Original Article

Effect of total flavonoids on expression of collagen, TGF-β1, and Smad 7 in hypertrophic scars

Ruzha Mulatibileke, Yang Yu, Zaiyang Zhang, Shaolin Ma

Department of Plastic Surgery, The First Affiliated Hospital of Xinjiang Medical University, Urumqi 830054, China

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Abstract: Objectives: This study was to explore the effect and mechanism of Total Flavonoids (TF) on hypertrophic scars (HS). Methods: HS was established in rabbits by creating round excisional wounds down to the bare cartilage on the ventral surface of the rabbit ears. After 15 days of wounding, the control group was injected with saline while the treatment group was treated with TF at different doses. Intralesional injections were performed every three days and four times in total. The HSs were harvested on the 27th day. Scar elevation index (SEI) was assessed after HE staining. Collagen deposition was observed by Masson’s trichrome staining. The expression of Type I and III collagen, TGF-β1 and Smad 7, were examined by real-time PCR, immunohistochemistry, and Western blot. Results: TF significantly reduced SEI in Groups C and D compared with that in the control group. The collagen was sparsely distributed in the experimental groups while it was dense and regularly arranged in the control group. Besides, the expressions of Type I and III collagen and TGF-β1 were much lower under a proper dose of TF treatment and Smad 7 expression was also enhanced. Conclusion: Intralesional injections of TF can alleviate dermal scarring probably by upregulating Smad 7 expression and thus suppressing the expression of Type I and III collagens and TGF-β1.

Keywords: Traditional Chinese medicine, total flavonoids, hypertrophic scar, TGF-β1, Smad 7

Introduction

Scarring is an inevitable natural process during wound healing. Normal wound-healing progress comprises three stages: inflammation, new tissue formation, and remodeling [1]. However, in some cases, a hypertrophic scar (HS) is formed, which is defined as an excessive scar tissue within the original wound. Despite the yet unknown detailed mechanism of HS, some factors are considered to be related to HS formation such as age, bacterial colonization, and skin stretch [2, 3]. Collagen is over-deposited in extracellular matrix (ECM) and the fibroblasts become over-proliferated during the remodeling phase of wound healing [4]. As a result, wound healing ends up with HS formation which may cause dysfunction, pain, aesthetic problems, etc [5]. Although several decades have been spent searching optimal treatments for HS, it remains an unmet challenge.

Several cellular signals are involved in the formation of HS among which transforming growth factor-β (TGF-β) is an important one. TGF-β, activated by Smad proteins in fibroblasts, interacts with TGF-β receptor and leads to an increase in the production of collagen in the ECM and cell proliferation [6]. However, Smad 7, as the unique negative feedback regulator of the TGF-β/Smads signal pathway, can prevent receptor-regulated Smads (R-Smads) phosphorylation by associating with the activated TGF-β1 receptors. Therefore, an overexpression of Smad 7 may possibly reduce TGF-β1 excretion and inhibit fibrinolysis [7].

Total Flavonoids (TF) are natural compounds commonly found in herbs, which is also used in modern clinical treatment because of its antioxidant, antithrombotic and anti-proliferative effects [8-12]. Our preliminary studies have shown that TF refined from traditional Chinese herbs has a potential preventive effect on HS formation both in experiments in rabbit in vivo and in human cells in vitro [13-16].

In this study, a HS model was established in rabbits. The effect of TF on HS was investigated.
and its role in regulating the expression of key factors in TGF-β/Smads signaling pathway was studied.

Materials and methods

Animals

The Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University approved all procedures of this experiment. Twenty female SPF New Zealand white rabbits (weighing 1.8 to 2.6 kg) were acquired from Laboratory Animal Center of Xinjiang Medical University (License No. SCXK 2003-001) and were single-housed under the same standard.

Preparation of rabbit ear HS model, treatment and sampling

On day 0, rabbits were anesthetized by intramuscular injection with 0.15 mL/kg Zoletile 50 (tiletamine hydrochloride and zolazepam hydrochloride) and 0.1 mL/kg Ketamine, respectively. Under sterile conditions, six full-thickness 8-mm diameter round excisional wounds were created down to the bare cartilage on the ventral surface of each ear [17]. To bare the cartilage, the epidermis, dermis, and perichondrium were removed under a surgical operating microscope [18]. During the excision, visible vessels were avoided and bleeding was treated by manually pressing with gauze. All of the wounds were then covered with a sterile adhesive dressing. A total of 240 wounds were created in 20 rabbits.

On day 15, successfully established HS models were divided into four groups along the vertical axis of each ear from left to right in 19 rabbits (one rabbit died on day 2 probably due to the anesthetization side effects) (Figure 1). Subsequently, 219 HSs (9 scars were excluded because of invagination) were treated by intratransosial injection of either normal saline (Group A, n=55) or different doses of TF solution (Group B, n=54, 2 mg/mL; Group C, n=56, 1 mg/mL; Group D, n=54, 0.5 mg/mL) [17, 19]. The injection was performed every three days and four times in total. In addition, since day 15 post-wounding, photographs of ears and scarring
areas were taken before each time of injection for qualitative changes in scar morphology [20]. On day 27, rabbits were sacrificed and 207 HSs (a rabbit died after the second injection) were harvested with a 3-mm unwounded margin (Figure 1E). From each group, 45 HSs were randomly collected.

**Histopathological analysis**

The 60 HSs (15 in each group) were fixed with formaldehyde, embedded in paraffin, cut into 4-μm sections, and stained for histopathological analysis.

After hematoxylin and eosin (HE) staining, the protuberant degree was quantified by measuring the scar elevation index (SEI) which represents the ratio of total scar area to the estimated area below the protuberant portion that had new tissue with the same height as the surrounding non-scarred dermis [17].

To analyze collagen deposition and arrangement, Masson’s trichrome staining was carried out using a quick Masson’s trichrome staining kit (Njjcbio, China), following the manufacturer’s instructions.

For immunohistochemistry, endogenous peroxidase was inactivated by 3% H₂O₂ incubation for 10 minutes at room temperature and then the sections were incubated with 1% citric acid for 10 minutes in microwave for antigen retrieval. After that, the sections were blocked with normal goat serum (ZSGB-Bio, China) for 15 minutes at 37°C and then incubated with primary antibodies of collagen I (Cat# ab90395; 1:1000 dilution; Abcam), collagen III (Cat# ab7778; 1:5000 dilution; Abcam), TGF-β1 (Cat# sc-146; 1:500 dilution; Santa Cruz Biotechnology), and Smad-7 (Cat# sc-365846; 1:500 dilution; Santa Cruz Biotechnology) at 4°C overnight. Finally, sections were incubated with goat anti-rabbit secondary antibody (Boster, China) or goat anti-mouse secondary antibody (Boster, China) for 30 minutes at 37°C, followed by DAB (Boster, China) coloration for 5 minutes and hematoxylin staining.

All histological images were obtained with an optical microscope connected to cell Sens software (Olympus, Japan) and all of the measurements were performed with its measurement function by two observers, blind to this experiment, and then averaged. The cell number was counted in five different microscopic regions (400×) for each section and then averaged.

**Real-time PCR**

Sixty (15 for each group) HS samples were ground into powders in liquid nitrogen and then total RNA was extracted using TRizol® (Invitrogen) according to the manufacturer’s instructions. RNA was reverse-transcribed into cDNA with a RevetAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). Real-time PCR was performed using the Maxima SYBR Green qPCR Master Mix (Thermo Scientific, USA) on the iCycler thermocycler (Bio-Rad). The primer sequences (synthesized by Sangon Biotech, China) are listed in Table 1. Real-time PCR was carried out as follows: initial denaturation for 10 minutes at 95°C and 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C. Quantification was always normalized to the internal control GAPDH and each template was repeated three times under the same condition.

**Western blot**

Briefly, 60 HS tissue samples (15 each group) were lysed with Pierce® RIPA buffer (Thermo Scientific, USA) and PMSF (100:1). The extracted proteins were quantified by Pierce® BCA Protein Assay Kit (Thermo Scientific, USA). Proteins were separated on 12% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, USA). Then, the membranes were incubated with primary antibodies of collagen I (Cat# ab90395; 1:500 dilution; Abcam), collagen III (Cat# ab7778; 1:1000 dilution; Abcam), TGF-β1 (Cat# sc-146; 1:200 dilution; Abcam), Smad-7 (Cat# sc-365846; 1:200 dilution; Abcam), and β-actin (Cat# sc-47778; 1:500 dilution; Santa Cruz Biotechnology) overnight at 4°C. After washing, HRP-
conjugated goat anti-rabbit or goat anti-mouse secondary antibody (Abbkine, USA) was added and incubated at room temperature for 2 hours. The protein bands were visualized with the ECL Kit (Thermo Scientific, USA) and densities of the bands were compared with that of β-Actin.

Statistical analysis

All data were analyzed using SPSS version 22 (SPSS, Inc., Chicago, IL, USA) and are presented as mean ± standard deviation (SD). Intergroup comparisons were analyzed by Student's t-test. One-way ANOVA was used to compare mean values of the four groups simultaneously, followed by Bonferroni's post hoc test or Tamhane's method when the variance was not homogenous. A P value < 0.05 was considered statistically significant.

Results

Effect of TF on gross scar formation

To dynamically observe the scar formation and the effect of TF on scar formation since day
TF on hypertrophic scar

15 post-wounding (Figure 1A), scars were observed every day and photographed before each injection. The intralesional TF injection groups showed obvious softness after the second injection compared with the control group (Figure 1B). Some HSs in Groups C and D almost resembled normal skin, which had lower protuberant heights (Figure 1C). Some vulnerable epidermis samples were observed in Group B, in a form of bleeding or ulceration. In addition, a rabbit showed slouched ears because of the scar contracture but the sides of rabbit ears in Groups C and D became erect again after the third injection (Figure 1D). These results indicate that TF may soften the scar and delay wound-healing.

Effect of TF on SEI and collagen

To assess the effects of TF on cell morphology, HE staining was performed. The cells of the control group were primarily spindle-shaped while those in the TF injection groups were round. The number of fibroblasts was lowest in Group C (Figure 2A). Furthermore, the quantitative analysis of SEI presented a remarkable decreasing in Groups C and D (Figure 2B). These results suggest that treatment with a proper dose of TF may inhibit the proliferation of fibroblasts and less decrease the protuberant degree of HSs.

The HS tissues were subjected to Masson staining to examine the effect of TF on collagen arrangement. It showed that the collagen was sparsely distributed and arranged in a net-like shape similar to the adjacent unwounded tissues in the TF treated groups, especially in Groups C and D, while the collagen of the control group was densely arranged, parallel to the epithelium and less mature with more fibroblasts (Figure 2C). Therefore, treatment with a proper dose of TF may lead to collagen arranging and depositing in a beneficial direction.

The effect of TF on the protein expressions of Type I and III collagens, TGF-β1 and Smad 7

In order to observe the expression of Type I and III collagens, TGF-β1 and Smad 7 in the HS tis-
sues, immunohistochemical staining was performed. There was less Type I and III collagen deposition in cellular mesenchyme after TF treatment compared with the control group (Figure 3A and 3B). Also, expression of TGF-β1 was obviously reduced in Group C and D (Figure 3C) while that of Smad 7 increased in the TF treated groups, especially in Group C (Figure 3D).

To further verify the effects of TF treatment on the protein expression of Type I and Type III collagens, TGF-β1 and Smad 7, Western blot was performed. The results showed that Type I and Type III collagen expressions remarkably reduced in the TF treated groups (Figure 4A and 4B, *P < 0.05). TGF-β1 protein expression was effectively suppressed in Group C and D, while a higher expression was detected in Group B (Figure 4C, *P < 0.05). The high expression of TGF-β1 protein in Group B (TF, 2 mg/mL) might be due to the damage of tissues and cells caused by the high dose of TF [13]. By contrast, Smad 7 protein expression significantly increased after TF treatment, among which Group D (TF, 0.5 mg/mL) showed the highest expression (Figure 4D, *P < 0.05).

Thus, it is assumed that TF might reduce collagen deposition and TGF-β1 expression but promote Smad 7 expression.
The effect of TF on the mRNA expression of Type I and III collagens, TGF-β1, and Smad 7

To detect the mRNA expression of Type I and III collagens, TGF-β1 and Smad 7, quantitative RT-PCR was carried out. The results showed that Type I collagen mRNA expression was suppressed in all the TF treated groups (Figure 5A, P < 0.05), especially in Group C (TF, 1 mg/mL). The mRNA expression of Type III collagen and TGF-β1 was inhibited in the TF treated groups, among which the effect in Group D (TF, 0.5 mg/mL) was the most significant (Figure 5B and 5C, P < 0.05). In contrast, mRNA expression of Smad 7, which is considered as a unique inhibitor of the TGF-β/Smad signaling pathway, in the TF treated groups was much higher than that of the control group (Figure 5D, P < 0.05). These results indicate that mRNA levels of collagen Type I and III, and TGF-β1 were downregulated after a proper dose of TF treatment, while Smad 7 was upregulated.

Discussion

Scar formation is the normal consequence of skin injury. It is thin and resembles normal skin under correct regulation. However, prolonged inflammation and chronic stimulation may cause excessive deposition of collagen and proliferation of fibroblasts, eventually leading to HS formation [6, 7, 21]. So far, the treatment for HS remains unsatisfying [22]. TF, one effective ingredient in traditional Chinese medicine, is found to have an anti-proliferative property to certain cells [11, 23, 24]. Preliminary studies have shown that total flavonoids (TF) extracted from ASMq might prevent HS formation [11-14]. The present study found that TF could soften HSs and decrease the SEI of the rabbit ear model in vivo.

Previous studies have suggested that wound-healing and scarring are closely related to TGF-β family members, which also regulate a wide spectrum of cellular functions such as prolifer-
TF on hypertrophic scar

Increased amounts of TGF-β have been found in HS and the scar-free healing in human fetal is considered as a result of TGF-β deficient [27]. Furthermore, it has been reported that TGF-β can mediate fibroblast proliferation, angiogenesis, ECM synthesis, and re-epithelialization in the wound-healing process [28, 29]. Overexpression of TGF-β1 and β2 were detected in HSs, whereas TGF-β3 was reported to have anti-fibrotic effects [7]. Particularly, TGF-β1 transcriptionally regulates various fibrosis-related proteins, including Type I and III collagens [30, 31]. It can also promote the transformation of fibroblasts to myofibroblasts, which are the major cells contributing to HS formation and characterized by an increased propensity to synthesize collagen and upregulation of cytokines [32, 33]. Therefore, it is speculated that inhibition of TGF-β1 activity would have potential benefits in suppressing HS formation.

In spite of the limited varieties of TGF-β receptors and Smads, there may be a greater versatility of the signaling possibilities than we used to expect. Combinatorial interactions between TGF-β receptors and Smads in oligomeric complexes allow substantial diversity and are complemented by various sequence-specific transcription factors cooperating with Smads, resulting in context-dependent transcriptional regulation [34-36]. In addition, other signaling pathways may also help to define the responses to TGF-β and it is evidently shown that TGF-β-related protein activation is not a linear signaling transduction pathway. These pathways that regulate Smad-dependent responses also induce Smad-independent responses [37]. On one hand, the TGF-β1-activated Smad-dependent pathways can cause cellular changes such as the synthesis and secretion of collagen, leading to increased scar formation. However, Smad-independent signaling pathways may improve healing. Therefore, inhibition of TGF-β1 transcription may reduce scarring but delay wound healing [38-40]. In this study, it was observed that treatment with a proper dose of TF could lessen scarring and delay healing. Meanwhile, expression of Smad 7 was upregulated and that of TGF-β1 was decreased.

The Smad family is divided into three distinct subfamilies: receptor-regulated Smads (R-Smads), common-partner Smads (Co-Smads), and inhibitory Smads (I-Smads). Smad 7, which acts as the I-Smads of TGF-β family signaling, can bind with TGF-β receptor and thus prevent the recruitment and phosphorylation of effector Smads and inhibiting TGF-β/Smads signaling [7, 37, 41]. The TGF-β/Smad signaling system is an auto inhibitory loop to control the intensity and duration of TGF-β signaling response. TGF-β stimulation could result in the export of Smad 7 from nucleus [36]. Therefore, the expression level of Smad 7 will influence TGF-β transcriptional responsiveness. Cells with a higher level of Smad 7 are more inclined to resist the pro-fibrotic actions of TGF-β. As this study indicated, TF treatment blocked the TGF-β/Smad signaling via increasing the expression of Smad 7 and thus the stimulation effect of TGF-β1 on collagen deposition in ECM was inhibited.

In conclusion, a rabbit ear HS model was established. A proper dose of TF could negatively regulate expression of Type I and III collagens and collagen deposition. TGF-β1 transcription was down regulated which might be partially due to Smad 7 upregulation. This study suggests that TF may have potential to prevent HS formation.

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Disclosure of conflict of interest

None.

Address correspondence to: Shaolin Ma, Department of Plastic Surgery, The First Affiliated Hospital of Xinjiang Medical University, 137 South Lyushan Road, Urumqi 830054, China. Tel: 0086-13639934408; E-mail: mashaolin9@outlook.com

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