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ORIGINAL ARTICLE

Vitamin A Derivative Etretinate Improves Bleomycin-induced Scleroderma

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ABSTRACT

Background: We recently demonstrated that the vitamin A derivative etretinate was clinically effective in the treatment of skin disorders in patients with systemic sclerosis (SSc). The aim of the present study is to investigate whether the oral treatment with etretinate improves sclerosis in bleomycin (BLM)-induced sclerotic skin mice.

Methods: BLM-induced sclerotic skin mice were treated orally with 10 mg/kg etretinate for 1 to 28 days. One control group received only the vehicle, 50 µl peanut oil, while another group received no agents. BLM-treated skin was removed and dermal thickness was measured histologically. Histopathological observation and TUNEL assay were also studied. Messenger RNA (mRNA) ratios for procollagen α 1 (I) chain to β actin from etretinate-treated and control mice were quantified at 1, 6, 14, and 28 days post-treatment, using quantitative RT-PCRs.

Results: There was a significant decrease in mean dermal thickness ($P < 0.05$) and changes in collagen bundles in the etretinate-treated mice group for a 28-day period compared to control groups. TUNEL assay showed that the density of TUNEL-positive cells in the dermis of etretinate-treated mice for a 14-day period was significantly increased ($P < 0.05$). The ratio of procollagen α 1 (I) chain to β actin mRNA from etretinate-treated mice for a 1-day period decreased significantly compared to that of the control mice, but the ratio from etretinate-treated mice for a 14-day period increased significantly ($P < 0.05$).

Conclusions: Etretinate improved BLM-induced scleroderma. These results suggest that etretinate can be applied to the treatment of SSc skin disorders.

KEY WORDS

apoptosis, bleomycin, collagen, etretinate, scleroderma

INTRODUCTION

Systemic sclerosis (SSc) is a connective tissue disorder characterized by the accumulation of an excessive extracellular matrix and vascular damage in various organs. The pathogenesis of SSc is not clear, however, involvement of SSc fibroblast action and related mechanisms on the maintenance of fibrosis have been suggested, especially *in vitro*. Expression of type I collagen, fibronectin, tissue inhibitors of metalloproteinase, and other enzymes are upregulated by skin fibroblasts in SSc patients.[1-4] Some cytokines such as interleukin-4 and -6, whose expression is upregulated in SSc patients, induce fibroblast mediated collagen production.[5] Transforming growth factor β and connective tissue growth factor play important roles in fibrosis or in the maintenance of fibrosis in SSc patients.[6,7]

Etretinate, a Vitamin A derivative, is also known as retinoic acid. It is typically used in the treatment of psoriasis, pustulosis palmaris et plantaris, and other conditions. Etretinate reduces the production of keratin, suppresses keratinization, and normalizes epidermal cell turnover. Some reports have indicated that retinoic acids inhibit collagen synthesis or the proliferation of normal and SSc human skin fibroblasts in culture.[8,9] In the serum of psoriasis patients treated with etretinate, propeptide type I procollagen levels

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are reduced.[10] Furthermore, retinoids are modulators of immune responses and apoptosis. In several case reports, systemic treatment with retinoic acids such as etretinate, tretinoin, and isotretinoin was associated with a decrease in skin thickness and improvements in skin disorders such as localized scleroderma and lichen sclerosus,[11] SSc,[12] scleroderma chronic graft-versus-host disease.[13] Another case study showed that topical retinoic acid treatment was effective in treating SSc and morphea skin sclerosis.[14] We recently showed that 5 out of 7 SSc patients treated systemically with etretinate and 3 out of 5 patients treated systemically with etretinate and corticosteroids, immunosuppressants, or D-penicillamine or other combinations demonstrated significantly improved skin disorders, and the mean modified Rodnan total skin thickness scores of 12 SSc patients treated with etretinate orally using ordinary doses decreased significantly.[15]

Bleomycin (BLM) is known to produce serious side effects including induction of induce pulmonary fibrosis. Yamamoto *et al.*[16] developed a mouse model of sclerotic skin using subcutaneous injections of BLM was established by In this model, dermal thickness and skin collagen was significantly increased compared to control and Fas/Fas ligand mediated apoptosis of infiltrating cells was suggested to be involved in the pathogenic mechanism.[16,17]

We investigated whether oral treatment of etretinate improved sclerosis in BLM-induced sclerotic skin model mice and whether the density of apoptotic cells in BLM-treated dermis and the expression level of procollagen messenger RNA (mRNA) changed. Furthermore we studied in detail the histopathological features using Masson-Trichrome staining in order to confirm whether the changes of dermal thickness were due to the changes of collagen bundles.

METHODS

MICE

We purchased specific pathogen-free 6-week-old female BALB/c mice weighing approximately 20 g from Japan SLC. Inc. (Hamamatsu, Japan). They were maintained in our laboratory animal center under standard conditions *at libitum* during all treatment procedures.

TREATMENTS

Etretinate was kindly provided as a gift from F. Hoffman-La Roche Ltd (Basel, Switzerland), and BLM was kindly provided as a gift from NIPPON KAYAKU CO., LTD. (Tokyo, Japan). Peanut oil was purchased from Sigma-Aldrich (St. Louis, MO, USA). All mice received daily doses of 10 μg BLM diluted in 100 μl phosphate-buffered saline (PBS) injected subcutaneously into a portion of shaved back skin for 4 weeks. Mice were divided into 3 groups. On the day following the final BLM treatment, one group was treated daily with oral 10 mg/kg etretinate diluted in 50 μl peanut oil (B→E). Another control group received only the vehicle, 50 μl peanut oil (B→P), while another group received no agents (B→Non). Treatments were continued for 1 to 28 days. Mice were sacrificed the day following these treatments, and 5 mm punch biopsies of the injected sites were examined. Dorsal skin from mice which had completed the entire BLM treatment was also removed as a starting point reference for the disease state (Day 0).

MEASUREMENTS OF DERMAL THICKNESS AND HISTOPATHOLOGICAL EXAMINATION

Biopsied skin of mice treated with etretinate, peanut oil, or without agents for 14 and 28 days, and mice in the group Day 0 was fixed in a 10% formalin solution, embedded in paraffin, and stained with Haematoxylin and Eosin and Masson-Trichrome by Narabyouri Research Co., Ltd (Nara, Japan). Dermal thickness was measured randomly in each section by ocular and objective micrometers purchased from KENIS LIMITED (Osaka, Japan). The histopathological changes were surveyed and assessed according to a qualitative scoring system. Each group consisted of 6 mice.

TUNEL ASSAY

To observe dermal changes in the density of apoptotic cells, sections of mice treated for 14 days and 28 days and in the group Day 0 were stained using a terminal deoxynucleotidyl transferase (TdT)-mediated dUNP nick end labeling (TUNEL) assay with DeadEnd Colorimetric TUNEL System from Promega Corporation (WI, USA). We counted TUNEL-positive cells randomly using ocular and objective micrometers. Each group consisted of 6 mice.

REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR)

For RT-PCR examinations, biopsied specimens were stored at −80℃. Day 0, etretinate treated, or negative control mouse specimens were examined after treatments of 1, 6, 14, 28 days. Total RNA was extracted from frozen biopsied specimens using a RNeasy® Mini Kit from QIAGEN K.K. (Tokyo, Japan). cDNA was synthesized from total RNA by reverse transcription. Quantitative PCRs were examined with a LightCycler Quicksystem 350S from Roche Diagnostics JAPAN (Tokyo, Japan). Specific primers and probes for mouse β actin and procollagen α 1 (I) chain were purchased from NIHON GENE RESEARCH LABORATORIES INC., (Miyagi, Japan). These were used in combination with Lightcycler-FastStart DNA Master Hybridization Probes from Roche Diagnostics JAPAN. Products were electrophoresed in 2% agarose gels using Tris-acetate-EDTA buffer with ethidium bromide. DNA fragments were examined under ultraviolet light and confirmed as single band targets. Each group consisted of 4 to 6