Original Article

Effect of transforming growth factor-β1 on the expression of peroxisome proliferator-activated receptor β and scar formation in rabbit ears

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Received June 5, 2017; Accepted November 3, 2017; Epub March 15, 2018; Published March 30, 2018

Abstract: Numerous studies have investigated the role of transforming growth factor (TGF)-β1 in wound healing and hypertrophic scarring; however, the precise pathological mechanisms of TGF-β1 in scar constitution remain unclear. In the present study, we arranged a wound healing model in rabbit ears, and then treated the wounds weekly with TGF-β1 to investigate the exact role of TGF-β1 in the hypertrophic scar. In the control group, wounds were conducted with the same dosage of saline. Changes in the histopathological characteristics of scars were examined, as well as changes in the expression of TGF-β1, peroxisome proliferator-activator receptor (PPAR)-β and collagen (types I and III) at day 7, 21, 30, 60, 90, 120 post-wounding. Immunohistochemical analysis, reverse transcription-quantitative PCR and western blot were used in the present study. Hematoxylin and Eosin (HE) Staining revealed significant differences in the histologic characteristics between TGF-β1 treated and saline-treated (control) scars. Exogenous TGF-β1 treatment was found to increase the expression of TGF-β1 at the early stage of scarring, as well as to increase the expression of PPAR-β and collagen I/III at all stages of scar formation. Comparison of the gene expression among PPAR-β, collagen and TGF-β1 revealed that TGF-β1 may induce PPAR-β and collagen expression at the early stage of scarring, while PPAR-β may be the major promoter of collagen at the later stage of scarring. In conclusion, the present study contributes toward an improved understanding of the biological activities of TGF-β1 during hypertrophic scar formation.

Keywords: Transforming growth factor-β1, PPAR-β, scar formation, collagen

Introduction

A precise series of overlapping processes including inflammation, granulation and remodeling were involved in wound healing [1]. During the inflammation process, capillary blood vessels are firstly disrupted, and then hemostatic cascade induced, paving the way for migration of various cells that participate in the wound healing process. From about day 4 to 5, the cell growth begins with the migration of fibroblasts into the wound matrix [2]. The fibroblasts are increased for maximum and replace the fibrin with a more robust matrix of collagen fibers in 2 to 4 weeks. Remodeling phase is a stage including reduction in fibroblast count, occlusion of blood vessels, and sclerosis of collagen fibers [2]. Any abnormal control mechanisms in the process would lead to a variety of ailments, including chronic wound healing [3], hypertrophic scar [4] and keloid [5]. Hypertrophic scarring is a manifestation of a dysfunctional response of the skin to surgical incision, thermal injury and traumatic injuries. The distinct characteristics of hypertrophic scars are an increased density of fibroblasts and an excessive deposition of collagen and glycoprotein in the lesions [6], with the symptoms such as itching, tenderness, pain and pigmentation. At present, there is no effective therapy for hypertrophic scarring, partly because the underlying mechanisms are poorly understood.

Transforming growth factor β (TGF-β) is a cytokine with multiple functions that plays a major role in regulation of cell growth, differentiation and synthesis of extracellular connective tissue [7]. An increasing amount of evidence
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Implicates that the TGF-β family exerts an important function in the pathogenesis of dermal scarring [8, 9]. TGF-β1 is the major signaling molecule participating in collagen synthesis, and net accumulation of collagen as the character of a hypertrophic scar [10]. Several cells, including fibroblasts, keratinocytes, and endothelial cells, have close association with the wound healing secrete TGF-β1 protein and express TGF-β1 receptors [11]. Increased expression of TGF-β1 and TGF-β2 was correlation with hypertrophic scarring [12], and treatment of wounds with anti-TGF-β antibody at the early stage led to delayed wound healing [8].

Peroxisome proliferator-activated receptors (PPARs), members of nuclear receptor family of ligand-activated transcription factors, were originally regarded as regulators of various metabolic pathways including metabolism, adipogenesis, trophoblast differentiation, cell migration and inflammation control [13]. According to the previous studies, PPARs has been regarded plays important role in the occurrence and development of many human diseases, such as diabetes, obesity, atherosclerosis, hypertension and cancer. PPAR-β, with an expression in series of cell lineages including keratinocytes, has been reported involved in wound healing, inflammatory responses, embryo implantation and lipid metabolism [14-16]. Emerging evidence demonstrated that PPAR-β exerts critical effect in the keratinocyte in response to skin injury induced inflammation. Besides, activation of PPAR-β induced by inflammation at the wound edge maintains a large amount of viable keratinocytes which are required for the re-epithelization [13, 17]. Given all these, we proposed that PPAR-β may represent a potential new therapeutic target for wound healing.

This study aimed to demonstrate the role of TGF-β1 on the hypertrophic scar in the rabbit ear model of wound healing and to clarify the mechanisms by which TGF-β1 affect the treatment for scar in wound healing.

Materials and methods

Model of wound healing and treatments

Male or female adult New Zealand white rabbits (pathogen-free) weighed from 1.5-2.0 kg were obtained from the Animal Centre, the Third Xiangya Hospital of Central-South University, Changsha, China. Wound healing model was generated following the previous study [18]. Anesthesia with intravenous injection of pentobarbital (30 mg/kg) was generated on the rabbits [18]. After removing the perichondrium, wounds with a diameter of 2 cm were generated on the ventral surface of the ear, down to bare cartilage. Then a subcutaneous injection was generated on the rabbits weekly with 200 µl normal saline containing TGF-β1, and as an internal control, the wounds on the other ear was injected weekly with 200 µl normal saline. Six animals were contained in each treatment group. This study was performed according to the animal use protocols approved by the Committee for Ethics of Animal Experiments of Xiangya Hospital of Central South University.

Scar formation and histopathological analysis

The specimens were first fixed overnight in 4% buffered formalin solution, then dehydrated, embedded in paraffin, sectioned and stained using hematoxylin and eosin (H&E) [12]. The epithelialization, cellularity, granulation tissue, collagen content and scar hypertrophy of the wounds in different groups were examined using light microscopy following the previous study [19]. Labwork 4.0 software (Gene Company Limited, Hong Kong, China) was used to measure the H&E sections under a high-power field. At least six values from different rat sections were used to calculate the average value. Values obtained from two independent observers blind to treatment conditions and averaged were used for all histological measurement.

Real-time reverse transcription (RT)-PCR

Trizol Reagent (Life Technologies, Carlsbad, CA, USA) was used to extract the total RNA according to the manufacturer’s instruction. RNA was converted into cDNA using reverse transcription kit (Life Technologies) in accordance with the manufacture’s instruction. The real-time PCR was performed by the SYBR Green PCR master Mix (Life Technologies) according to the following conditions: 95°C for 5 min followed by 40 cycles of amplification at 95°C for 10 s, 59°C for 20 s, and 72°C for 30 s. PPAR-β primer was shown as follows: forward (5’-CCAGGTGACCGTCGTGAAG-3’); reverse (5’-
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AGAATGATGGCTGCGATGAAC-3’). GAPDH was used as an internal reference. The forward primer of GAPDH (5’-AGAGCACCAGAGGAGGAGA-CGA-3’) and reverse primer (5’-TGGGATGGAA- ACTGTGAAGAGG-3’).

Western blot

Cell Lysis Reagent (Sigma-Aldrich, USA) was used to extract the whole cell according to the manual, and then a BCA assay (Pierce, USA) was performed to quantify the protein. SDS-PAGE (10%) was used to separate the protein samples, and Western blot assay was used to detect the protein content. The antibodies used in the Western blot assays are as follows: polyclonal (rabbit) anti-PPAR-β antibody (1:200, Santa Cruz Biotechnology, USA), goat anti-rabbit IgG (1:10000, Pierce) secondary antibody conjugated to horseradish peroxidase and ECL detection systems (SuperSignal West Femto, USA).

Statistical analysis

Experimental results are presented as mean ± SD. Comparisons between two groups were conducted using two-tail Student’s T-test The level of significance was based on the probability of p <0.05 and p < 0.01.

Results

TGF-β1 promotes hypertrophic scar formation in rabbit ear model

Histologically analysis showed that TGF-β1 promotes hypertrophic scar formation in rabbit ear model (Figure 1A and 1B). Scars observed a thicker epithelium in the TGF-β1 treated group compared with control group from day 7. High fibroblast and capillary counts occurred in the TGF-β1 treated group, on day 21. From day 30, the dermis layer of the scars significantly thickened in response to TGF-β1 treatment, compared to control scars. Besides, in addition to the dense collagen fibers, collagen bundles were inordinate, which were patchily arranged in the profound dermis and nodular, circular, or whirled in the superficial dermis (Figure 1A and 1B). In control groups, cells’ number increased with the basal layer of the epidermis in the scars flattened under the normal saline treatment for 60 day. The dermis layer was not significantly thickened, and collagen fibers were well arranged, with few cells (Figure 1A and 1B). On day 90 after TGF-β1 conduction, the dermis layer of the scars in TGF-β1 treated group was significantly thickened, collagen fibers became thicker with increased densities, cells and microvessels significantly increased compared to the control group.

Exogenous TGF-β1 amplified its own expression at the early stage of hypertrophic scar

In this study, the position and expression of TGF-β1 was analyzed using immunohistochemical (IHC) techniques. As shown in Figure 2A and 2B, the protein expression of TGF-β1 was observed in epidermis and fibroblasts. TGF-β1 was highly expressed in both the TGF-β1-treated group and the control group at day 7 and 21, but it was gradually reduced from day 30 to day 120. Moreover, exogenous TGF-β1 significantly increased its expression on day 7, but had few influence on its expression from day 21 to day 120 (Figure 2A-C).
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PPAR-β expression in hypertrophic scar

PPAR-β protein is related to hypertrophic scar formation. Figure 3 shows that mRNA of PPAR-β increased gradually, and reached its peak at 90 days after post-wounding. Exogenous TGF-β1 resulted in increased levels of PPAR-β protein at all stages of scar formation. The levels of PPAR-β protein at day 60, 90 and 120 after post-wounding were significantly elevated over the control group. The results were presented as exhibited in Figure 3B of three independent experiments with Western blot assay (Figure 3B).

Effect of TGF-β1 on collagen I and III

In the previous studies, both collagen I and collagen III have been regarded as the components of the bulk of the scar ECM. Next, the protein content of collagen I and III was determined to investigate the exact effects of TGF-β1 on matrix synthesis. As we have supposed, the expression level of collagen I was decreased on day 21 while slightly increased on day 30 in the control group (Figure 4A, 4E); the expression level of collagen III expression was markedly decreased on day 21 but increased on day 30 in the control group (Figure 4B, 4F).
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The density of the collagen was keenly increased in response to TGF-β1 treatment, compared to the control group as exhibited using IHC staining (Figure 4C, 4D).

**Discussion**

Hypertrophic scars, with an abnormal collagen metabolism, an increased abundance of extracellular matrix components, are major complications of abnormal wound healing significantly affecting patients and physicians worldwide. Several previous studies have regarded TGF-β1 as an important pathologic factor in excessive scar formation [20, 21], making it a potential target of scar reduction treatment. TGF-β1, released from platelets after degranulation at the injury site, provides the early signals for the activation and infiltration of macrophages and neutrophils, spearheads of the inflammatory phase [22]. TGF-β1 has been reported to cause the differentiation of fibroblasts to myofibroblasts during wound healing [20]. Neutralizing antibodies to TGF-β1 have been shown to decrease scarring in adult rat incisional wounds [23]. In a model of a human fetal wound repair, neither TGF-β1 mRNA up regulation nor TGF-β1 protein were detected in human fetal skin after wounding and non-scarring fetal skin is relatively TGF-β1 deficient when compared to scarring adult skin [24]. In this study, TGF-β1 could be detected by the IHC in rabbit ears hyperplastic scars tissue from day 7 to 120, and the TGF-β1 expression levels peaked in the early scar and decreased gradually at the late stage. Similar to our studies, the effect of TGF-β1 on the early stage of wound healing was reported in the studies of...
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Mytien and Hakvoort [25, 26], demonstrating the TGF-β1 expression levels peak at early stage and then decrease later. According to all these, the major role of TGF-β1 seemed to be in early stage of scar formation process rather than in the mature stages. The promotion of TGF-β1 expression at early stages of scars in wound healing might be associated with the early inflammatory stage during scarring, along with clot formation, inflammatory cells increasing and prominent angiogenesis; the alternations above could be the origins of TGF-β1, which is derived from degranulated platelets, macrophages, lymphocytes, and endothelial cells. At the scar maturity, these alternations are gradually depleted or even disappear, explaining the lower expression level of TGF-β1 at this stage [27]. Exogenous TGF-β1 could induce TGF-β1 mRNA expression in human fetal fibroblasts with scar forming [28]. In this work, the exogenous TGF-β1 also amplified its own expression, but the induction occurred only in the early scar (Figure 2), indicating that TGF-β1 might be a good inductor of itself and could increase TGF-β1 expression rapidly.

PPAR-β, a nuclear receptor, plays a major role during the process of tissue injury and wound repair [13, 29, 30]. Recent study revealed that PPAR-β cooperated with TGF-β1 and participated in wound healing processes [31], PPAR-β-mediated induction of ECM proteins through the TGF-β1/Smad3 signaling-dependent or -independent pathway plays a critical role in the wound healing regulation [31]. In this study, the expression levels of PPAR-β were found to increase during the early stages, which was consistent with that of TGF-β1. We speculated that TGF-β1 might be a major inductor of PPAR-β at the early stages of scarring. Contrarily to TGF-β1 expression, PPAR-β expression was up-regulated continually at the later stage both in the control group and the TGF-β1 treated group. We speculated that other factors effected PPAR-β expression at the late mature stage.

Excessive collagen synthesis and subsequent deposition are involved in hypertrophic scars. The abundant deposition of matrix proteins (pre-dominantly collagen I and collagen III) by peripheral cells was associated with the enhanced tensile strength of healing wounds. As shown in Figure 4, collagen III expressed abundantly at the early stages of scarring and tended to down-regulate at the late mature stages. Similar tendency observed in collagen I expressed. Recent studies shows that TGF-β1 plays the role as the irritation of these ECM components at the edge of wounds [32], and blocking of the TGF-β1/Smad signaling pathway could suppress the production of collagen [33]. Moreover, it has reported that PPAR-β promoted the expression of collagen I and III in the process of wound healing [31]. In this study, collagen I and III expression were evidently up-regulated by exogenous TGF-β1, indicating that TGF-β1 played an important role on collagen expression at the early stage of scarring. Both collagen I and III expression levels peaked at day 90, indicating that PPAR-β might promote collagen expression at the later stages.

In conclusion, the data reported here demonstrates the expression levels of TGF-β1, PPAR-β and collagen at different stages of scar formation. We indicated that TGF-β1 when highly expressed may play a critical role in the expression of PPAR-β and collagen at the early stages of scarring, and PPAR-β might induce collagen expression at the late stages of scar. These results elucidate the role of TGF-β1 and PPAR-β in different stages of hypertrophic scarring and therefore may aid in the development of therapies for hypertrophic scarring.

Disclosure of conflict of interest

None.

Abbreviations

TGF-β1, Transforming growth factor-β1; PPAR, Peroxisome proliferator-activator receptor; HE, Hematoxylin and Eosin.

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