

# Botulinum Toxin Type A Inhibits Connective Tissue Growth Factor Expression in Fibroblasts Derived From Hypertrophic Scar

Zhibo Xiao · Miaobo Zhang · Ying Liu · Lihong Ren

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## Abstract

**Background** Botulinum toxin type A (BTXA) can inhibit the growth of hypertrophic scars, but the molecular mechanism for this action is unknown. In addition to reducing the tension around the wound by stimulating temporary denervation, a growing body of evidence suggests that BTXA is involved in regulating the cell cycle and decreasing transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) expression in the fibroblasts of hypertrophic scars. Connective tissue growth factor (CTGF) is a downstream regulator of TGF- $\beta$ 1 function and an independent mediator of scarring and fibrosis. The effects of BTXA on CTGF in hypertrophic scar still are unknown. This study aimed to explore the effect of BTXA on CTGF in fibroblasts derived from hypertrophic scar and to elucidate its actual mechanism further.

**Methods** Fibroblasts isolated from tissue specimens of hypertrophic scar were treated with BTXA. The difference in proliferation between treated and nontreated fibroblasts was analyzed by flow cytometry. Proteins of CTGF were checked using Western blot in fibroblasts with and without BTXA.

**Results** The proliferation of the fibroblasts treated with BTXA was slower than that of the fibroblasts that had no BTXA treatment ( $p < 0.01$ ), which showed that BTXA effectively inhibited the growth of fibroblasts. Compared with fibroblasts that received no BTXA treatment, BTXA at  $1 \text{ U}/10^6$  cells decreased the expression of CTGF by  $49.2\% \pm 12.5\%$  ( $p < 0.01$ ), and BTXA at  $2.5 \text{ U}/10^6$  cells decreased the expression of CTGF by  $56.9\%$  ( $p < 0.01$ ).

**Conclusion** These results suggest that BTXA effectively inhibited the growth of fibroblasts derived from hypertrophic scar and in turn caused a decrease in CTGF protein, providing theoretical support for the application of BTXA to control hypertrophic scarring.

**Keywords** Botulinum toxin type A · Connective tissue growth factor · Fibroblasts · Hypertrophic scar · Transforming growth factor- $\beta$ 1

Hypertrophic scar is a hyperproliferative disorder of the dermal collagen caused by sophisticated mechanisms [1, 2]. Patients with hypertrophic scars often experience severe physical complications (deformities, restricted range of motion, pain, and pruritus) and psychological problems (cosmetic concern). Because the pathogenesis of hypertrophic scar has not been fully understood, clinical treatment remains problematic. Although numerous treatments are currently available including surgical excision, steroid injection, radiation therapy, laser, and pressure therapy, these methods cannot always provide good therapeutic results. Hence, alternatives are needed.

The optimal treatment for hypertrophic scar is based on a good understanding of molecular processes. A better understanding of the pathophysiology of hypertrophic scar holds great promise for the development of novel therapeutic strategies. It is well known that hypertrophic scar is attributed partly to an imbalance in the cellular dynamics of fibroblasts caused by an overabundance of fibroblast proliferation and a lack of fibroblast apoptosis, which results in excessive deposition of extracellular matrix [3–7].

Botulinum toxin, a potent neurotoxin derived from *Clostridium botulinum*, exists in various serotypes (A through G). Botulinum toxin type A (BTXA) is available

Z. Xiao (✉) · M. Zhang · Y. Liu · L. Ren  
Department of Plastic Surgery, The Second Affiliated Hospital  
of Harbin Medical University, Harbin City, P.R.China  
e-mail: xiaozhibodoctor@yahoo.com.cn

for clinical use in many countries. For more than 20 years, the application of BTXA has proved to be safe and effective in the treatment of various disorders, including blepharospasm and hyperfunctional facial lines. [8–12] In addition to producing a flaccid paralysis of striated muscle for 2 to 6 months, recent reports show BTXA improving the symptoms of hypertrophic scar [13–15]. However, the molecular mechanism of this improvement remains unknown.

Excessive expression of connective tissue growth factor (CTGF) is the important factor in the formation of hypertrophic scar. Not only does CTGF regulate cellular adhesion and growth. It also causes excessive deposition of collagen [16–20]. Recent publications have shown the close relationship between CTGF and hypertrophic scar. Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) stimulates the expression of CTGF, and CTGF in turn can affect collagen deposition.

We previously published articles reporting that BTXA could inhibit fibroblast growth and reduce TGF- $\beta$ 1 expression in fibroblasts derived from hypertrophic scar [21, 22]. The close connection between TGF- $\beta$ 1 and CTGF in molecular networks caused us to consider the influence of BTXA on CTGF expression in fibroblasts derived from hypertrophic scar.

These findings led to the hypothesis that BTXA may reduce the expression of CTGF protein in fibroblasts derived from hypertrophic scar. The current study aimed to observe the action of BTXA in CTGF expression and to explore preliminarily the molecular mechanism of BTXA's regulation of CTGF in fibroblasts derived from hypertrophic scar.

## Materials and Methods

### Fibroblast Culture and Treatment

Tissue biopsies of hypertrophic scar were obtained from eight different patients. The protocols for human tissue sampling were approved by the Health Research Ethics Board of the authors' hospital. Informed consent was obtained from each patient.

With the patient under local anesthesia, biopsy specimens were taken using a 6-mm punch. The tissue samples were immediately placed in ice cold supplemented Dulbecco modified Eagle medium (DMEM) and transported to the laboratory for processing.

Each excised sample was rinsed with phosphate-buffered saline (PBS), minced into 1-mm pieces, and digested in 0.25% collagenase for 2 h and in 0.25% trypsin for 10 min at 37°C. The sample was placed in DMEM supplemented with 10% fetal bovine serum (FBS), and after

shaking, suspended cells were collected. The cells were cultured in DMEM with 10% FBS until confluent. Sometimes the cells were stored under liquid nitrogen. Only the cells from passages 3 to 5 were used in this study.

Cells were divided into three groups: cells not treated (control) and cells treated for 24 h with BTXA (Lanzou Biocompany of China) in respective concentrations of 1 U/10<sup>6</sup> cells and 2.5 U/10<sup>6</sup> cells. As defined previously by in vivo experiments, 1 ng of BTXA in Swiss-Webster mice is approximately 30 U of BTXA.

### Measurement of Fibroblast Proliferation

#### Flow Cytometry Analysis

Cell cycle distribution was analyzed by flow cytometry (Beckman Coulter Electronics, Norton Shores, MI, USA). We used fluorescence-activated cell sorter (FACS) flow cytometry to observe cell cycle distributions. Cells were harvested by treatment with trypsin/ethylenediaminetetraacetic acid (EDTA), pelleted by centrifugation, and then washed in PBS. Next, the cells were resuspended in cold 70% ethanol and maintained at 4°C for 16 h. Subsequently, the cells were centrifuged, washed in PBS, and resuspended in 300  $\mu$ l of binding buffer and 10  $\mu$ l of 50  $\mu$ g/ml propidium iodide solution together with 5  $\mu$ l/sample RNase (10 mg/ml stock). The cells then were incubated at 37°C for 30 min before FACS analysis. The percentage of cells in the G0 to G1, G2 to M, and S phases of the cell cycle was autoanalyzed.

Data are expressed as the percentage of cells in the G0 to G1, G2 to M, and S phases. The percentages of cells in the different phases of the cell cycle were quantified using SPSS 11.0 software (Harbin Medical University of Heilongjiang Province, China).

#### Western Blot Analysis for CTGF Expression

In the experiments, adherent cells were rinsed with PBS and lysed using UDC buffer (8 mol/l urea, 10 mmol/l dithiothreitol, 4% 3-([3-cholami-dopropyl]dimethylammonio)1-propanesulfonate). Equal amounts of protein (20 mg per lane) were loaded on 15, 10, or 4–20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Proteins were electrophoretically separated, then transferred to immobilon membranes (Millipore, Billerica, MA, USA). The membranes were probed overnight at 4°C with the following primary antibodies: rabbit anti-CTGF (Torrey Pines Biolabs, Houston, TX, USA) and mouse anti- $\beta$ -actin (Chemicon, Temecula, CA, USA).

After washing, the membranes were incubated with appropriate secondary antibodies (Li-Cor) for 1 h at room temperature. The protein antibody complexes were

**Table 1** Proliferation of fibroblasts from hypertrophic scar measured by flow cytometry (n = 8, X ± S)

Fibroblasts	Distribution of different phases in the cell cycle (%)		
	G0–G1	S	G2–M
No BTXA	34.21 ± 2.05	19.18 ± 1.93	47.17 ± 1.85
BTXA (1 U/10 <sup>6</sup> cells)	58.14 ± 1.64	8.67 ± 1.43	34.65 ± 1.37 <sup>a</sup>
BTXA (2.5 U/10 <sup>6</sup> cells)	61.12 ± 1.45	9.34 ± 1.57	30.46 ± 1.25 <sup>a</sup>

BTXA botulinum toxin type A

Data are presented as mean ± standard deviation

<sup>a</sup>  $p < 0.01$ , compared with “No BTXA”

visualized using the Odyssey direct infrared fluorescence imaging system (Li-Cor). In addition, the authors noted a possible difference in the number of fibroblasts between the BTXA-treated group and the no-BTXA group when Western blot was planned due to the different growth speed caused by BTXA.

To eliminate the effects of the different cell densities in the BTXA-treated group and the no-BTXA group, cell counting was performed before CTGF protein analysis using Western blot. Fibroblasts from the BTXA-treated group and the no-BTXA group were seeded into 96-well plates, and every well was seeded by  $3 \times 10^6$  fibroblasts, which made the different groups have the same cell density when Western analysis was performed.

#### Statistical Analysis

All data are presented as mean ± standard deviation. The Western blot bands were quantified by densitometry, and protein expression was normalized by the loading control ( $\beta$ -actin) expression. Statistical evaluation of all the data was performed using one-way analysis of variance followed by Dunnett's *t* test for between-group comparisons. The level of significance was considered to be  $p$  values less than 0.05.

## Results

### BTXA-Inhibited Fibroblast Proliferation

Cell cycle distribution can be quantitatively measured by analyzing DNA content flow cytometrically. The data are expressed as the percentage of cells in the G0 to G1 phase, G2 to M phase, and S phase. Approximately 34% of the fibroblasts without BTXA were distributed in the G0 to G1 phase, 19% in the G2 to M phase, and 47% in the S phase. In the fibroblasts treated with 1 U/10<sup>6</sup> cells and 2.5 U/10<sup>6</sup> cells, the percentages of fibroblasts were respectively 58 and 61% in the G0 to G1 phase, 8 and 9% in the G2 to M phase, and 34 and 30% in the S phase. For more details, see

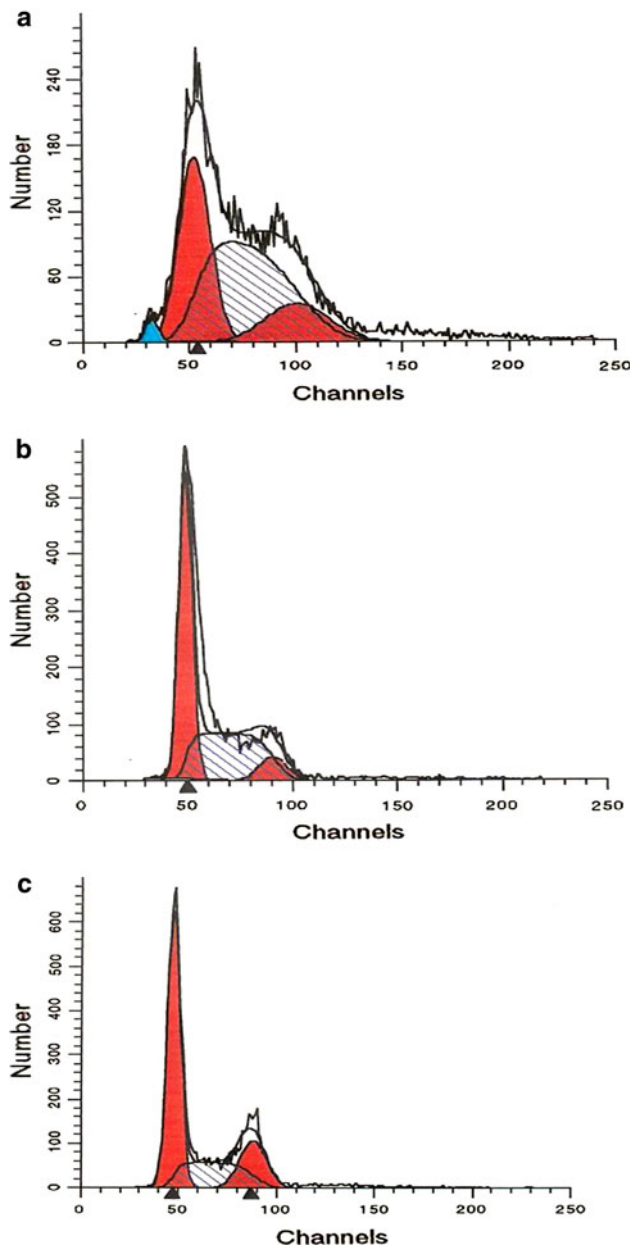
Table 1 and Fig. 1, which summarize the original data. The cell cycle distributions did not differ significantly between the fibroblasts with no BTXA (1.0 U/10<sup>6</sup> cells) and those treated with BTXA (2.5 U/10<sup>6</sup> cells) ( $p < 0.01$ ). No significant difference was observed between the fibroblasts with BTXA 1.0 U/10<sup>6</sup> cells and those treated with BTXA 2.5 U/10<sup>6</sup> cells ( $p > 0.05$ ). These results showed that fibroblast proliferation was significantly inhibited by BTXA when fibroblasts were treated with BTXA concentrations of 1.0 U/10<sup>6</sup> cells and 2.5 U/10<sup>6</sup> cells.

### BTXA Reduced Expression of CTGF Protein

The BTXA concentrations of 1.0 U/10<sup>6</sup> cells and 2.5 U/10<sup>6</sup> cells inhibited the proliferation of fibroblasts derived from hypertrophic scar. We next evaluated the effects of BTXA on the expression of CTGF. Compared with fibroblasts receiving no BTXA treatment, BTXA at 1.0 U/10<sup>6</sup> cells decreased the expression of CTGF by  $49.2 \pm 12.5\%$  ( $p < 0.01$ ), and BTXA at 2.5 U/10<sup>6</sup> cells decreased the expression of CTGF by 56.9% ( $p < 0.01$ ). No significant reduction in CTGF was found between the fibroblasts with BTXA of 1.0 U/10<sup>6</sup> and those with BTXA of 2.5 U/10<sup>6</sup> cells ( $p > 0.05$ ) (Fig. 2). These results showed that BTXA had significant effects on downregulation of CTGF expression. The experiments were conducted with cells from eight different patients, and consistent results were obtained.

## Discussion

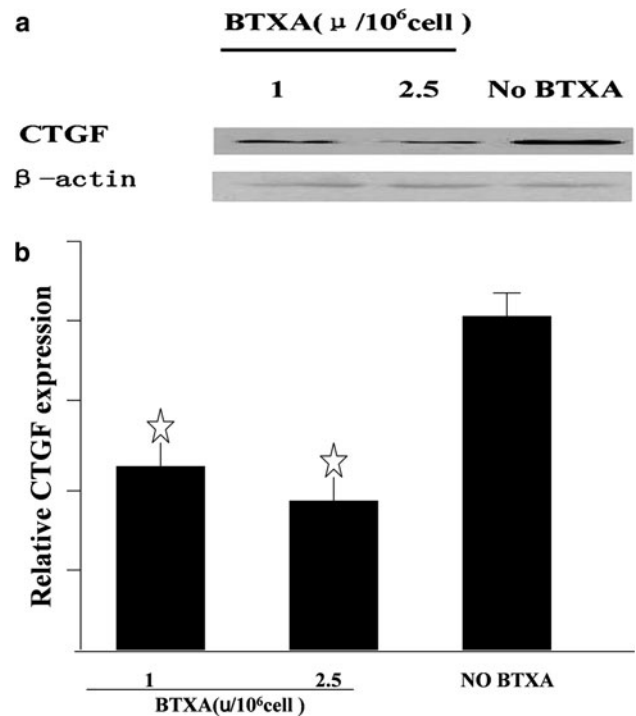
Abnormal proliferation of fibroblasts plays an important role in the formation and development of hypertrophic scar. Because the biologic behavior of fibroblasts derived from hypertrophic scar is poorly understood, treatment options are not always effective [1, 23–25]. Recent findings have shown that BTXA can improve the appearance of hypertrophic scar [14, 21, 22, 26], but the molecular mechanism still is unknown. Besides the reason that BTXA eliminates the tension around the wound by stimulating temporary denervation [27, 28], the more sophisticated network of



**Fig. 1** Botulinum toxin type A (BTXA) inhibited the proliferation of fibroblasts derived from hypertrophic scar. Cell cycle analysis was performed by flow cytometry. **a** In the fibroblasts without BTXA, approximately 34% of fibroblasts were distributed in the G0 to G1 phase, 19% in the G2 to M phase, and 47% in the S phase. **b** In the fibroblasts with a BTXA concentration of 1 U/10<sup>6</sup> cells, the percentage of fibroblasts was 58% in the G0 to G1 phase, 8% in the G2 to M phase, and 34% in the S phase. **c** In the fibroblasts with a BTXA concentration of 2.5 U/10<sup>6</sup> cells, the percentage of fibroblasts was 61% in the G0 to G1 phase, 9% in the G2 to M phase, and 30% in the S phase

molecular regulation between BTXA and hypertrophic scar has not been fully elucidated. Therefore, further investigation is needed.

In a previous article, we reported that BTXA can affect both the cell cycle distribution and TGF- $\beta$ 1 expression in



**Fig. 2** Botulinum toxin type A (BTXA) reduced the expression of CTGF in fibroblasts derived from hypertrophic scar. Fibroblasts treated with the different doses of BTXA (0, 1, and 2.5  $\mu$ /10<sup>6</sup> cells) for 24 h are shown. **a** Representative Western blots of connective tissue growth factor (CTGF) and  $\beta$ -actin. Levels of CTGF were quantified relative to  $\beta$ -actin expression to correct for loading differences and normalized to untreated levels. **b** Data are expressed as mean  $\pm$  standard deviation of three to six experiments ( $p < 0.01$ )

fibroblasts derived from hypertrophic scar [21, 22]. As a downstream regulator of TGF- $\beta$ 1 function, CTGF is an independent mediator of scarring and fibrosis.

One study showed increased CTGF in fibroblasts derived from hypertrophic scar [20]. The expression of CTGF protein was affected by the expression of TGF- $\beta$ 1 in hypertrophic scar, but its response to BTXA treatment is unknown. The authors hypothesized that BTXA could reduce CTGF expression in fibroblasts of hypertrophic scar.

Our aim was to investigate further the potential use of BTXA for the prevention of hypertrophic scar by exploring the influence of BTXA on CTGF expression in fibroblasts derived from hypertrophic scar. In this study, we observed that BTXA inhibited cell proliferation and simultaneously decreased CTGF expression in fibroblasts derived from hypertrophic scar. On the basis of these findings, we inferred that the reduction of CTGF expression caused by BTXA might be attributed to the effects of BTXA on fibroblast proliferation and that inhibition of cell proliferation might in turn lead to a decrease in CTGF.

In this study, the concentrations of BTXA were 1.0 and 2.5 U/10<sup>6</sup> cells, and these concentrations of BTXA were

sufficient to inhibit fibroblast proliferation. The important observation of the current study was that BTXA negatively regulated CTGF protein and fibroblast proliferation at the same time. Although the precise mechanism behind these findings remains to be determined, it should be strongly mentioned that BTXA played the important role in the pathogenesis of hypertrophic scar. Thus, the ability of BTXA to reduce CTGF expression and inhibit cell proliferation may be related to clinical improvement in hypertrophic scar.

To the best of our knowledge, this article is the first to report the influence of BTXA on the expression of CTGF in fibroblasts derived from hypertrophic scar. Although this article presents the preliminary results of our study, these findings could help to explain the molecular mechanism of BTXA-based therapies for hypertrophic scar.

Some limitations of this study cannot be ignored. Although BTXA could simultaneously affect the expression of CTGF protein and cell proliferation in the fibroblasts derived from hypertrophic scar, we have hardly ascertained the reason why BTXA could regulate the expression of the cytokine in fibroblasts derived from hypertrophic scar. Additionally, we could not know whether the BTXA effect on CTGF was specific to hypertrophic scar-derived fibroblasts.

Our research about this problem is ongoing. Our experimental plan will be completed gradually. In the next experiment, we will explore the effect of BTXA on normal fibroblasts and keloid-derived fibroblasts. Therefore, much work needs to be done to discover the molecular network of the relationship between BTXA and the cytokine in fibroblasts derived from hypertrophic scar and normal skin fibroblasts. We plan to study the effect of BTXA on the CTGF signaling pathway in normal skin fibroblasts and fibroblasts of hypertrophic scar, which may help to explain the molecular mechanism for the effects of BTXA on CTGF in the fibroblasts derived from hypertrophic scar.

## Conclusion

Our findings support the notion that BTXA can inhibit cell proliferation and reduce CTGF expression in fibroblasts derived from hypertrophic scar at the same time, which can partly explain the molecular mechanism of the effects that BTXA has on the biologic behavior of fibroblasts derived from hypertrophic scar. Our results provide a new theoretical basis for the use of BTXA to treat hypertrophic scars.

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**Conflict of interest** None.

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