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Immunopharmacology and inflammation

Anti-inflammatory cytokine TSG-6 inhibits hypertrophic scar formation in a rabbit ear model

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Hematoxylin (PubChem CID: 10603)
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Ethanol (PubChem CID: 702)
Sodium citrate (PubChem CID: 6224)
Xylene (PubChem CID: 6850715)
Isopropanol (PubChem CID: 3776)
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ABSTRACT

Hypertrophic scars are characterized by excessive fibrosis and extracellular matrix (ECM) deposition and can be functionally and cosmetically problematic; however, there are few satisfactory treatments for controlling hypertrophic scars. The inflammatory cells and cytokines involved in excessive inflammation during wound healing facilitate fibroblast proliferation and collagen deposition, leading to pathologic scar formation. TSG-6 exhibits anti-inflammatory activity. This study examined the effect of recombinant TSG-6 on inflammation in hypertrophic scars using a rabbit ear model. Six 7-mm, full-thickness, circular wounds were made on the ears of 12 rabbits. TSG-6 and PBS were intradermally injected into the right and left ear wounds, respectively. The methods of TEM and TUNEL were used to detect fibroblast apoptosis. The expressions of inflammatory factors: IL-1β, IL-6 and TNF-α, were detected by immunohistochemistry and real time polymerase chain reaction. Collagen I and III expression detected by immunohistochemistry and Masson's trichrome staining and SEI (scar elevation index) was used to evaluate the extent of scarring. TSG-6 injection mitigated the formation of a hypertrophic scar in the rabbit ear. TSG-6-treated wounds exhibited decreased inflammation compared with the control group, as evidenced by the lower levels of IL-1β, IL-6, TNF-α and MPO. The SEI and the synthesis of collagens I and III were significantly decreased in the TSG-6-treated scars compared with control scars. The apoptosis rate was higher in the TSG-6-treated scars. TSG-6 exhibited anti-inflammatory effects during the wound healing process and cicatrization and significantly diminished hypertrophic scar formation in a rabbit ear model.

1. Introduction

Scar formation is an inevitable result of wound healing. In causing pain, pruritus and contractures, pathological scars significantly affect physical and psychological aspects of quality of life. Hypertrophic scarring, a type of pathological scarring, is a fibrotic skin disease that is characterized by the abundant production and deposition of extracellular matrix, and is usually caused by excessive wound repair. The healing process of wound including the formation of scar is crucial to restore the skin barrier when cutaneous integrity is violated. The formation of a blood clot acts as a provisional wound matrix which releases multiple cytokines that attract and guide inflammatory cells, endothelial cells, fibroblasts, and keratinocytes. Active angiogenesis creates new capillaries, allowing nutrient delivery to support fibroblast proliferation. Myofibroblast coming from the growth of activated fibroblasts can synthesize and deposit extracellular (ECM) components, which replace the provisional matrix and cover the surface of the wounds. The abnormal biological behavior of activated fibroblasts plays a major role in the formatting and developing process of hypertrophic scars, and facilitates both wound contraction and collagen deposition. These fibroblasts have a higher capacity for proliferation, cytokine production, and collagen synthesis than normal fibroblasts. Numerous studies have demonstrated that apoptosis and apoptosis related processes are playing an important role in wound healing. The wound healing process with overabundant extracellular matrix production and abnormal extracellular matrix may result in a raised hypertrophic scar. Because clinical management remains problematic, a more efficient therapy is needed.

Skin wound healing is a programmed process that includes inflammation, proliferation, maturation and reshaping. When skin

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suffers an injury of a certain depth, the early inflammatory cascade is activated, in which numerous inflammatory cells infiltrate the damaged area and simultaneously release cytokines. The inflammatory response functions as an anti-infection immune barrier and can stimulate collagen synthesis to repair the wound. Therefore, moderate inflammation is advantageous for wound healing. However, cytokines, such as IL-1β, IL-6 and TNF-α, released by inflammatory cells in excessive inflammation, can promote fibroblast proliferation and the synthesis of extracellular matrix (fibrous connective tissue and collagens I and III), inhibit collagenase activity and increase the production of collagenase inhibitors. These events result in abnormal collagen metabolism and composition and ultimately lead to pathological scar formation (Shaw et al., 2010; Su et al., 2010; van der Veer et al., 2009; Viera et al., 2010). Mast cells, which play an important role in acute inflammatory reaction, are also scattered widely in the dermal collagen of pathological scars, and MPO is a neutrophil specific enzyme (Wilgus and Wulff, 2014).

TSG-6 (tumor necrosis factor, alpha-stimulated gene-6 protein) is encoded by the TNFAIP6 (tumor necrosis factor, alpha-induced protein 6) gene, which is a new gene isolated by differential screening of a cDNA library prepared from tumor necrosis factor-treated human diploid FS-4 fibroblasts (Lee et al., 1990). TNFAIP6 expression can be induced by numerous signaling molecules, principally TNF-α and IL-1 (Klampfer et al., 1994). TSG-6 contains a hyaluronan-binding link domain and can form a stable covalent complex with inter-alpha-inhibitor (IαI), which is important in the protease network associated with inflammation. This anti-inflammatory effect of TSG-6 has been confirmed in many studies (Getting et al., 2002; Lin et al., 2013; Nagyeri et al., 2011). In 2010, Tan showed that the anti-inflammatory factor TSG-6 was differentially distributed in keloid scars, normal scars and unscarred skin (Tan et al., 2011). However, the effect of TSG-6 on scar tissue formation is rarely reported in the past few years.

Here, we demonstrated that injection of TSG-6 into ear wounds remarkably inhibited the inflammatory reaction and significantly diminished hypertrophic scar formation in a rabbit ear model.

2. Materials and methods

2.1. Animals and reagents

Twelve young adult New Zealand White rabbits (no sex restriction) aged 3–4 months weighting between 2.5 and 3.0 kg were used. The animals were obtained from the Animal Care Center of Anhui Medical University. The animals were housed under standard conditions and fed ad libitum under an experimental protocol approved by the Anhui Medical University Animal Care and Use Committee. The rhTSG-6 was purchased from R&D Systems (R&D Systems, Inc., Minneapolis, Mini Sota, USA).

2.2. Animal hypertrophic scar model and treatment

The hypertrophic scars in the rabbit ears were established by the method as described previously (Morris et al., 1997). The animals were anesthetized with sodium pentobarbital (30 mg/kg), and then, 6 identical, 7-mm, full-thickness, circular wounds were created down to the cartilage on the ventral surface of each ear using a biopsy punch under sterile conditions. A curette was used to remove the epidermis, dermis and perichondrium in each wound. However, the hemostasis method in this work was different from the occlusive dressing method described in paper (Morris et al., 1997). In this work, hemostasis was obtained by applying pressure after creating the wounds. Then, to observe the scar healing process, the wounds were exposed to air and the secretions were removed every day. The results show that there is no difference about the rabbit ear model using those two preparing methods. The infected or necrotic wounds were excluded from this work. The rabbits were divided into three detection groups: early, middle and late. The scar tissue samples in the early detection group (4 rabbits, $n=24$ wounds for PBS and 24 wounds for TSG-6) were harvested on day 21 after wounding; the middle detection group (4 rabbits, $n=24$ wounds for PBS and 24 wounds for TSG-6) on day 28; and the late detection group (4 rabbits, $n=24$ wounds for PBS and 24 wounds for TSG-6) on day 42. The right ear of each rabbit served as the experimental group, and the left ear served as the control group. The edge and center of the right or left ear wound on a rabbit was injected with rhTSG-6 (0.001–1 μg in 10 μl of PBS) or the same volume of PBS on post-wounding days 0 (injection immediately after injury), 5, 10 and 15 with a 10 μl microsyringe. The rabbits were sacrificed by excessive anesthetic, and the wounds were harvested at the designated time for each group.

2.3. Histological examination

The scars were bisected through the maximum point and immediately fixed in 10% formalin, paraffin-embedded, cut into 5 μm sections and stained with hematoxylin and eosin. Light microscopy (Eclipse 80i Microscope, Nikon Corporation, Tokyo, Japan) with a calibrated lens reticle was used to quantitatively measure the degree of scar hypertrophy. Scar elevation was quantified by measuring the scar elevation index as described previously by Morris DE (Morris et al., 1997). A scar elevation index of 1 indicates the absence of a newly formed hypertrophic scar, and an index >1 denotes hypertrophic scar formation. All scars were examined in a blinded fashion.

2.4. Transmission electron microscopy

The hypertrophic scars were fixed in 2% glutaraldehyde and 2% paraformaldehyde for 2 h, rinsed with PBS three times, conventionally dehydrated, embedded in paraffin and cut into 0.1 μm sections; the ultra-thin sections of these scars were stained with uranyl acetate and lead citrate and examined with a transmission electron microscopy (TEM; JEM-1230 electron microscope, JEOL Ltd., Tokyo, Japan). The wounds in the late detection group (post-wounding day 42) were evaluated by TEM.

2.5. Enzyme-linked immunosorbent spot analyses

MPO protein levels were measured using a rabbit MPO ELISA Kit (CSB-E13689Rb, Cusabio Biotech Co. Ltd., Hubei, China) according to the manufacturer's instructions. The optical density of each well was measured with a spectrophotometer (NanoDrop Spectrophotometer, Thermo Fisher Scientific Inc., Wilmington, USA) at 450 nm. MPO levels in the samples were determined based on a reference curve prepared from the reagent blank and the MPO reference solution provided in the kit.

2.6. Immunohistochemistry

Immunohistochemistry was performed to detect collagens I and III, IL-1β, IL-6 and TNF-α. The scars from the rabbits' ears were fixed in 10% paraformaldehyde for 48 h. Paraffin-embedded sections (4 μm) of the scars were deparaffinized and rehydrated in 100%, 95% and 70% ethanol and subsequently incubated in double-distilled water for 2 min. For antibody staining, the sections were incubated with primary rabbit antibody at 37 °C for 1 h. The following primary antibodies were used in this assay: collagen I antibody (Catalog number orb10442, Biorbyt, South San Francisco,