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Smad ubiquitination regulatory factor 2 expression is enhanced in hypertrophic scar fibroblasts from burned children

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Abstract

Transforming growth factor- β 1 (TGF- β 1) plays a key role in hypertrophic scar formation. A lot of studies have shown that TGF- β 1 stimulates fibroblast proliferation, collagen production, and α -smooth muscle actin (α -SMA) expression, inhibits matrix degradation and eventually leads to scar formation. Smad proteins are important intracellular mediators of TGF- β 1 signaling, and Smad ubiquitination regulatory factor 2 (Smurf2), an ubiquitin ligase for Smads, plays critical roles in the regulation of TGF- β 1/Smad signaling. It was reported that Smurf2 was abnormally expressed during the process of liver fibrosis and lung fibrosis. Hypertrophic scarring is a fibroproliferative disorder of the dermis that occurs following wounding. However, little is known about the expression of Smurf2 in hypertrophic scarring. We hypothesized that TGF- β 1 signaling cannot be disrupted after wound epithelialization probably due to abnormal expression of Smurf2 in hypertrophic scar fibroblasts. In the present study, we found that hypertrophic scar fibroblasts exhibited increased Smurf2 protein and mRNA levels compared with normal fibroblasts, and the expression of Smurf2 gradually increased in hypertrophic scar fibroblasts after TGF- β 1 stimulation. Furthermore, we transfected Smurf2 siRNA into hypertrophic scar fibroblasts, and we found that silencing the expression of Smurf2 in hypertrophic scar fibroblasts dramatically reduced TGF- β 1 production, inhibited TGF- β 1-induced α -SMA expression and inhibited TGF- β 1-induced collagen I synthesis. Our results suggest that the enhanced expression of Smurf2 is involved in the progression of hypertrophic scarring.

Keywords

Smad ubiquitination regulatory factor 2; TGF- β 1; Hypertrophic scar

1. Introduction

Hypertrophic scarring is a common complication of wound healing in patients, with thermal and other injuries penetrating the dermis. It is characterized by exuberant, firm scar tissue that is erythematous and pruritic. The bulky and inelastic qualities of the scar, as well as the

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frequent occurrence of contractures, can severely restrict the mobility of joints and extremities, constrict orifices, immobilize structures such as eyelids, and drastically compromise cosmetic appearance [1]. Treatment for hypertrophic scarring is problematic, with no single modality producing uniformly satisfactory results.

Transforming growth factor- β 1 (TGF- β 1) plays a key role in hypertrophic scar formation [2]. A lot of studies have shown that TGF- β 1 stimulates fibroblast proliferation, collagen production, and α -smooth muscle actin (α -SMA) expression, inhibits matrix degradation and eventually leads to scar formation [3, 4, 5, 6].

Smad proteins have been identified as important components of the TGF- β 1 signaling pathway [7]. TGF- β 1 signals through the heteromeric complex of TGF- β 1 type I receptor (T β RI) and TGF- β 1 type II receptor (T β RII), which are transmembrane serine–threonine kinase receptors. Activation of the receptor complex occurs when type II receptor kinase transphosphorylates the glycine–serine domain of type I kinase. The activated type I kinase associates transiently with and also phosphorylates receptor-regulated Smad2 and Smad3 (R-Smads). When they are phosphorylated, R-Smads dissociate from the receptor, bind to Smad4, and then enter the nucleus, where they activate target gene transcription [8].

Smad ubiquitination regulatory factor 2 (Smurf2), an ubiquitin ligase for Smads, plays critical roles in the regulation of TGF- β 1/Smad signaling by targeting Smad-associating proteins for ubiquitin degradation [9]. It was reported that Smurf2 was abnormally expressed during the process of liver fibrosis [10] and lung fibrosis [11]. Recent studies have shown that Smurf2 expression was increased in renal fibrosis [12, 13]. Hypertrophic scarring is a fibroproliferative disorder of the dermis that occurs following wounding. However, little is known about the expression of Smurf2 in hypertrophic scarring. We hypothesized that TGF- β 1 signaling cannot be disrupted after wound epithelialization probably due to abnormal expression of Smurf2 in hypertrophic scar fibroblasts.

This study was first conducted to investigate the difference of Smurf2 transcript and protein expressions between normal dermal fibroblasts and hypertrophic scar fibroblasts from burned children, and then we transfected Smurf2 siRNA into hypertrophic scar fibroblasts, in order to investigate whether Smurf2 genetic silencing affected TGF- β 1 production, α -SMA expression and collagen I synthesis in hypertrophic scar fibroblasts from burned children.

2. Materials and methods

2.1 Cell culture

The fibroblast was established as a primary cell line from normal skin or hypertrophic scar tissue obtained from three children recovering from severe burns. Each patient demonstrated extensive areas of hypertrophic scarring as manifested by raised, erythematous, pruritic, thickened, and non-compliant scars confined to the site of injury. Scarring resulted in functional impairment in the form of restricted range of motion, and an operation was needed. The three hypertrophic scar samples were obtained between 3 months and 6 months after thermal injury. The sample 1 was from the right elbow of a 6-year-old male child. The sample 2 was from the left elbow of a 5-year-old female child, and the sample 3 was from the right knee of an 8-year-old male child. Exemption to use operative specimens that would otherwise be discarded was approved by the Institutional Review Board at the University of Texas Medical Branch, Galveston, TX.

Using a sterile technique under a laminar flow hood, the dermal specimen was minced into approximately 1-mm³ fragments with a sterile scalpel blade on a Petri dish. The specimens

were washed in phosphate-buffered saline (PBS) solution with a combination of 1% penicillin, streptomycin sulfate, and amphotericin B (GIBCO, Invitrogen). The specimens were then placed in 75-mm³ tissue culture flasks (T75; Falcon, Becton, Dickinson and Company, Franklin Lakes, NJ) with 10 mL of culture medium (15 % fetal calf serum in Dulbecco-modified Eagle medium with 1% penicillin-streptomycin sulfate-amphotericin B [GIBCO]). The specimens were then maintained in a humidified incubator at 37°C with a 5% carbon dioxide atmosphere. After 24 hours, the medium was changed to 7 mL of culture medium. The medium was then changed every 2 days until fibroblasts were seen growing outward from the explanted tissue under light microscopy. At that time, the tissue was removed. With sufficient outgrowth of fibroblasts, cells were subcultured into 75-mm² culture flasks. Culture medium was changed every 3 days. Successive cultures were passed at confluence. Experiments were performed with early passage cells (passages 3–6). Cells at the same passage were used to examine the expression of Smurf2. Normal dermal fibroblasts from the same patients were used as a control for hypertrophic scar fibroblasts, which would confirm that the hypertrophic scar fibroblasts act differently than normal fibroblasts.

2.2 Western blot analysis

Cells were washed with sterile PBS twice and then 100 µl cell lysis buffer (35 mM Tris–HCl, pH 7.4, 0.4 mM EGTA, 10 mM MgCl₂, 100 µg/ml aprotinin, 1 µM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, and 0.1% Triton X-100) was added. Lysates were centrifuged at 10,000g for 20 min. The protein concentration in the cell lysates was measured using the Bio-Rad Protein Assay Kit. Cell lysate samples heated at 80 °C for 10 min before loading (35 µg/lane), and 4–12% SDS–polyacrylamide gel electrophoresis was performed. After SDS–PAGE, proteins were transferred onto a PVDF membrane (Bio-Rad). The membrane was blocked for 1 h at room temperature with 5% skim milk in TBS–Tween and incubated overnight at 4 °C with rabbit anti-human Smurf2 polyclonal antibody (Santa Cruz, CA, USA), mouse anti-human α-SMA (α-smooth muscle actin) monoclonal antibody (Sigma-Aldrich, USA), and rabbit anti-human GAPDH monoclonal antibody (Cell Signaling, USA). After incubation with HRP-conjugated anti-mouse-IgG or anti-Rabbit-IgG, an ECL Western blots detection system (Amersham Biosciences, Piscataway, New Jersey) was used according to the manufacturer's instructions. The resulting radiographs were scanned, and relative densities of the bands were determined by a scanning transmission densitometer (Applied Imaging, Santa Clara, CA, USA). The expression levels of Smurf2 and α-SMA were normalized to the corresponding GAPDH determined as an internal control. Data presented are from means of triplicate from one experiment that was replicated on three separate occasions (n = 3).

2.3 RNA isolation and reverse transcriptase PCR (RT-PCR) analysis

Total RNA was prepared using the RNAqueous kit (Ambion, Austin, TX, USA), according to the manufacturer's instructions. The RT-PCR was performed using the AccessQuick RT-PCR System (Promega Biotech, Madison, WI, USA) according to the manufacturer's instructions. Briefly, 1 µg of RNA was reverse-transcribed. The primer sets used were as follows: for Smurf 2, forward 5'-CGCTTGATCCAAAGTGAAT-3' and reverse 5'-GGTTGATGGCATTGGAAAGA-3'; for α-SMA, forward 5'-AGGAAGGACCTCTATGCTAACAAT-3' and reverse 5'-AACACATAGGTAACGAGTCAGAGC-3'; for Collagen I, forward 5'-CCAAATCTGTCTCCCCAGAA-3' and reverse 5'-TCAAAAACGAAGGGGAGATG-3'; for β-actin, forward 5'-ACTTAGTTGCGTTACACCCTTTCT-3' and reverse 5'-TTCATACATCTCAAGTTGGGGGAC-3'. Each RT-PCR included a cDNA synthesis and pre-denaturation cycle at 95°C for 5 min; the cDNA was amplified for 28 cycles involving a denaturation step at 95°C for 1 min; a primer annealing step at 55°C for 60 s; and an

extension step at 72°C for 1 min. The PCR products were analyzed by electrophoresis on a 2% agarose gel containing ethidium bromide and visualized and photographed under UV light. In all experiments, two control reactions, one containing no mRNA and another containing mRNA but no reverse transcriptase or Taq, were included. Image capture and density analysis of bands were performed using the SynGene™ gel documentation system (SynGene-Synoptics, Cambridge, UK). The level of mRNA expression was presented as the ratio of the band density to β -actin. Data are presented as means of results from three experiments each performed in duplicate ($n = 3$).

2.4 RNA interference

Normal and hypertrophic scar fibroblasts were plated in 6-well plates (8×10^4 cells per well), cultured for 24 h, and transfected with Smurf2 siRNA (100 nM final concentration, Santa Cruz, CA, USA) using DharmaFECT™ (Dharmacon, Lafayette, CO, USA) according to the manufacturer's instruction. The target sequences of Smurf2 siRNA used in this study were sense 5'-CCACUUUGUUGGACGAAUAtt-3' and antisense 5'-UAUUCGUCCAACAAAGUGGtt-3'. One scrambled siRNA (Dharmacon, Lafayette, CO, USA) was used for a non-target control, and transfection medium without siRNA was used as control. After 72 h from siRNA transfection, cultures were treated with 10 ng/ml recombinant human TGF- β 1 (R&D Systems, USA) for 6 h. Efficiency of Smurf2 knockdown was assessed by Western blot and RT-PCR.

2.5 Measurement of TGF- β 1 levels by ELISA

Normal and hypertrophic scar fibroblasts were plated in 6-well plates (8×10^4 cells per well), and transfected with Smurf2 siRNA. The scrambled siRNA was used for a negative control. After 72 h from siRNA transfection, cell-free supernatants were collected, and cells were counted in duplicate using phase-contrast microscopy and a hemacytometer. The TGF- β 1 levels (active + latent forms of TGF- β 1) were measured in supernatants using a solid-phase ELISA with TGF- β 1 ELISA kits for humans (R&D Systems, USA), according to the manufacturer's instructions. The antibodies used in the ELISA kit are able to detect TGF- β 1 in its active form, but latent TGF- β 1 complex has approximately 15% cross-reactivity with this assay; thus, samples were activated by acidification (HCl) before ELISA to determine the amount of latent TGF- β 1 in the supernatants. Samples with a concentration exceeding the limits of the standard curve were repeated after dilution. Eight replicate wells were used to obtain all data points, and all samples were performed in duplicate and averaged.

2.6. Statistical analysis

Data were expressed as mean $\pm$ SEM. Statistical evaluation of the continuous data was performed by one-way analysis of variance, followed by Dunnett's t-test for between-group comparisons. The level of significance was considered to be $P < 0.05$.

3. Results

3.1 Increase in Smurf2 protein and mRNA in hypertrophic scar fibroblasts

Normal fibroblasts and hypertrophic scar fibroblasts were lysed and analyzed by Western blot. The relative expression level of Smurf2 was normalized to GAPDH in the same sample. As shown in Fig. 1, the expression of Smurf2 protein in hypertrophic scar fibroblasts was higher than that in normal fibroblasts ($P < 0.05$).

The expression of Smurf2 gene in normal fibroblasts and hypertrophic scar fibroblasts was monitored by semi-quantitative RT-PCR. The expression levels of Smurf2 mRNA were normalized to β -actin used as an internal control. As shown in Fig. 2, the expression of

Smurf2 mRNA in hypertrophic scar fibroblasts was higher than that in normal fibroblasts ($P < 0.05$).

3.2 Increase in Smurf2 protein and mRNA in normal and hypertrophic scar fibroblasts after TGF- β 1 stimulation

Normal fibroblasts and hypertrophic scar fibroblasts were treated with TGF- β 1 (10 ng/ml) for 5 min, 15 min, 30 min, 1 h, 2 h, and 12 h. Smurf2 protein level was examined by Western blot analysis. The relative expression level of Smurf2 was normalized to GAPDH in the same sample. As shown in Fig. 3, the expression of Smurf2 protein in normal and hypertrophic scar fibroblasts gradually increased in a time-dependent manner after TGF- β 1 stimulation.

Smurf2 mRNA level was examined by semi-quantitative RT-PCR. As shown in Fig. 4, the expression of Smurf2 mRNA gradually increased in a time-dependent manner after TGF- β 1 stimulation.

3.3 Silencing the expression of Smurf2 in hypertrophic scar fibroblasts reduced TGF- β 1 production

Normal and hypertrophic scar fibroblasts were transfected with Smurf2 siRNA for 72 h, and supernatants were then collected. The TGF- β 1 concentration in cell supernatant measured by ELISA was divided by the number of viable cells to yield graphs of TGF- β 1 concentration per cell (Fig. 5). TGF- β 1 concentration per cell in hypertrophic scar fibroblasts was higher than that in normal dermal fibroblasts without Smurf2 siRNA transfection ($P < 0.05$), and TGF- β 1 concentration per cell was significantly decreased in the siRNA Smurf2-transfected hypertrophic scar fibroblasts ($P < 0.05$). The result suggested that knocking down the expression of Smurf2 resulted in attenuation of the autocrine production of TGF- β 1 in hypertrophic scar fibroblasts.

3.4 Genetic silencing of Smurf2 inhibited TGF- β 1-induced α -SMA protein and mRNA expression in normal and hypertrophic scar fibroblasts

Normal and hypertrophic scar fibroblasts were transfected with Smurf2 siRNA for 72 h, and then treated with 10 ng/ml TGF- β 1 for 6 h. These cell lysates were subjected to Western blot analysis. The relative expression level of Smurf2 and α -SMA was normalized to GAPDH in the same sample. As shown in Fig. 6A, The Smurf2 protein expression significantly decreased in normal and hypertrophic scar fibroblasts after Smurf2 siRNA transfection for 72 h ($P < 0.05$), with or without TGF- β 1 stimulation. This suggested that the siRNA to Smurf2 was effective in decreasing Smurf2 protein levels in normal and hypertrophic scar fibroblasts.

As shown in Fig. 6B, the addition of TGF- β 1 to normal and hypertrophic scar fibroblasts resulted in a marked increase in the expression of α -SMA protein ($P < 0.05$), but upon genetic silencing of Smurf2 in normal and hypertrophic scar fibroblasts, TGF- β 1 was not able to increase the expression of α -SMA protein, and a significant decrease in the expression of α -SMA protein was observed in Smurf2 siRNA transfection group ($P < 0.05$), compared with control or non-target control group with TGF- β 1 stimulation.

Normal and hypertrophic scar fibroblasts were transfected with Smurf2 siRNA for 72 h, and then treated with 10 ng/ml TGF- β 1 for 6 h. These cell lysates were subjected to RT-PCR analysis. The relative expression level of Smurf2 and α -SMA mRNA was normalized to β -actin mRNA. As shown in Fig. 7A, The Smurf2 mRNA expression significantly decreased in normal and hypertrophic scar fibroblasts after Smurf2 siRNA transfection for 72 h ($P <$

0.05), with or without TGF- β 1 stimulation. This suggested that the siRNA to Smurf2 was effective in decreasing Smurf2 mRNA levels in normal and hypertrophic scar fibroblasts.

As shown in Fig. 7B, the addition of TGF- β 1 to normal and hypertrophic scar fibroblasts resulted in a marked increase in the expression of α -SMA mRNA ($P < 0.05$), but upon genetic silencing of Smurf2 in normal and hypertrophic scar fibroblasts, TGF- β 1 was not able to increase the expression of α -SMA mRNA, and a significant decrease in the expression of α -SMA mRNA was observed in Smurf2 siRNA transfection group ($P < 0.05$), compared with control or non-target control group with TGF- β 1 stimulation.

These results indicated that Genetic silencing of Smurf2 inhibited TGF- β 1-induced α -SMA protein and mRNA expression in normal and hypertrophic scar fibroblasts.

3.5 Genetic silencing of Smurf2 inhibited TGF- β 1-induced collagen I synthesis in normal and hypertrophic scar fibroblasts

Normal and hypertrophic scar fibroblasts were transfected with Smurf2 siRNA for 72 h, and then treated with 10 ng/ml TGF- β 1 for 6 h. These cell lysates were subjected to RT-PCR analysis. The relative expression level of Collagen I mRNA was normalized to β -actin mRNA. As shown in Fig. 8, the addition of TGF- β 1 to normal and hypertrophic scar fibroblasts resulted in a marked increase in the expression of Collagen I mRNA ($P < 0.05$), but upon genetic silencing of Smurf2 in normal and hypertrophic scar fibroblasts, TGF- β 1 was not able to increase the expression of Collagen I mRNA, and a significant decrease in the expression of α -SMA mRNA was observed in Smurf2 siRNA transfection group ($P < 0.05$), compared with control or non-target control group with TGF- β 1 stimulation. The results indicated that Genetic silencing of Smurf2 inhibited TGF- β 1-induced collagen I synthesis in normal and hypertrophic scar fibroblasts

4. Discussion

Results of wound healing research over the past decades have demonstrated that TGF- β 1 plays an essential role in hypertrophic scar formation. TGF- β 1 stimulated normal human dermal fibroblasts to synthesize collagen [3, 4]. Ghahary et al. [14] reported that TGF- β 1 mRNA was 61% greater in hypertrophic scar tissue than in normal skin. Tredget et al. [15] reported that hypertrophic scar tissues and fibroblasts produce more TGF- β 1 mRNA and protein than normal skin and cells. The *in vitro* cultured fibroblasts derived from the hypertrophic scar also expressed TGF- β 1 mRNA in a level significantly higher than that of the normal fibroblasts [16]. Intense immunostaining of TGF- β 1 had also been reported in hypertrophic scar [17]. The scar formation in adult rodent wounds could be inhibited by neutralizing wound TGF- β 1 with anti-TGF- β 1 antibody [18, 19]. Thus, TGF- β 1 is the driving force behind the excessive scar formation seen in hypertrophic scar.

TGF- β 1 signaling is transduced by intracellular mediators known as Smad [8]. Upon TGF- β 1 stimulation, Smad2 and Smad3 are phosphorylated at serine residues. Once phosphorylated, Smads 2/3 dissociate from the receptor, bind to Smad4, and then enter the nucleus where they activate target gene transcription, such as α -SMA expression and collagen I synthesis. Smad7 is an intracellular antagonist for TGF- β 1 signaling, and it is known to associate with activated T β RI and hinder the activation of R-Smads by preventing their interaction with activated T β RI and consequent phosphorylation [20, 21]. In the nucleus, activated Smad can interact with various general transcriptional co-activators such as p300, resulting in transcriptional activation. Alternatively, they form transcriptionally inactive complexes with co-repressors such as c-Ski (Sloan-Kettering Institute proto-oncogene), SnoN (ski-related novel gene N). Thus, the relative levels of Smad

transcriptional co-repressors present inside the cell could determine the ultimate outcome of a TGF- β 1 response [22, 23].

Smurf2 (Smad ubiquitination regulatory factor-2) is an E3 ubiquitin ligase that plays a pivotal role in regulating TGF- β 1 signaling via selectively targeting key components of the Smad pathway for degradation. The Smurf2 is of particular importance to TGF- β 1 signaling [24], as Smads also function as adapters that recruit the Smurf2 to various pathway components including the TGF- β 1 receptor complex and the transcriptional repressor, SnoN, and thereby regulate the degradation of these Smad-associating proteins. Thus, by controlling the level of positive and negative regulators of the pathway, Smurf2 provide for complex and fine control of signaling output [25]. Recent studies have demonstrated that Smurf2 is involved as E3 ubiquitin ligases for Smad7 [26, 27] and transcriptional corepressor SnoN [28] that targets them for ubiquitination and degradation.

It was reported that the Smurf2-mediated inhibitory effect on TGF- β 1 signaling was impaired in scleroderma fibroblasts [29, 30]. Gene expression profiling analyzed by microarray revealed that Smurf2 was abnormally expressed during the process of rat liver fibrosis [10] and adult lung fibrosis [11]. Taken together, it seems conceivable to consider that the role of Smurf2 on the TGF- β 1 signaling may be modified in disease processes such as fibrosis. Recent studies have shown that Smurf2 expression was increased in renal fibrosis, and the reduction of inhibitory Smad7 [12] and transcriptional co-repressors SnoN/Ski proteins [13, 31] resulting from increased Smurf2 ubiquitination degradation is involved in the progression of renal fibrosis. However, little is known about the expression of Smurf2 in hypertrophic scarring. In this paper we reported that the expression of Smurf2 protein and mRNA in hypertrophic scar fibroblasts was higher than that in normal fibroblasts, and the expression of Smurf2 protein and mRNA in normal and hypertrophic scar fibroblasts gradually increased in a time-dependent manner after TGF- β 1 stimulation.

Schmid et al. [32] found that more TGF- β 1 receptors I and II were expressed in the granulation tissues than in the normal skin at both mRNA and protein levels. However, the expression levels decreased gradually in the normal healing excisional wounds during the granulation tissue remodeling. By contrast, the receptor expression in the hypertrophic scar maintained at high levels up to 20 months post-injury, suggesting that a persistent autocrine loop of TGF- β 1 may exist and contribute to hypertrophic scar formation. Recently, hypertrophic scar fibroblasts persistence is thought to occur as a consequence of the autocrine production and activation of TGF- β 1 [1, 33]. In this study we transfected Smurf2 siRNA into hypertrophic scar fibroblasts, and found that Smurf2 genetic silencing decreased the per-cell concentration of TGF- β 1 in hypertrophic scar fibroblasts. Our result suggested that Smurf2 was an essential element in the mechanism driving autocrine TGF- β 1 production, and knocking down the expression of Smurf2 resulted in disruption of TGF- β 1-dependent autocrine loop. It could be speculated therefore that Smurf2 genetic silencing may lead to improved wound healing by reducing the level of autocrine TGF- β 1 production, bringing TGF- β 1 production somewhat closer to that present in normal fibroblasts.

Cutaneous wound healing involves a complex phenotypic modulation of the fibroblast compartment, which results in an accumulation of myofibroblasts at the wound site [34, 35]. It has been shown that the number of myofibroblasts successively diminishes during normal granulation tissue remodeling but that this cell population persists at high densities in hypertrophic scars [36]. In hypertrophic scars, myofibroblasts were distributed throughout the fibrotic tissue [32]. Hypertrophic scar fibroblasts persistence is thought to occur as a consequence of the autocrine production and activation of TGF- β 1 [1, 33]. α -SMA has been known to be a specific marker protein in myofibroblast differentiation. Hypertrophic scar fibroblasts were constitutively myofibroblastic, expressing α -SMA persistently, and TGF-

β 1 is important in the transition from fibroblast to myofibroblast [37, 38]. α -SMA was upregulated in normal fibroblasts to levels comparable to hypertrophic scar fibroblasts control with addition of TGF- β 1 [39]. As increased expression of TGF- β 1 directly induces α -SMA expression [5], In the present study, our results showed that the expressions of α -SMA protein and mRNA were increased after TGF- β 1 stimulation, and Smurf2 genetic silencing prevented the increases in α -SMA expression that were observed with TGF- β 1 stimulation in hypertrophic scar fibroblasts. The results suggested that Smurf2 was required to express α -SMA in hypertrophic scar fibroblasts after TGF- β 1 stimulation.

It was reported that scar fibroblast selectively increased the biosynthesis of type I collagen in the process of wounding healing [40, 41]. TGF- β 1 stimulates the expression of collagen gene in fibroblasts [42, 43, 44]. In the present study, our results showed that the synthesis of collagen I was increased after TGF- β 1 stimulation, and Smurf2 genetic silencing inhibited the increase in collagen I synthesis that was observed with TGF- β 1 stimulation in hypertrophic scar fibroblasts. The results suggested that Smurf2 was required to synthesize collagen I in hypertrophic scar fibroblasts after TGF- β 1 stimulation.

In summary, we showed that hypertrophic scar fibroblasts exhibited increased Smurf2 protein and mRNA levels compared with normal fibroblasts, and Smurf2 was upregulated by TGF- β 1 and, in turn, was required for production of TGF- β 1 in hypertrophic scar fibroblasts; Genetic silencing with Smurf2 in hypertrophic scar fibroblasts dramatically reduced the autocrine production of TGF- β 1, inhibited TGF- β 1-induced α -SMA expression and inhibited TGF- β 1-induced collagen I synthesis. These results suggest that the enhanced expression of Smurf2 is involved in the progression of hypertrophic scarring. Alteration of the concentration of TGF- β 1 in hypertrophic scarring, by Smurf2 genetic silencing, could possibly modify the wound environment and convert it from one of excessive scarring to one in which the normal processes of extracellular matrix accumulation during repair terminates appropriately. It could be a new, promising therapeutic approach for improving skin wound healing and inhibiting progression of fibrotic conditions by disrupting TGF- β 1-dependent autocrine loop.

Acknowledgments

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Fig. 1. The expression of Smurf2 protein in normal and hypertrophic scar fibroblasts (Abbreviation: Fb, Fibroblasts). Normal fibroblasts and hypertrophic scar fibroblasts were lysed and analyzed by Western blot. The relative expression level of Smurf2 was normalized to GAPDH in the same sample. Three pairs of normal fibroblasts and hypertrophic scar fibroblasts were examined and data are shown as means $\pm$ SEM (n = 3) of three experiments performed in triplicate on separate occasions. * $P < 0.05$ as compared with normal fibroblasts. The expression of smurf2 protein in hypertrophic scar fibroblasts was higher than that in normal fibroblasts.

Fig. 2. The expression of Smurf2 mRNA in normal and hypertrophic scar fibroblasts (Abbreviation: Fb, Fibroblasts). The expression of Smurf2 gene in normal fibroblasts and hypertrophic scar fibroblasts was monitored by semi-quantitative RT-PCR. The expression levels of Smurf2 mRNA were normalized to β-actin used as an internal control. The mRNA values are expressed as relative units calculated according to the following formula: density of the Smurf2 / density of the β-actin. Three pairs of normal fibroblasts and hypertrophic scar fibroblasts were examined and data are shown as means ± SEM (n = 3) of three experiments performed in triplicate on separate occasions. * P < 0.05 as compared with normal fibroblasts. The expression of Smurf2 mRNA in hypertrophic scar fibroblasts was higher than that in normal fibroblasts.

Fig. 3. The expression of Smurf2 protein in normal and hypertrophic scar fibroblasts after TGF-β1 stimulation

Normal fibroblasts and hypertrophic scar fibroblasts were treated with TGF-β1 (10 ng/ml) for 5 min, 15 min, 30 min, 1 h, 2 h, and 12 h. Smurf2 protein level was examined by Western blot analysis. The relative expression level of Smurf2 was normalized to GAPDH in the same sample. Data are presented as means of results from three experiments each performed in duplicate (n = 3). * P < 0.05 as compared with control. The expression of smurf2 protein in normal and hypertrophic scar fibroblasts gradually increased in a time-dependent manner after TGF-β1 stimulation.

Fig. 4. The expression of Smurf2 mRNA in normal and hypertrophic scar fibroblasts after TGF-β1 stimulation

Normal fibroblasts and hypertrophic scar fibroblasts were treated with TGF-β1 (10 ng/ml) for 5 min, 15 min, 30 min, 1 h, 2 h, and 12 h. Smurf2 mRNA level was examined by RT-PCR. The relative expression level of Smurf2 mRNA was normalized to β-actin in the same sample. Data are presented as means of results from three experiments each performed in duplicate (n = 3). * P < 0.05 as compared with control. The expression of smurf2 mRNA gradually increased in a time-dependent manner after TGF-β1 stimulation.

Fig. 5. The TGF- β 1 concentration per cell in normal dermal fibroblasts (NDFb) and hypertrophic scar fibroblasts (HSFb) with Smurf2 siRNA transfection

Normal and hypertrophic scar fibroblasts were transfected with Smurf2 siRNA for 72 h, and supernatants were then collected. The TGF- β 1 concentration in cell supernatant was measured by ELISA. Data were represented mean $\pm$ SEM (n = 8). Significance by * $P < 0.05$ with respect to fibroblast without Smurf2 siRNA transfection; # $P < 0.05$, significant difference between hypertrophic scar fibroblasts and normal dermal fibroblasts. TGF- β 1 concentration per cell in hypertrophic scar fibroblasts was higher than that in normal dermal fibroblasts without Smurf2 siRNA transfection, and silencing the expression of Smurf2 in hypertrophic scar fibroblasts reduced TGF- β 1 production.

Fig. 6. Smurf2 siRNA blocked TGF-β₁-induced α-SMA expression in normal and hypertrophic scar fibroblasts
 (Abbreviation: C, Control; NTC, Non-target Control). Normal and hypertrophic scar fibroblasts were transfected with Smurf2 siRNA for 72 h, and treated with 10 ng/ml TGF-β₁ for 6 h. These cell lysates were subjected to Western blot analysis. The relative expression level of Smurf2 and α-SMA was normalized to GAPDH in the same sample. Data are presented as means of results from three experiments each performed in duplicate (n = 3). * P < 0.05 as compared with control; # P < 0.05, significant difference between before and after TGF-β₁ stimulation. (A) The Smurf2 protein expression decreased in normal and hypertrophic scar fibroblasts after Smurf2 siRNA transfection for 72 h, with or without TGF-β₁ stimulation. (B) The α-SMA expression increased after TGF-β₁ stimulation in Control and Non-target Control group, but in Smurf2 siRNA transfection group, TGF-β₁ was not able to increase the expression of α-SMA.

Fig. 7. Smurf2 siRNA blocked TGF-β₁-induced α-SMA mRNA expression in normal and hypertrophic scar fibroblasts

(Abbreviation: C, Control; NTC, Non-target Control). Normal and hypertrophic scar fibroblasts were transfected with Smurf2 siRNA for 72 h, and treated with 10 ng/ml TGF-β₁ for 6 h. These cell lysates were subjected to RT-PCR analysis. The relative expression level of Smurf2 and α-SMA mRNA was normalized to β-actin mRNA in the same sample. Data are presented as means of results from three experiments each performed in duplicate (n = 3). * P < 0.05 as compared with control; # P < 0.05, significant difference between before and after TGF-β₁ stimulation. (A) The Smurf2 mRNA expression decreased in normal and hypertrophic scar fibroblasts after Smurf2 siRNA transfection for 72 h, with or without TGF-β₁ stimulation. (B) The α-SMA mRNA expression increased after TGF-β₁ stimulation in Control and Non-target Control group, but in Smurf2 siRNA transfection group, TGF-β₁ was not able to increase the expression of α-SMA mRNA.

Fig. 8. Smurf2 siRNA blocked TGF-β1-induced Collagen I mRNA expression in normal and hypertrophic scar fibroblasts
(Abbreviation: C, Control; NTC, Non-target Control). Normal and hypertrophic scar fibroblasts were transfected with Smurf2 siRNA for 72 h, and treated with 10 ng/ml TGF-β1 for 6 h. These cell lysates were subjected to RT-PCR analysis. The relative expression level of Collagen I mRNA was normalized to β-actin mRNA in the same sample. Data are presented as means of results from three experiments each performed in duplicate (n = 3). * P < 0.05 as compared with control; # P < 0.05, significant difference between before and after TGF-β1 stimulation. The Collagen I mRNA expression increased after TGF-β1 stimulation in Control and Non-target Control group, but in Smurf2 siRNA transfection group, TGF-β1 was not able to increase the expression of Collagen I mRNA.