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Permalink
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Journal
Archives of Dermatological Research, 311(10)

ISSN
0340-3696

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Publication Date
2019-12-01

DOI
10.1007/s00403-019-01975-0

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Archives of Dermatological Research
https://doi.org/10.1007/s00403-019-01975-0

ORIGINAL PAPER

Botulinum toxin type A suppresses pro-fibrotic effects via the JNK signaling pathway in hypertrophic scar fibroblasts

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Received: 20 January 2019 / Revised: 1 July 2019 / Accepted: 30 August 2019
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Abstract

Hypertrophic scar is a dermal fibroproliferative disease characterized by the overproduction and deposition of extracellular matrix, and the hyperproliferation and enhanced angiogenesis of fibroblasts, along with their enhanced differentiation to myofibroblasts. Botulinum toxin type A shows potential for prevention of hypertrophic scar formation; however, its effectiveness in attenuating skin fibrosis and the related mechanism are unclear. In this study, human scar fibroblasts were cultured and stimulated with botulinum toxin type A, and the changes in fibroblast proliferation, migration, and protein expression of pro-fibrotic factors were evaluated with colorimetric, scratch, and enzyme-linked immunosorbent assays and western blotting, respectively. Botulinum toxin type A treatment decreased the proliferation and migration of human scar fibroblasts compared with those of untreated controls. Protein expression levels of pro-fibrotic factors (transforming growth factor β1, interleukin-6, and connective tissue growth factor) were also inhibited by botulinum toxin type A, whereas the JNK phosphorylation level was increased. Activation of the JNK pathway demonstrated the inhibitory effects of the toxin on human scar fibroblast proliferation and production of pro-fibrotic factors, suggesting that the suppressive effects of botulinum toxin type A are closely associated with JNK phosphorylation. Overall, this study showed that botulinum toxin type A has a suppressive effect on extracellular matrix production and scar-related factors in human scar fibroblasts in vitro, and that regulation of JNK signaling plays an important role in this process. Our results provide a theoretical basis, at the cellular level, for the therapeutic use of botulinum toxin type A.

Keywords Botulinum toxin type A · Fibroblasts · JNK signaling · Hypertrophic scar

Introduction

Hypertrophic scars (HSs) result from a fibroproliferative disorder that is clinically characterized by elevated, erythematous, inelastic scarring with relatively slow and incomplete regression compared with normal scars [23]. Unlike an HS, a keloid scar projects beyond the original wound margin and does not spontaneously regress [9]. Histologically, HSs are mostly composed of type III collagen fibers that are oriented parallel to the epidermal surface [9], and are thus characterized by excessive collagen deposition with varying numbers of fibroblasts. The pathogenesis of HS is associated with excessive fibroblast proliferation and overproduction of extracellular matrix (ECM), especially collagen [19]. Transforming growth factor β1 (TGF-β1) is considered to play a role in the molecular mechanism of fibrotic disease and hypertrophic scarring by participating in cell growth regulation, differentiation, adhesion, and apoptosis [2], and also by stimulating skin fibroblasts to promote ECM production.

Gil Soon Park and Min Kyun An contributed equally to this work.

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Published online: 09 September 2019

The wound tension that results in pathological scar formation by enhancing fibroblast migration and reducing apoptosis can potentially be suppressed by botulinum toxin (BTX), a potent neurotoxin from *Clostridium botulinum*, which indirectly blocks neuromuscular transmission [12]. Indeed, BTX injection for traumatic and iatrogenic wounds of the face or neck can reduce mechanical distortion to induce chemoimmobilization and reduce inflammation during the wound healing phase. The major effects of BTX include inhibition of muscle contraction and chronic inflammatory stimuli, which consequently reduces scar formation [6–8, 28]. Moreover, a recent study showed that BTX injections inhibited the formation of HSs and collagen fibrils in a rabbit ear model [20].

Despite several reports that BTX can inhibit HS formation, the detailed molecular mechanism remains poorly understood, as several cytokines and growth factors are involved in the wound healing and HS formation processes. Early research on the molecular mechanism of BTX focused on TGF-β1. BTX treatment of HS-derived fibroblasts could reduce the proliferation of the cells along with the expression level of TGF-β1 [30]. And the expression level of connective tissue growth factor (CTGF) is decreased in HSs, with consequent inhibition of the differentiation of fibroblasts originated from scar tissues [14, 31].

During wound healing, increased TGF-β1 improves tissue regeneration, and a persistent increase in TGF-β1 activates several intracellular signals such as Smads and the mitogen-activated protein kinase (MAPK) pathway [5, 24]. BTX treatment contributes to the inhibition of capsule formation through the TGF-β/Smad signaling pathway [15]. BTX has also been reported to regulate the intracellular levels of ECM proteins through the MAPK pathway and thus inhibit the formation of HS by inhibiting or activating this pathway [4, 10, 18, 22]. However, the specific role of the MAPK pathway members in BTX-induced pro-fibrotic factor expression has not been determined.

Accordingly, in this study, we investigated the inhibitory effect of BTX on HS-derived fibroblasts (HSFs) in vitro, and explored the possible associated molecular mechanism by examining the influence of BTX on cell proliferation, cell migration, protein expression of scar-related factors, and intracellular signaling.

Materials and methods

Patients and ethical approval

HS tissues were obtained from 11 patients who underwent surgical scar revision at Hallym University Medical Center (Republic of Korea). All patients had confirmed HSs with induration and erythema, the tissues were obtained at least 1 year after the initial trauma or surgery, and the site was confirmed to show slow or incomplete regression. We excluded keloid scars whose boundaries exceeded the original wound margin. All patients provided written informed consent for inclusion of their tissues in this study before the surgery. The study was approved by the Ethics Committee of Hallym University Medical Center (IRB No: 2016I120) and was performed in accordance with the guidelines of the Declaration of Helsinki.

Primary cell culture

Human-derived HSFs were prepared according to a previously reported standard procedure [27]. Briefly, the HS tissues from patients were cut into 4-mm sections, and incubated in high-glucose Dulbecco's modified Eagle medium (DMEM; GIBCO, Grand Island, NY, USA) supplemented with 20% fetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin to favor the growth of fibroblasts over keratinocytes; fibroblasts appeared 7–10 days after the first outgrowth of keratinocytes. The obtained HSFs were maintained in DMEM supplemented with 10% FBS at 37 °C in 5% (v/v) CO_2. Only cells at passage 3–6 were used in subsequent experiments. To reduce the influence of individual variation, human HSFs were prepared from the tissues of 11 patients, and 3 cell samples were randomly selected for analysis (designated HSF4, HSF5, and HSF6).

Cell proliferation and viability assay

To investigate the influence of BTX on cell proliferation, the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) uptake assay was performed. HSFs were seeded and starved upon reaching 70–80% confluence for 24 h in DMEM with 0.5% FBS. The cells were then left untreated (control) or treated with 8 units/mL of BTX (ALLERGAN, Irvine, CA, USA) for 24 h or 48 h at 37 °C, after which 20 µL of MTS reagent was added. Cell proliferation was evaluated based on the absorbance at 490 nm detected using a 96-well plate reader (Dynex Revelation, Dynex Ltd., Billingshurst, UK). For cell viability evaluation, trypan blue staining (GIBCO) was conducted according to the manufacturer's instructions.

Cell migration assay

An in vitro scratch wound assay was performed to evaluate cell migration. The cells were seeded and attached onto culture inserts contained in 35-mm µ-Dishes (Ibidi GMBH, Martinsried, Germany) with DMEM supplemented with 10% FBS until they reached 100% confluence. After starvation with DMEM supplemented with 0.5% FBS for 24 h, a scratch was made in the middle of the

insert. A defined 500-μm cell-free gap was created, with no leakage during cultivation and no material left behind. The cell patches were then overlaid with BTX-containing starvation medium for 0, 12, 24, and 48 h. Digital photographs of scratched areas were captured using a light microscope (Olympus CKX41, Olympus, Tokyo, Japan).

Enzyme-linked immunosorbent assay (ELISA) of pro-fibrotic protein expression

The HSF4 cells (4×10^5) were seeded in 60-mm cell culture plates (SPL Life Science, Yeoju, Republic of Korea) in DMEM supplemented with 10% FBS. After starving the cells with DMEM containing 0.5% FBS for 24 h, they were treated with 8 units/mL of BTX for 48 h. Thereafter, the culture supernatant was collected for the ELISA. The cells were homogenized and stored overnight at − 20 °C. After two freeze–thaw cycles to break the cell membranes, the homogenates were centrifuged for 5 min at 5000×*g* at 2–8 °C. The supernatant was assayed using the human CTGF ELISA Kit (CUSABIO Biotech, Wuhan, Hubei, China). The production of soluble human IL-6 and TGF-β1 was tested by ELISA using a commercial kit (Quantikine; R&D Systems, Minneapolis, MN, USA). Human pro-collagen I α1/COLA1 was also measured by ELISA (R&D DuoSet Kit) according to the manufacturer's instructions. Measurements were obtained in triplicates.

Western blotting

To investigate the mechanism of pro-fibrotic factor suppression by BTX, the cells from sample HSF4 were pretreated for 1 h with 2.5 μM of the c-Jun N-terminal kinase (JNK) inhibitor SP600124 (Calbiochem, San Diego, CA, USA). The cells were lysed in boiling sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer [62.5 mmol/L Tris (pH 6.8), 1% SDS, 10% glycerol, and 5% β-mercaptoethanol]. The cellular lysates were boiled for 5 min, separated by SDS-PAGE, and transferred onto an Immobilon membrane (Millipore, Merck KGaA, Darmstadt, Germany). The blocked membranes were incubated at room temperature for 2 h with antibodies specific for phosphorylated JNK (p-JNK) or total JNK (Cell Signaling, Danvers, MA, USA). The membranes were then washed three times with Tris-buffered saline with Tween 20 and incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies. Visualization of protein bands was performed using ECL (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA).

Statistical analyses

All data were analyzed using the GraphPad Prism 5 package (GraphPad Software Inc., La Jolla, CA, USA). The statistical analyses were performed by two-way ANOVA followed by a Bonferroni post hoc test to compare values among groups and by paired Student's *t* tests. The results with P values < 0.05 were considered statistically significant, and the data are presented as mean ± standard error of the mean.

Results

BTX decreases HSF proliferation

As shown in Fig. 1, cell proliferation significantly decreased after 48 h of BTX treatment compared with that of the

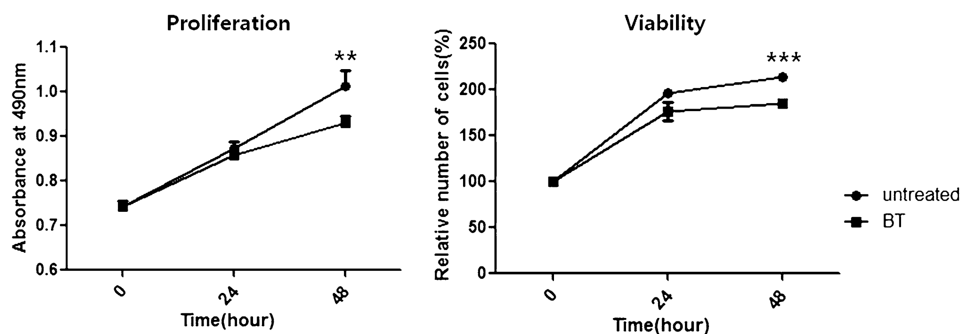

Fig. 1 Effect of botulinum toxin type A (BTX) on proliferation and viability of cultured human hypertrophic scar fibroblasts. The cells were seeded at a density of 5×10^3 cells/well in 96-well plates. After serum-starvation for 24 h, the proliferation of cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay at 24 or 48 h after the addition of BTX to the culture medium. The relative number of cells after BTX treatment compared with untreated ones is shown. Circles represent untreated cells and squares represent cells treated with 8 units/mL BTX. The significant difference in cell number between untreated fibroblasts and those treated with 8 units/mL of BTX is indicated by asterisks; $^{**}P < 0.01$; $^{***}P < 0.001$. The data are expressed as mean ± SD. *BT* BTX treated

untreated control ($P < 0.01$), but not at 24 h ($P > 0.05$). For further confirmation, the cells were analyzed by trypan blue assays, and similar results were obtained ($P < 0.001$).

BTX suppresses HSF migration

To examine the effects of BTX on HSF migration, confluent monolayers were scratched and incubated with BTX for 0, 12, 24, and 48 h. As shown in Fig. 2, at 12 h, 32.3% and 8.9% of the areas were filled with migrated HSFs in the

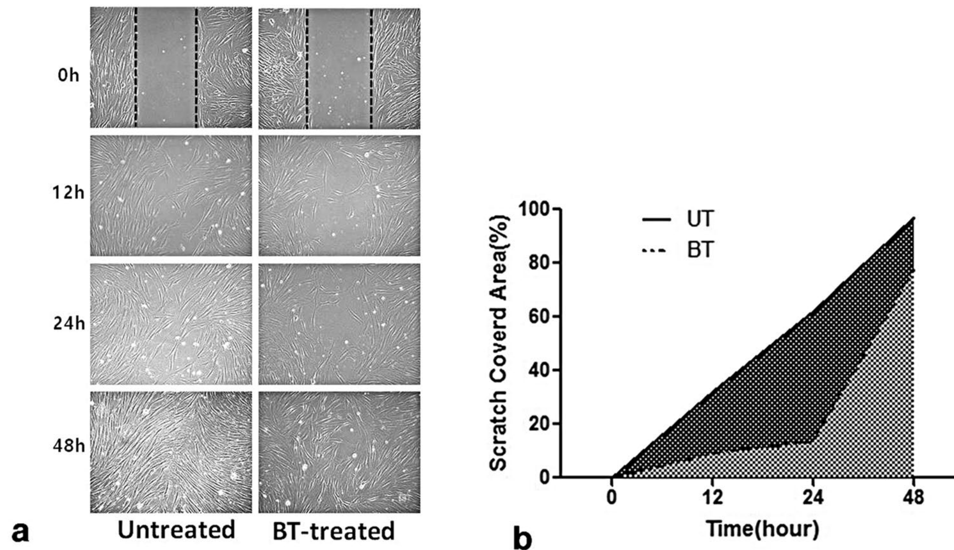

Fig. 2 Effect of botulinum toxin type A (BTX) on the migration of cultured human hypertrophic scar fibroblasts. The cells were seeded onto culture insert-containing 35-mm μ-Dishes. After insert removal, confluent cells were scratched and the cells were cultured with DMEM only or DMEM with 8 units/mL of BTX for 0, 12, 24, or 48 h, as indicated. **a** Photograph of in vitro cell migration observed using a microscope. Original magnification: ×40. **b** The quantitative analysis of cell migration rate at 12, 24, or 48 h post-scratch. Covered scratched area (%) was evaluated using WimScratch software. The full line represents untreated cells and the dotted line represents cells treated with 8 units/mL of BTX. *UT* BTX untreated, *BT* BTX treated

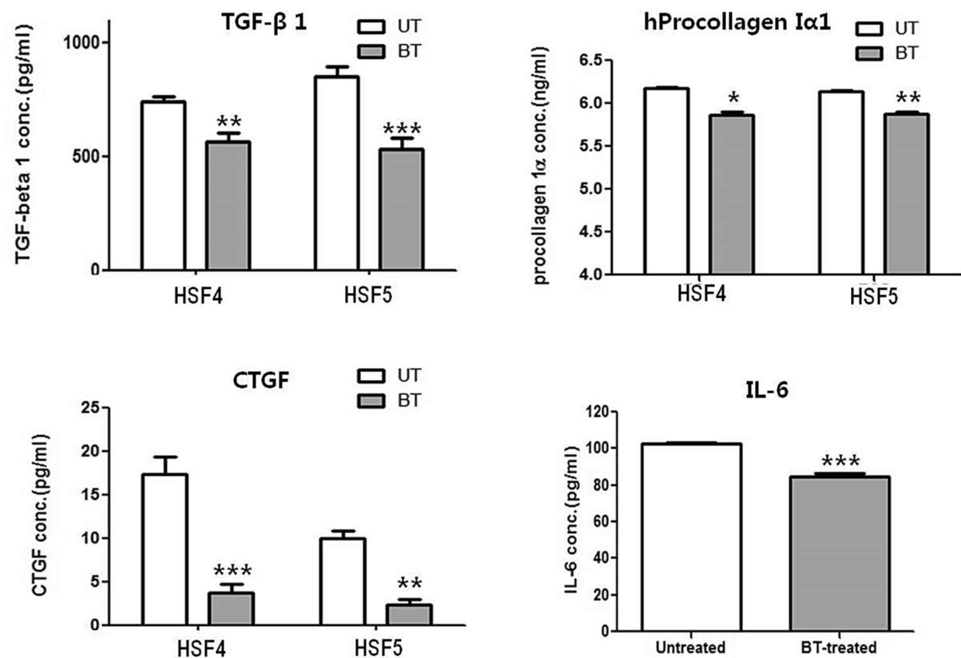

Fig. 3 Effect of botulinum toxin type A (BTX) on protein expression of scar-related ECM and pro-fibrotic factors in cultured human hypertrophic scar fibroblasts. The cells were cultured without serum for 24 h, followed by the addition of 8 units/mL of BTX to culture media for 48 h. The protein expression level was assessed by enzyme-linked immunosorbent assay. Each treatment was performed in triplicate and the data are presented as mean ± SEM. Two-way ANOVA, Bonferroni's post hoc test, compared with the control in each group; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. *UT* BTX untreated, *BT* BTX treated

control and BTX-treated groups, respectively. After 24 and 48 h, 62.1% and 97.3% of the areas were filled in the control group, whereas 13.7% and 77.3% of the areas were filled in the BTX-treated group, respectively. These results showed that BTX inhibited HSF cell migration, and that this inhibition was most effective between 12 and 24 h.

BTX downregulates the expression of scar-related ECM and pro-fibrotic proteins in HSFs

We further evaluated the expression of scar-related ECM and pro-fibrotic proteins by ELISA after 48 h of BTX treatment to cells of the samples. Although the concentration of pro-fibrotic proteins in HSFs could vary depending on the progression of each HS or other factors specific to each patient, there was a significant difference between the BTX-treated and untreated groups in all comparisons consistently across the samples. In particular, BTX treatment significantly inhibited the expression of TGF-β1, hPro-collagen Iα1, CTGF, and IL-6 regardless of the patient source (Fig. 3). Thus, BTX can exhibit a suppressive effect on the expression of scar-related ECM and pro-fibrotic proteins in HSFs.

BTX activates the JNK signaling pathway in HSFs in vitro

As shown in Fig. 4a, the p-JNK level in HSF4 significantly increased with BTX treatment and reached the maximum at 4 h. The JNK activity then decreased at 8 h, but recovered at 24 h. In contrast to the change in p-JNK over time, the total JNK level remained constant with BTX treatment. Densitometric quantification showed a marked increase (3.1-fold) in the p-JNK level with BTX treatment at 4 h compared with that in the control group, and then decreased to nearly the control level at 8 h after BTX treatment (1.1-fold). At 24 h, JNK phosphorylation was reactivated (3.1-fold) and was maintained at a high level (2.3-fold) until 48 h. Similar results were obtained in further experiments performed under the same conditions with HSF5 and HSF6 (Fig. 4b). These results demonstrated that BTX induces JNK activation in HSFs in vitro for up to 4 h.

BTX suppresses the fibrotic effect by activating the JNK pathway in HSFs in vitro

To further understand the mechanism of BTX-induced JNK activation, we used a selective inhibitor of the JNK pathway; 2.5 μM SP600125 was added to the HSF4 cells and incubated for 30 min before BTX treatment. Figure 5a shows that SP600125 markedly suppressed BTX-induced JNK phosphorylation, which occurred maximally at 4 h. The MTS assay further showed that the significant decrease of proliferation due to BTX treatment at 48 h ($P < 0.01$) was reversed with treatment of SP600125 ($P < 0.05$) (Fig. 5b). Moreover, BTX-mediated suppression of pro-fibrotic protein expression was rescued by treatment of SP600125 at 48 h (Fig. 5c), with a significant difference between the BTX-treated group and BTX plus inhibitor-treated group for CTGF ($P < 0.05$), IL-6 ($P < 0.001$), and hPro-collagen Iα1 ($P < 0.01$).

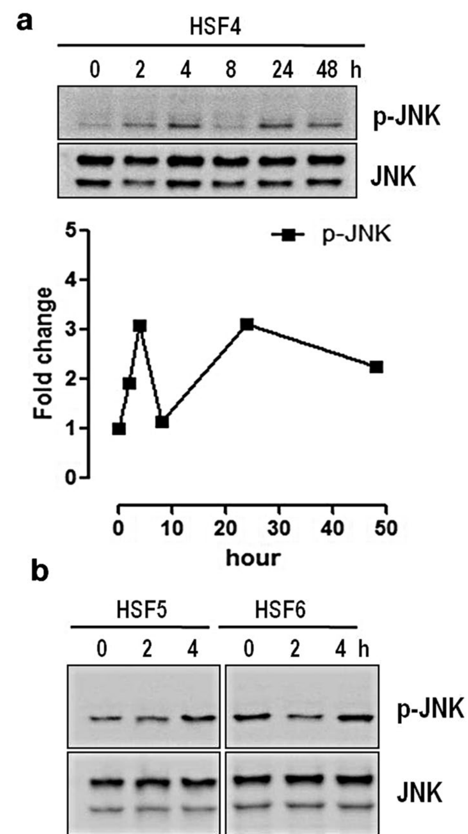

Fig. 4 Effects of botulinum toxin type A (BTX) on the activation of the JNK signaling pathway in cultured human scar fibroblasts. **a** The HSF4 cells were cultured without serum for 24 h, followed by the addition of 8 units/mL of BTX to culture media for 0, 2, 4, 8, 24, or 48 h. The cell lysates were analyzed by western blotting with anti-phospho-JNK antibodies. The graph shows the band density of p-JNK relative to the consistent total JNK protein concentration. **b** To elucidate the effects of BTX on patient-specific cell cultures from hypertrophic scars, HSF5 and HSF6 cells were assessed under the same conditions. Similar results were obtained in the two different experiments. *P-JNK* phosphorylated JNK

Discussion

BTX is a neurotoxic protein produced by *C. botulinum*, which irreversibly blocks acetylcholine release at the neuromuscular junction to achieve muscle paralysis [11]. Several studies have shown that BTX injection around a wound

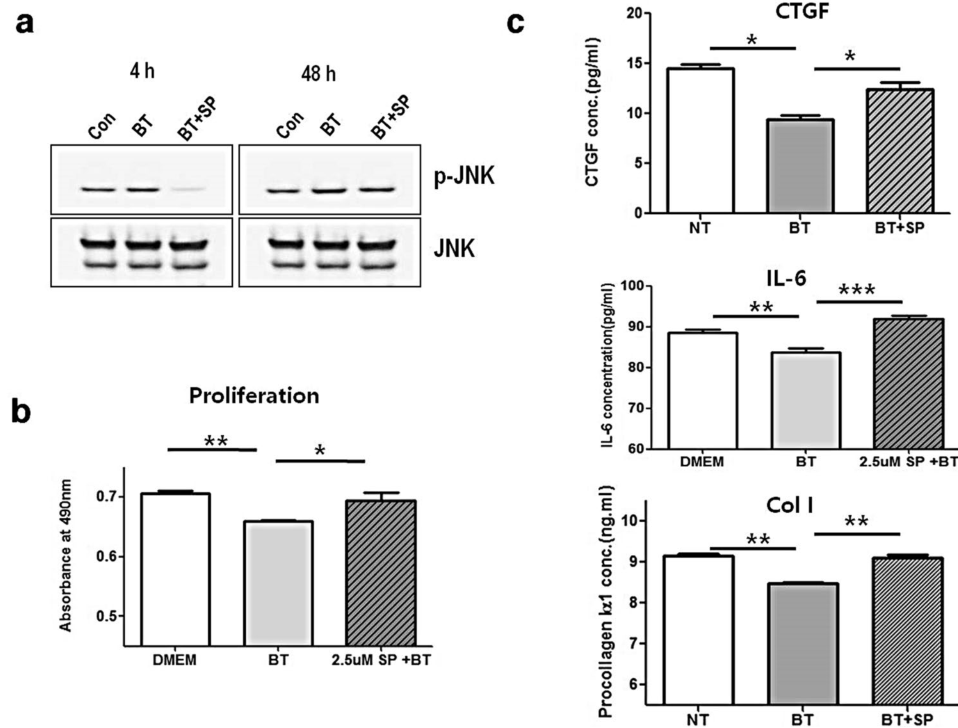

Fig. 5 JNK inhibitory effect on BTX-induced cellular proliferation and expression of pro-fibrotic proteins in cultured hypertrophic scar fibroblasts. **a** To explore the underlying mechanism of the suppressive effect of BTX, the protein level of p-JNK was analyzed by western blotting in HSF4 in the presence of SP600125 (4 h or 48 h). **b** The proliferation rate of HSF4 stimulated with 8 units/mL BTX in the presence of SP600125 was measured by the MTS assay at 48 h. **c** The level of pro-fibrotic protein-inducing CTGF and IL-6 as well as hPro-collagen Iα1 in HSF4 was determined by the enzyme-linked immunosorbent assay at 48 h. Each treatment was performed in triplicate and the data are presented as mean ± SEM. Paired student's *t* test, compared with the control in each group; $*P<0.05$; $**P<0.01$; $***P<0.001$. *Con* control, *BT* BTX treated, *SP* SP600125, *NT* not treated

could reduce the formation of scar hyperplasia [29, 31]. A recent study further showed that BTX effectively improved the appearance of HSs and inhibited the formation of collagen fibrils and fibroblasts in a rabbit ear model, with efficacy similar to that of conventional triamcinolone acetonide treatment [20]. Furthermore, the combination of BTX and triamcinolone for intralesional therapy resulted in a significantly improved therapeutic effect compared to that observed with monotherapy [3]. BTX injection also improved the facial scar quality during the early postoperative period [17]. Collectively, these previous studies showed that BTX not only reduces local muscle tension to inhibit scar formation, but also modulates cytokine production or release to inhibit cell proliferation and differentiation, which was confirmed in the present study. This indicates that emerging therapies such as BTX-based treatment can be used to augment current therapies.

Although the suppressive effect of BTX on HSs has been demonstrated, the associated mechanism has remained unclear. Recently, Austin et al. [1] reviewed the literature on the cellular response of HSs to BTX, highlighting that BTX downregulates the expression of TGF-β and profibrotic factors such as CTGF, IL-6, IL-8, and α-SMA, decreases fibroblast proliferation, and modulates collagen activity in pathologic scarring. Our results confirmed that BTX decreases the expression level of TGF-β1 in HSFs along with fibroblast proliferation. This suggests that inhibition of fibroblast proliferation and migration by BTX is associated with TGF-β1 signaling.

The direct stimulation of CTGF by TGF-β has been demonstrated in HSFs and during wound healing in vivo, accompanied by coordinated upregulation of TGF-β followed by CTGF expression [13]. CTGF is a central mediator of tissue remodeling and fibrosis, and its inhibition can reverse the process of fibrosis. Indeed, we found a significant difference in the level of CTGF protein between the BTX-treated and untreated control groups. Further studies should investigate whether the significant downregulation of CTGF expression by BTX has direct effects on the ability of TGF-β1 to promote ECM production and other mechanisms involved in this process.

IL-6 is an important marker of inflammatory tissue reactions and wound healing processes by modulating immune responses, and is thus an essential factor for timely wound

healing [21]. Overexpression of IL-6 leads to increased keratinocyte activity, which can favor scarring [26]. Moreover, the IL-6-STAT3 pathway mediates the ECM and proliferation of fibroblasts from HSs [25]. Therefore, we hypothesized that BTX-mediated IL-6 modulation might suppress HSF proliferation. Indeed, BTX significantly reduced the expression of IL-6 in HSFs.

TGF-β/Smad is the most well studied pathway for HS formation. Moreover, the MAPK pathway has been reported to be involved in TGF-β signaling, but the exact mechanisms are not clear [22]. Adipose tissue-derived stem cells were shown to decrease collagen deposition and scar formation in vitro, ex vivo, and in vivo by regulating p38/MAPK signaling [18]. Inhibition of ERK and JNK with loureirin B downregulated the expression of Col1 and fibronectin in TGF-β1-stimulated fibroblasts, and decreased the protein levels of p-ERK and p-JNK in cultured HS tissue ex vivo [10]. Olaeanolic acid induced apoptosis via the mitochondrial death pathway in HSFs by triggering p38 and JNK signaling [4]. However, activation of JNK might depend on the cell type, various stimuli, and the activity of other signaling pathways. Kim et al. [16] found that BTX-stimulated RAW264.7 cells induced nitric oxide and tumor necrosis factor-α production through Toll-like receptor 2–mediated signal transduction via activation of ERK, JNK, and p38. Here, we found that JNK was activated by BTX treatment, whereas the phosphorylation levels of p38 MAPK kinase and ERK1/2 were not affected by BTX. When blocking the JNK pathway, the inhibitory effects of BTX on the proliferation of HSFs and the production of pro-fibrotic factors were reversed via the down regulation of p-JNK. These results suggest that the suppressive effect of BTX is closely associated with activation of the JNK pathway. Therefore, we speculate that the phosphorylation of JNK in cells treated with BTX may critically contribute to suppression of fibrotic effects. Nevertheless, further investigation is needed to determine how fibroblast-associated fibrosis and inflammation factors act on the JNK pathway and mediate the suppressive effects of BTX.

In summary, we investigated the molecular mechanism driving the bioactivities of BTX, including proliferation and migration of cells, and the expression of fibrosis-related ECM and pro-fibrotic growth factors derived from HSFs in vitro. We confirmed that BTX affects the expression of scar-related cytokines and growth factors as well as the proliferation and migration of HSFs. Moreover, we demonstrated that the JNK signaling pathway likely plays an important role in the molecular mechanism driving these effects. These results can be exploited as a potential therapeutic strategy.

Funding This research was supported by AmorePacific Grant in 2015.

Compliance with ethical standards

Conflicts of interest None declared.

Research involving human participants and/or animals All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

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