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2-Methoxyestradiol inhibits bleomycin-induced systemic sclerosis through suppression of fibroblast activation

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SUMMARY

Background*:* The most dominant feature of systemic sclerosis (SSc) is fibrosis, which is caused by overproduction of collagen by fibroblasts. 2-Methoxyestradiol (2-ME) has exhibited disease-modifying activity in animal models of rheumatoid arthritis and autoimmune encephalomyelitis and inhibitory effect in cell proliferation and collagen synthesis. Therefore, we hypothesized that 2-ME may exhibit antifibrotic effect in SSc.
Objective: To investigate the antifibrotic effect of 2-ME in SSc.
Methods*:* We established a bleomycin-induced SSc mice model by injection with bleomycin daily for 21 days. 2-ME (100 mg/kg/d) was simultaneously administered for 14 days. On the end of Week1 (W1), W2, W3 and W4, skins and lungs were collected for histological examination and analysis of hydroxyproline content and mRNA level of α1(I) procollagen (COL1A1) and COL1A2. In skin fibroblasts derived from SSc patients and healthy subjects treated with 2-ME (1, 5, or 25 μM), we examined cell proliferation, expression of α-smooth muscle actin (SMA) and mRNA level of COL1A1, COL1A2, COL3A1, matrix metalloproteinase(MMP)-1 and tissue inhibitors of MMP (TIMP)-1.
Results*:* We found reduced dermal thickness and lung fibrosis and decreased hydroxyproline content and mRNA level of COL1A1 and COL1A2 in skin and lung in SSc mice treated with 2-ME. In cell study, we observed a dose- and time-dependent inhibitory effect on proliferation of SSc fibroblasts by 2-ME. We also detected reduced α-SMA expression, decreased mRNA level of COL1A1, COL1A2, COL3A1 and TIMP-1, and increased mRNA level of MMP-1 in SSc fibroblasts treated with 2-ME.
Conclusion*:* 2-ME could suppress SSc tissue fibrosis, which may be attributable to its inhibitory effect on the excessive proliferation, differentiation and production of collagen in fibroblasts. 2-ME is rising as a prospective agent for control of fibrosis in SSc.

1. Introduction

Systemic sclerosis (SSc), also known as scleroderma, is a connective tissue disease that possesses three cardinal clinical features, including excessive deposition of extracellular matrix (ECM), vascular damage and inflammation/autoimmunity [1]. Although the pathogenesis of SSc is still unclear, it is characterized by the pathologic remodeling of connective tissues in skin and internal organs caused by overproduction of ECM, especially the production of collagen by SSc fibroblasts [2]. In our previous study using skin fibroblasts clones obtained from SSc patients and healthy controls, we found that the overproduction of collagen in SSc fibroblasts may result from the abnormally activated transcription of type I procollagen [3]. Therefore, cultured fibroblasts could be used as an in vitro model for the study of the SSc characteristics. In a recent summary of experimental models of SSc, Avouac et al. suggest that the bleomycin-induced sclerotic mice model, a frequently used model, is suitable for studying the prevention or the treatment of fibrosis [4]. Currently, there is still no cure for SSc, and the possibility of modifying or reversing fibrosis of skin and internal organs remains very low [5]. Therefore, there is an urgent need for additional and novel treatment for SSc patients, such as treatment to alleviate pathological fibrosis.

2-Methoxyestradiol (2-ME), a natural endogenous metabolite of 17β-estradiol with low affinity for estrogen receptor, exerts stronger antimitotic and anti-angiogenic effects than estradiol via

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an estrogen receptor-independent pathway [6]. Therefore, it has few side effects of estrogen and is well tolerated with considerable potential clinical applications [6]. The anti-tumor activity of 2-ME has been proved in phase I and II trials for treatment of various solid malignancies [7,8]. Disruption of microtubule dynamics and tubulin depolymerization by binding with tubulin at the colchicine-binding site has been recognized as one of the underlying anti-tumor activity [9,10]. Several recent studies on 2-ME have been focused on its disease-modifying activity to inhibit connective tissue diseases, finding its anti-inflammation [11] and immunomodulation activity [12,13], as well as inhibition of vascular remodeling [14] in animal models of rheumatoid arthritis, autoimmune encephalomyelitis and pulmonary hypertension. In addition, 2-ME has been shown to inhibit proliferation and collagen synthesis in human and rat leiomyoma cells [15]. Based on these pieces of evidence and the known role of collagen deposition in orchestrating the tissue fibrosis in SSc, we hypothesized that 2-ME may interfere with the process of fibrosis in SSc.

In this study, we examined the inhibition effect of 2-ME on tissue fibrosis and collagen deposition in bleomycin-induced SSc mice model, and analyzed the hydroxyproline content and mRNA level of type I procollagen in skin and lung. We further investigated whether 2-ME inhibits fibroblasts proliferation and differentiation into myofibroblasts in SSc fibroblasts. We also examined the collagen production and degradation in SSc fibroblasts. Our study thus examines a rationale for the use of 2-ME as an antifibrotic agent for the treatment of SSc.

2. Material and methods

2.1. Induction of skin and lung fibrosis in mice and the administration of 2-ME

Female C3H/He mice (totally $n = 96$), aged 6 weeks weighing 20–25 g, were obtained from Vitalriver Laboratory Animal Center (Beijing, China) and were maintained under pathogen-free conditions. The animal protocol was approved by the Committee of Animal Care and Use of Fudan University. Bleomycin powder (Nihon Kayaku, Tokyo, Japan) was dissolved in phosphate-buffered saline (PBS, 10 mM, pH = 7.4) at a concentration of 0.2 g/L. Using a 27-gauge needle, 100 μL of filter-sterilized bleomycin ($n = 72$) or PBS ($n = 24$) was injected subcutaneously into the shaved back of mice daily for 21 days to establish SSc (bleomycin) or control (PBS) mice models.

A stock solution of 2-ME (Gracia, Chengdu, China)was prepared in dimethylsulfoxide (DMSO, Sigma Aldrich, Poland) and then diluted in saline (0.5%, v/v) for administration. Saline containing the same concentration of DMSO as that in 2-ME administration was used as vehicle. From the first day of bleomycin injection, 48 mice in the above bleomycin group were chosen randomly and 2-ME at 100 mg/kg/d (bleomycin + 2-ME, $n = 24$) or vehicle (bleomycin + -vehicle, $n = 24$) was injected intraperitoneally daily for 14 days. Based on the half-life of bleomycin in rodents (30–120 min) [16], 2-ME or vehicle was administered 8 h after bleomycin administration to avoid possible interference with bleomycin disposition.

At the end of Week1 (W1), W2, W3 and W4 after initiating bleomycin/PBS injection, the four groups of mice (bleomycin, PBS, bleomycin + 2-ME and bleomycin + vehicle, six per group per time point) were euthanized by CO_2 asphyxiation. The back skins and lungs were excised and fixed immediately by 4% formalin for histological examination or snapped frozen into liquid nitrogen.

2.2. Histologic evaluation

Formalin-fixed skin and lung tissues were paraffin-embedded and stained with hematoxylin and eosin (H&E). Masson's trichrome staining kit (Yuanye, Shanghai, China) was used for demonstration of collagen. All sections were examined by a Leica DFC 280 light microscope. Five randomly selected independent microscopic fields from skin or lung sections of each mouse (Masson's trichrome stain, ×100 magnification) were counted to ensure the representative and homogeneous of histological data. Using Leica Q Win Plus Image Analysis System (Cambridge, UK), dermal thickness (measured from the epidermal–dermal junction to dermal–fat junction) was determined. Percentage of lung tissue fibrosis was evaluated by counting the number of pixels corresponding with stained collagen areas.

2.3. Measurement of hydroxyproline content

Hydroxyproline is a modified amino acid uniquely found in a high percentage in collagen. Therefore, we analyzed the hydroxyproline content in the punch biopsy (4 mm) samples obtained from shaved dorsal skin and right lung of each mouse as a quantitative measurement of collagen deposition. As described previously [17], hydroxyproline test kit (Jiancheng Biology Engineering, Nanjing, China) was used for the analysis according to the manufacturer's instructions. Absorbance of each sample was read at 550 nm by using a spectrophotometer. Results were expressed as total hydroxyproline amount (in micrograms) per skin punch sample or per mg of wet lung weight. A standard curve was performed for all hydroxyproline measurements by using known quantities of hydroxyproline.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR) of COL1A1 and COL1A2

Total RNAs of liquid nitrogen–frozen skin and lung samples from mice were extracted using TRIzol (Invitrogen, USA) and reverse transcribed to generate cDNA according to the manufacturer's instructions. mRNA levels of COL1A1 and COL1A2 in skin and lung were detected by real-time RT-PCR using SYBR Green Master Mix (TaKaRa, Japan). The PCR condition is 95 °C for 4 min and 40 cycles of 95 °C for 30 s and 60 °C for 20 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. Primers are shown in Table 1. A relative quantification was

Table 1
The primers for target genes in RT-PCR analysis.

Target gene	Primer sequence (5′–3′)	
Mouse COL1A1	Forward	GGTCTTGGTGGTTTTGTATTCG
	Reverse	AACAGTCGCTTCACCTACAGC
Mouse COL1A2	Forward	GCCACCATTGATAGTCTCTCC
	Reverse	CACCCCAGCGAAGAACTCATA
Mouse GAPDH	Forward	GGTGAAGGTCGGTGTGAACG
	Reverse	CTCGCTCCTGGAAGATGGTG
Homo COL1A1	Forward	CCTGGAAAGAATGGAGATGATG
	Reverse	ATCCAAACCACTGAAACCTCTG
Homo COL1A2	Forward	AGCAGGTCCTTGGAAACCTT
	Reverse	GAAAAGGAGTTGGACTTGGC
Homo COL3A1	Forward	GCTGGCTACTTCTCGCTCTG
	Reverse	TCCGCATAGGACTGACCAAG
Homo MMP-1	Forward	TGAAAAGCGGAGAAATAGTGG
	Reverse	GAGGACAAACTGAGCCACATC
Homo TIMP-1	Forward	ATACTTCCACAGGTCCCACAAC
	Reverse	GGATGGATAAACAGGGAAACAC
Homo GAPDH	Forward	AGGTCGGAGTCAACGGATTTG
	Reverse	GTGATGGCATGGACTGTGGT