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Usnic acid inhibits hypertrophic scarring in a rabbit ear model by suppressing scar tissue angiogenesis

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ABSTRACT

Hypertrophic scarring is a common condition in the Chinese population; however, there are currently no satisfactory drugs to treat the disorder. Previous studies showed that angiogenesis plays an important role in the early phase of hypertrophic scarring and inhibition of angiogenesis has been reported as an effective strategy for anti-hypertrophic scar therapy. A recent study showed that usnic acid (UA), an active compound found mainly in lichens, inhibited tumor angiogenesis both in vivo and in vitro. To investigate the therapeutic effects of UA on hypertrophic scarring and to explore the possible mechanism involved, a rabbit ear hypertrophic scar model was established. Scars were treated once a week for four weeks with UA, DMSO or triamcinolone acetonide acetate. Histological evaluation of hematoxylin and eosin staining indicated that UA significantly inhibited hypertrophic scar formation, with obvious reductions in scar height and coloration. The scar elevation index (SEI) was also evidently reduced. Masson's trichrome staining showed that UA significantly ameliorated accumulation of collagen tissue. Immunohistochemical analysis of CD31 expression showed that UA significantly inhibited scar angiogenesis. In vitro, UA inhibited endothelial cell migration and tube formation as well as the proliferation of both human umbilical vein endothelial cells and scar fibroblast cells. These results provide the first evidence of the therapeutic effectiveness of UA in hypertrophic scar formation in an animal model via a mechanism that involves suppression of scar angiogenesis.

1. Introduction

Hypertrophic scarring is a common proliferative disorder of dermal fibroblasts characterized by collagen overexpression and excessive extracellular matrix (ECM) deposition in healing wounds elicited by deep burns, inflammatory reactions, and trauma [1]. This condition has been a major concern for patients and a challenge to surgeons for centuries; therefore, effective strategies to inhibit hypertrophic scar formation are highly important.

Currently, there are varied therapeutic approaches to the management of hypertrophic scarring, such as surgical excision, silicon containing dressings, pressure therapy, intralesional corticosteroid injection, laser therapy, and radiation [2,3]. However, no individual treatment modality appears to give satisfactory results; therefore, novel strategies for the prevention and treatment of hypertrophic scar formation are worthy of further investigation.

Angiogenesis plays an essential role in the wound-healing process, and reports show that hypertrophic scars contain more microvessels than the normal dermis [4–9]. We have used an anti-angiogenesis approach to inhibit hypertrophic scar formation [4,10] and there are many other reports of the effectiveness of anti-angiogenic therapy for the reduction of hypertrophic scarring [11,12]. In a recent study of human tissue samples, Zheng et al. observed markedly more microvessels in early and proliferative scars than in normal skin [13]. These reports suggest that anti-angiogenesis could be an effective strategy for early intervention of hypertrophic scar.

Usnic acid (UA, Fig. 1A), a dibenzofuran derivative, is an active compound isolated mainly from lichens [14]. Previous studies have shown that UA exhibits several biological properties, such as anti-viral, anti-microbial, anti-inflammatory, anti-proliferative [15], and anti-tumor activities via different mechanisms in various cell types [16–18]. Previous research showed that use of reconstituted bovine type-I collagen-based films containing liposome-loaded UA improved burn healing in rats [19]. In addition, Bruno et al. demonstrated the wound repair properties of (+)-UA enamines both in vitro and in vivo [20]. In accordance with this, our previous study showed that UA inhibits angiogenesis in vivo and in vitro, and inhibits tumor xenograft growth and angiogenesis by suppressing the VEGFR2-mediated signaling pathway

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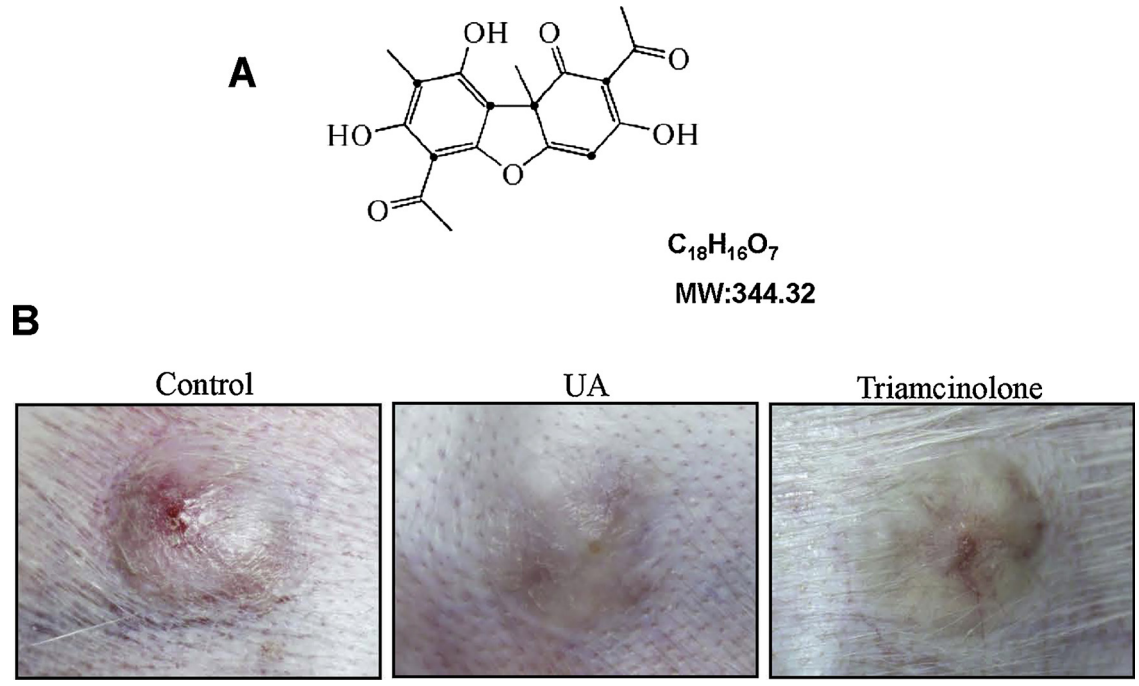

Fig. 1. Usnic acid (UA) inhibits hypertrophic scar formation in a rabbit ear hypertrophic scarring model.
(A) Chemical structure of UA with the molecular weight of 344.32. (B) Four 1 cm circular wounds were created on the ventral surface of each ear by removal of the skin, subcutaneous tissues, and perichondria. When all wounds were completely healed, each wound was injected with 2 mg UA in 50 µl DMSO, 0.5 mg Triamcinolone (50 µl), or 50 µl DMSO solvent control once per week for 4 weeks starting on day 23 post-wounding. Thirty-five days after the completion of treatment, the gross appearance of scar tissues in the control and treated groups was observed.

[21]. Therefore, in view of the close relationship between angiogenesis and scar, we speculate that UA might be a potential agent capable of reducing hypertrophic scar formation.

In this study, we investigated the functional roles of UA in hypertrophic scar formation in a rabbit ear hypertrophic scaring model. We found that UA significantly restrained hypertrophic scar formation, and inhibited scar angiogenesis. We also found that UA inhibited human umbilical vascular endothelial cell (HUVEC) proliferation, migration and tube formation in vitro. In addition, UA inhibited hypertrophic scar fibroblast (HSFB) cell proliferation.

2. Materials and methods

2.1. Animals, reagents, and cell lines

Male and female New Zealand white rabbits were purchased from the Animal Center, The Fourth Military Medical University, Xian, China. All experimental animals were caged individually and maintained in accordance with the current regulations and standards of the United States National Institutes of Health. The experimental protocol was approved by the Animal Investigation Committee of the Institute of Biomedical Sciences, The Fourth Military Medical University.

UA (98% pure, catalog number: 329967) was obtained from Sigma–Aldrich (St. Louis, MO, USA). UA was stored in a light-proof container at −20 °C and dissolved in DMSO (Sigma–Aldrich). The anti-CD31 antibody (ab9498) used for immunohistochemistry was purchased from Abcam (Cambridge, UK).

HUVECs were obtained ScienCell Research Laboratories (San Diego, CA, USA) and cultured in completed endothelial cell medium.

The hypertrophic scar fibroblast (HSFB) cells were derived from hypertrophic scar tissues from patients who underwent surgical excision at Xijing Hospital with informed consent. None of the samples obtained in this study had been treated previously. For HSFB isolation, the tissue samples were washed with phosphate-buffered saline (PBS) containing penicillin (100 U/mL), and streptomycin (0.1 mg/mL). The epidermis and fat was excised from the tissue, and the remaining tissue was cut into small pieces (1 mm^3), which were then seeded into 75 cm^2 tissue culture flasks containing 2 mL of Dulbecco's modified Eagle's medium (DMEM; Hyclone Inc., Logan, UT, USA) supplemented with 20% fetal bovine serum (FBS). The cells were incubated for 24 h to allow cell adherence before 5 mL of DMEM supplemented with 10% FBS was added. When the primary fibroblasts reached 80%–90% confluence, the cells were released by trypsin digestion and passaged for continuous culture. Fibroblasts derived from scar tissues were maintained in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 0.1 mg/ml streptomycin. All cultures were maintained at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2. The cells were passaged at 80%–90% confluence and then sub-cultured at the same density for two more passages. Cells from passages three to seven were harvested for the use in this study.

2.2. Rabbit ear hypertrophic scarring model

The rabbit ear model of cutaneous hypertrophic scarring was established according to the procedure previously described with a minor modification [4]. Briefly, New Zealand white rabbits were anesthetized with 1% (10 g/L) pentobarbital sodium at 40 mg/kg and four identical, 1-cm full-thickness circular wounds were created on the ventral surface of each ear using a 1-cm biopsy punch. The perichondrium in each wound bed was completely removed from the cartilage using a surgical blade. The wounds were exposed to air and cleaned for removal of secretions for 3 days. Any wound showing signs of infection or necrosis was excluded from the study.

2.3. Treatment of hypertrophic scars with UA

Ko et al. suggested that timing plays an essential role in scar therapy and treatment after re-epithelialization correlates with more substantial reductions in scar hypertrophy [22]. In our rabbit ear hypertrophic scarring model, re-epithelialization was complete by day 23 post-