be better to examine the efficacies of the treatments at later stages, on preexisting fibrosis, in addition to the early stages of dermal fibrosis. Secondly, the lack of negative controls is a limitation of the experimental procedure. Thirdly, the angiogenic process could have been evaluated. And finally, analyzing serum levels of cytokines may be another limitation of the present study, since dermal fibrosis is localized and restricted to the area of injections in the BLM-induced scleroderma model. Methods for detecting local expression of cytokines could be more appropriate.

Immune activation, vasculopathy and increased fibrotic activity are the key items of scleroderma pathogenesis; however, the pathogenesis of scleroderma is still unclear. Vasculopathy associated Raynaud's phenomenon and capilleroscopic abnormalities are the initial signs of scleroderma, and they may be detected even in the preclinical periods of the disease. These abnormalities are accepted to stimulate the angiogenesis; however, compensatory angiogenesis is insufficient in scleroderma. The present study documents that *in vivo* anti-VEGF applications ameliorated dermal fibrosis in the model of BLM-induced experimental scleroderma. Thus, it may be suggested that VEGF acts on pathological roles in dermal fibrosis instead of stimulating angiogenesis or that angiogenic process deteriorates dermal fibrosis instead of building new vessels. In conclusion, the actions of VEGF and angiogenesis on scleroderma are required to be evaluated by the further studies.

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