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Original Research

High temperature requirement factor A1 (HTRA1) regulates the activation of latent TGF-β1 in keloid fibroblasts

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Abstract: Scar treatments are considered a major issue in the plastic surgery field. Activation of the transforming growth factor-β (TGF-β)-mediated signaling pathway plays a key role in the scar pathogeneses, and high temperature requirement factor A1 (HTRA1) inhibits TGF-β1 activation in tumor cells. Our study aims to investigate the role of HTRA1 in the pathogenesis of scars. The mRNA levels of HTRA1 was evaluated by real time PCR, HTRA1 protein expression was determined using western blot and immunohistochemistry, and a luciferase assay was applied to measure dynamic changes of TGF-β1 activity. We found that the expression of HTRA1 was significantly elevated in keloid tissues, compared to normal skin, and TGF-β1 mRNA levels slightly increase in the keloid tissue. Furthermore, active TGF-β1 protein levels and Smad2 phosphorylation significantly increased in the keloid tissue. Treatment with the latent TGF-β1 or recombinant human HTRA1 (rhHTRA1), alone or in combination, increased Smad2 phosphorylation levels in keloid fibroblasts and active TGF-β1 contents of associated supernatants. Our results suggest that HTRA1 is involved in the pathogenesis of scars through regulating activation of latent TGF-β1 in keloid fibroblasts, and our study reveals that HTRA1 is a novel target that regulates scar formation.

Key words: Pathological scar; HTRA1; TGF-β; Smad2.

Introduction

Pathological scars, which are products of excessive wound healing, are classified into keloids and hypertrophic scars and are considered an abnormal hyperplastic skin fiber disease that results from the excessive deposition of extracellular matrix, particularly type I and III collagen (1, 2). Patients with cutaneous scars suffer from deformed appearance and contracted neogenetic tissue, which lead to both physiological and psychological burdens (3). Therefore, it is crucial to elucidate the pathogenesis of the pathological scars and develop potential therapeutic interventions for patients with scar tissue.

It has been shown that transforming growth factor-β1 (TGF-β1) is involved in scar formation. TGF-β1 activates the expression of multiple fibrogenic genes, including type I and III collagen, connective tissue growth factor, and fibronectin (4-6). Upon binding to the TGF-β1 receptor, the TGF-β1/TGF-β1 receptor complex triggers several signaling cascades, including the mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinases (ERK), and Jun N-terminal kinase (JNK) pathways (6). Smad2 is an intracellular protein that is transduces extracellular signals from TGF-β to the nucleus and subsequently activates downstream gene transcription. TGF-β/Smad2 signaling has been shown to play critical roles in a wide range of biological activities, such as cell growth, differentiation, extracellular matrix (ECM) production, and apoptosis (7,8). Thus, it is critical to control the expression level of TGF-β1 during the reparation of pathological scars.

High temperature requirement factor A1 (HTRA1), a member of the HTRA protein family, consists of a trypsin-like serine protease domain, a PDZ domain, an IGFBP/mac25-like domain, and a kazal-type inhibitor domain (9, 10). Importantly, it has been shown that HTRA1 is involved in the activation of TGF-β1 signaling (11, 12). However, the interaction between HTRA1 and TGF-β1 and the roles of these proteins in scar formation have not been elucidated. Thus, our study aims to explore the role of HTRA1 in the scar pathogenesis.

Materials and Methods

Samples

This study was approved by the Ethics Committee of the First Hospital of China Medical University. Informed consent was obtained from included patients. A total of 26 matched keloid and normal skin tissue samples were collected.

Cell culture

Subcutaneous tissue was removed and samples were incised into small pieces, after which they were digested with type I collagenase at 37 °C for 4-6h prior to centrifugation. Next, fibroblasts were collected and seeded into cell culture dishes in an incubator with 5% CO_2 at 37°C. When cell confluence was greater than 90%, cells were trypsinized and passaged. After three or four passages, fibroblasts were used for experiments.

Real time PCR

Total RNA was extracted and reversely transcribed

into cDNA using a cDNA synthesis kit (Stratagene), according manufacturer's recommendations. The PCR reaction was performed on the ABI 7300 Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA), and the amplification program consisted of an initial denaturation at 94°C for 5 seconds, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 40 seconds at 72°C, and a final extension at 72°C for 10 minutes. The relative expression of each gene was calculated using 2(-ΔΔCt).

Western blot

Tissues or fibroblasts were lysed with lysis buffer and protein concentrations were determined using a BCA protein assay kit (Preice, NJ, USA). Protein samples were loaded onto a 10% SDS-PAGE gel and transferred onto a PVDF membrane. Thereafter, membranes were blocked with 5% non-fat milk in PBS for 2 h at room temperature. The membrane was then incubated with primary antibody overnight, at 4°C, washed three times in TBST, and incubated with horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. Protein bands were detected using an enhanced chemiluminescence kit (ECL, Amersham), and band density was quantified using Quantity-One software (Bio-Rad Laboratories).

Immunohistochemistry

4-mm paraffin sections were deparaffinized, rehydrated, and heated in a microwave oven. Sections were incubated with the primary antibody at 37°C for 60 min, and then with secondary antibodies at 37°C for 30 min. After DAB coloration, images of cells were viewed and captured (200x) on an Olympus microscope.

Luciferase assay

For determination of TGF-β1 concentration, the human PAI-1 promoter sequence was cloned and inserted into the GL4-Luc plasmid, and recombinant plasmids were transfected into HEK293T cells. After 5h, luciferase activity was determined using the Promega Dual Luciferase System, according to manufacture's instruction.

Statistical analysis

Data are presented as mean ± SD and analyzed by SPSS 18.0 (SPSS Inc., Chicago, IL, USA). Comparisons between groups were analyzed using t-tests or ANOVA. p-values less than 0.05 were considered statistically significant.

Results

Expression of HTRA1 and TGF-β1 in keloid tissue and normal skin

First, we collected 26 keloid tissues samples, with matched normal skin controls, and examined the expression of HTRA1 and TGF-β1 by real time PCR and western blot. Compared with normal tissue, the expression of HTRA1 was significantly elevated in keloid tissues, at both mRNA and protein levels (Fig. 1A and C). With regards to TGF-β1, we found that TGF-β1 transcripts exhibited a slight increase in keloid tissue compared with normal skin (Fig. 1B), and active TGF-β1

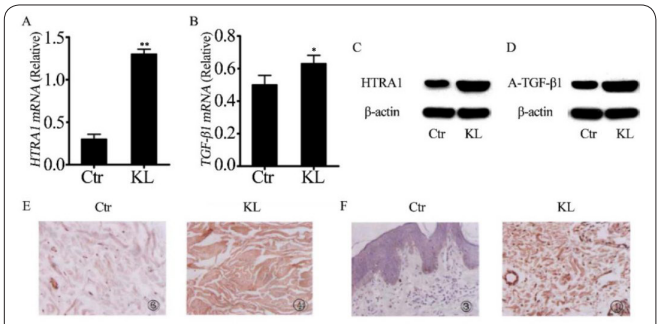

Figure 1. Expression of HTRA1 and TGF-β1 in keloid tissue and normal skin. The expression levels of HTRA1 in keloid tissue and normal skin were determined by real time PCR (A), western blot (C), and immunohistochemistry (E and F). In addition, TGF-β1 mRNA and active TGF-β1 protein levels were measured by real time PCR (B) and western blot (D), respectively. * P<0.05, ** P<0.01.

protein levels were significantly increased in keloid tissue, compared with normal skin (Fig. 1D). Additionally, immunohistochemical analysis revealed that HTRA1 was substantially elevated in keloid tissue compared with normal tissue (Fig. 1E and F), and taken together, these results suggest a potential link between HTRA1 and TGF-β1 in keloid tissue.

TGF-β1 activates Smad2 in the keloid tissue

To explore the status of the TGF-β1 signaling pathway, we examined phosphorylation levels of Smad2 in the keloid tissue. Western blot analysis revealed that Smad2 phosphorylation was significantly elevated in keloid tissue compared with the normal tissue (Fig. 2A and B), which suggests that elevated levels of active TGF-β1 enhances Smad2 signaling in keloid tissue.

HTRA1 is involved in the activation of latent TGF-β1

Active TGF-β1 can specifically bind to the promoter of human plasminogen activator inhibitor-1 (PAI-1) and activate transcription. Thus, we inserted the PAI-1 promoter sequence into a luciferase reporter plasmid and investigated dynamic changes in TGF-β1 activity (Fig. 3A). After transfection into HEK293T cells, we found that active TGF-β1 specifically triggered PAI-1 promoter-mediated luciferase expression (Fig. 3B). On the contrary, latent TGF-β1 failed to activate luciferase expression (Fig. 3B).

Keloid fibroblasts were next isolated and treated with latent TGF-β1 and recombinant human HTRA1

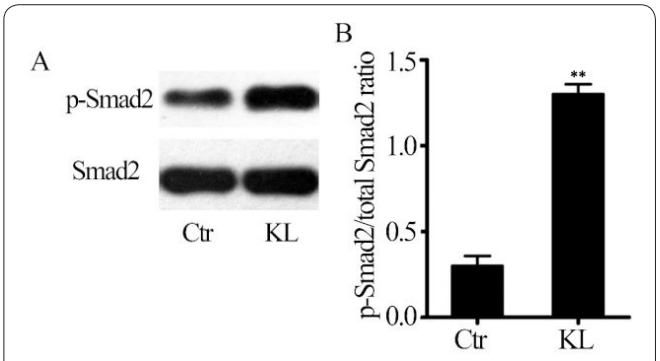

Figure 2. TGF-β1 up-regulates Smad2 in keloid tissue. Western blot was applied to detect the phosphorylation level of Smad2 and total Smad levels in keloid tissue and normal skin (A). Ratio of p-Smad2/total Smad2 (B). * P<0.05, ** P<0.01.

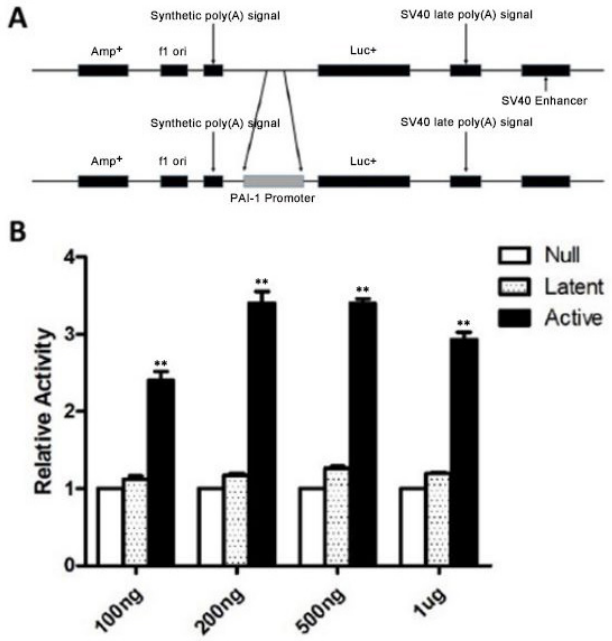

Figure 3. Active TGF-β1 induces PAI-1-mediated luciferase gene expression. (A) Illustration luciferase reporter vector construction. (B) Recombinant plasmids were transfected into HEK293T cells, and luciferase activity was determined in cells exposed to different concentrations of the active TGF-β1 (10 ng, 200 ng, 500 ng, 1 ug). * P<0.05, ** P<0.01.

(rhHTRA1), alone or in combination. After 24h, we performed western blot and luciferase reporter analyses to detect active TGF-β1 and Smad2 phosphorylation levels. We found that the both Smad2 phosphorylation and active TGF-β1 levels were significantly elevated in the fibroblasts upon exposure to rhHTRA1 or latent TGF-β1 alone (Fig. 4A and B). Additionally, co-treatment of fibroblasts with rhHTRA1 and latent TGF-β1 further enhanced Smad2 phosphorylation and active TGF-β1 production (Fig. 4A and B). These findings suggest that HTRA1 is involved in the activation of extracellular TGF-β1 in fibroblasts.

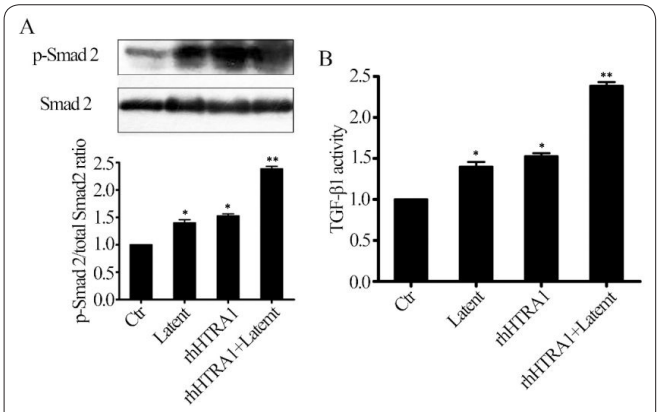

Figure 4. HTRA1 regulates the activation of latent TGF-β1 in fibroblasts. Fibroblasts were treated with latent TGF-β1 and rhHTRA1 alone or in combination. The phosphorylation level of Smad2 and the total amount of Smad2 in fibroblasts were measured by western blot (A, upper panel). Ratio of p-Smad2/total Smad2 (A, lower panel). The luciferase reporter assay was applied to detect levels of active TGF-β1 in fibroblasts treated with latent TGF-β1 and rhHTRA1 alone or in combination (B). * P<0.05, ** P<0.01.

Discussion

Scars lead to a deformed appearance and result in physiological and psychological problems for patients (13). While the pathogenesis of scars has not been fully elucidated, the present study demonstrates that HTRA1 is involved in scar formation through the regulation of the latent TGF-β1 levels.

TGF-β1 is a multi-functional cytokine that is involved in the regulation of collagen metabolism, fibroblast growth, and extracellular matrix deposition (14, 15). Regulation of TGF-β1/Smad signaling and TGF-β1 expression is critical for controlling the wound scarring process. HTRA1 (also called PRSS11) is a member of the mammalian HTRA serine protease family and contains a highly conserved protease domain (9, 10). There is increasing evidence that HTRA1 plays an important role in regulating various physiological and pathological processes, including tumor progression, Alzheimer's disease, skeletal development, and osteoarthritis (16-18). For example, a single nucleotide polymorphism in the HTRA1 promoter is associated with the age-related macular degeneration (19). Although several HTRA1 substrates have been identified, including biglycan, aggrecan, fibronectin, fibromodulin, decorin, and TGF-β (20, 21), the relationship between HTRA1 and TGF-β in scar pathogenesis has not been investigated. In our study, we found that HTRA1 levels were significantly elevated in keloid tissues, compared to normal skin. Moreover, TGF-β1 mRNA and active TGF-β1 protein levels were slightly and significantly increased, respectively, in keloid tissue. Collectively, our findings suggest a potential link between HTRA1 and TGF-β1 in the keloid tissue.

As a secreted protein, HTRA1 has been shown to bind to TGF-β family members, including TGFβ1, growth differentiation factor 5, and bone morphogenetic protein 4 (22,23). Particularly, the interaction between HTRA1 and these factors alters the TGF-β/Smad signaling pathway (24). Our study found that the phosphorylation level of Smad2 was significantly elevated in keloid tissue, which suggests that TGF-β1 activates Smad2 signaling.

TGF-β is the strongest inducer of PAI-1 expression in multiple cell types (15, 25, 26), and therefore we inserted the PAI-1 promoter sequence into a luciferase reporter vector to investigate dynamic changes in TGF-β1 activity. Using this system, we found that active TGF-β1, but not latent TGF-β1, specifically induces PAI-1-mediated luciferase expression. Our data shows that this luciferase reporter system can be applied to detect active TGF-β1 activity, and we subsequently made use of this platform to investigate the treatment of keloid fibroblasts with latent TGF-β1 and rhHTRA1, alone or in combination, for 24h. We found that treatment with latent TGF-β1 and rhHTRA1, alone and in combination, increased Smad2 phosphorylation and active TGF-β1 levels in fibroblasts, which suggests that HTRA1 is involved in latent TGF-β1 activation in fibroblasts.

Our study found that HTRA1 expression is elevated in keloid tissue and stimulates the activation of TGF-β1 in fibroblasts. Our study suggests that HTRA1 could serve as a novel target for controlling scar formation.

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Competing interests
None.

Ethics approval and consent to participate
This study was approved by the Ethics Committee of the First Hospital of China Medical University. Informed consent was obtained from all patients.

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