Botulinum Toxin A for the Treatment of Keloids

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Abstract
Introduction: Keloids are the result of excessive scar tissue formation. Besides their poor aesthetic appearance, keloids can be associated with severe clinical symptoms such as pain, itching, and rigidity. Unfortunately, most therapeutic approaches remain clinically unsatisfactory. Recently, injections with botulinum toxin A (BTA) were proposed for the treatment of established keloids in a clinical trial. In this study, we aimed to verify the effects of intralesional BTA for the treatment of therapy-resistant keloids using objective measurements. In addition, the underlying molecular mechanisms were investigated using cultured keloid-derived fibroblasts. Materials and Methods: Four patients received BTA (doses varying from 70 to 140 Speywood units per session) injected directly into their keloids every 2 months for up to 6 months. Differences in height and volume were evaluated clinically and measured with a 3-D optical profiling system. Keloid-derived fibroblasts were treated with different concentrations of BTA, and expression of collagen (COL)1A1, COL1A2, COL3A1, TGF-β1, TGF-β2, TGF-β3, fibronectin-1, laminin-β2, and α-SMA was determined by real-time quantitative PCR. MTT and BrdU assays were used to analyze the effects of BTA on fibroblast proliferation and metabolism. Results: Intralesional administration of BTA did not result in regression of keloid tissue. No differences in expression of ECM markers, collagen synthesis, or TGF-β could be observed after BTA treatment of keloid fibroblasts. In addition, cell proliferation and metabolism of keloid fibroblasts was not affected by BTA treatment. Conclusion: The suggested clinical efficiency of intralesional BTA for the therapy of existent keloids could not be confirmed in this study. Based on our data, the potential mechanisms of action of BTA on keloid-derived fibroblasts remain unclear.
matory period with a specific immune cell infiltrate present in the scar tissue of keloids resulting in increased fibroblast activity with greater and more sustained extracellular matrix (ECM) deposition [2].

In the developed world alone, a total of 100 million patients develop scars each year as a result of 55 million elective surgeries and 25 million operations after accidental trauma [3]. A recent survey performed in the USA confirmed that many patients are disappointed with the resulting scars irrespective of gender, age, or ethnicity [4]. As demonstrated in this survey, many patients would highly value any opportunity to improve or minimize scarring following surgery or trauma [4]. However, most therapeutic approaches remain clinically unsatisfactory, probably due to poor knowledge of the complex mechanisms underlying the process of excessive scarring.

Reduction of the muscular tensile force during scar formation as well as restoration of the balance between fibroblast proliferation and cellular apoptosis may represent a novel therapeutic option for the aesthetic improvement of postsurgical scars [5]. Botulinum toxin A (BTA) paralyzes local muscles and reduces skin tension caused by muscle pull, thereby decreasing scar tension and subsequent inflammation in wound edges [6]. Indeed, Gassner et al. [7] could demonstrate that BTA injections into the musculature adjacent to the wound within 24 h of wound closure resulted in enhanced wound healing and less noticeable scars compared to placebo. Recently, intralosional injections with BTA were also proposed for the treatment of established keloids in a prospective, uncontrolled study [8]. BTA was injected into the lesions at 3-month intervals for a maximum of 9 months at a concentration of 35 units/ml. Total doses ranged from 70 to 140 units per session. At the 1-year follow up, three of the 12 included patients demonstrated excellent results, 5 patients had good results, and 4 patients had fair results. This therapy did not fail in any of the patients. When analyzing clinical symptoms, scar regression was noted from the periphery in all of the patients, followed by flattening of the lesions. Within the follow-up period of 1 year, no signs of recurrence were noted in any of the patients. As a mechanism, the same group could then demonstrate that BTA was able to reduce the expression of TGF-β1 protein in fibroblasts derived from scars and significantly reduced their proliferating rate [9].

As BTA promised to be very effective in the treatment of pathologic scars, this study aimed to verify the effects of intralosional BTA on keloids resistant to any previous therapy using objective measurements. In addition, the potential underlying molecular mechanisms of BTA treatment on keloid tissue were investigated using cultured keloid-derived fibroblasts.

Material and Methods

Patients

Four patients (2 male, 2 female) suffering from keloids for longer than 2 years resistant to any previous therapy (e.g., kryotheraphy, intralesional corticosteroids, silicone sheets) and presenting to the scar clinic of the Department of Dermatology and Allergy, Ludwig Maximilian University, Munich, from May to July 2011, were included in the present study after signing informed consent forms. Keloid scars were defined clinically as itchy, raised, erythematous tumors projecting beyond the original wound margins. A detailed patient history including duration, any predisposing factors such as trauma, infection, or any inflammatory skin disorder, any prior treatment implemented, and family history was obtained. In all 4 patients, any prior treatment was discontinued for at least 6 months before starting treatment with intralosional BTA. For treatment, BTA (Azzalure, 125 Speywood units diluted in 0.5 ml saline) in a total dose varying from 70 to 140 Speywood units per session was injected directly into the keloid tissue every two months using a 24-gauge needle for a maximum of 6 months.

3-D Optical Profiling

3-D optical profiling (Primos 3D Imaging; GFMesstechnik GmbH, Vectra 3D) was used to objectively measure the differences in height of the keloid scars at baseline and after 2–4 treatment sessions.

Fibroblast Culture and BTA Treatment

Primary fibroblasts were isolated from tissue biopsies obtained from keloids from 3 independent patients after signing informed consent. Different patients with keloids were chosen for fibroblast isolation as an additional, partial keloid excision for fibroblast isolation could have negatively influenced the outcome of BTA treatment. However, keloids chosen for fibroblast isolation were comparable to those that were treated with BTA injections with respect to body location and the skin color of the respective patients. First, subcutaneous fat was removed from keloid specimens and washed in PBS. For separation of the dermis, specimens were incubated overnight at 4°C in sterile Dispase II (Roche) solution (4 mg/ml). The next day the epidermis was detached and the dermis was minced into pieces of 2 mm² in sterile cell culture dishes. Explants were gently covered with Dulbecco’s modified Eagle’s medium (PAA Laboratories) supplemented with 10% fetal bovine serum and penicillin (100 U/ml), and streptomycin (100 µg/ml) (PAA Laboratories), and incubated in a humidified incubator at 37°C and 5% CO2. Fibroblasts were grown out of the tissue and after 3–4 weeks cells were detached by trypsin and replated. Passages 3–6 of keloid-derived fibroblasts were seeded at a density of 1 × 104 cells in 12-well plates. After 48 h, the cells were placed in serum-free medium for at least 24 h before starting the experiments. Medium was changed to DMEM with 1% fetal bovine serum, and BTA (Azzalure, 5–10 U/ml) was added to cultured cells for different time periods.
Real-Time Quantitative RT-PCR

For gene expression analyses cells were harvested after 24 h and total RNA was isolated using an RNA Miniprep Kit (Zymo Research). cDNA synthesis was performed with 1 μg of total RNA using a DyNAmo cDNA Synthesis Kit (Finnzymes). Expression of collagen (COL1A1, COL1A2 and COL3A1, TGF-β1, TGF-β2, TGF-β3, fibronectin-1, laminin-β2, α-SMA, and PCNA (proliferating cell nuclear antigen) was determined by quantitative PCR (qPCR) using Evagreen PCR Master Mix (BioRad) as specified by the manufacturer. All primers were synthesized by Qiagen, and β-actin was used as housekeeping gene for normalization. All PCR assays were run in the Bio Rad CFX96 Real-Time System.

Cell Proliferation Assays

Fibroblast proliferation was determined by BrdU assay (Roche Applied Science). Cells were incubated with BTA for 72 h and BrdU added to the cell culture for the final 24 h. Incorporated BrdU was then detected by an anti-BrdU antibody labeled with HRP. Absorbance was measured at 370 nm using a microplate reader.

The MTT assay was performed using an MTT cell proliferation kit (R&D Systems). After 72 h of incubation with different concentrations of BTA, 30 μl of MTT reagent were added to the medium and the cells were incubated for another 4 h at 37°C. After addition of the solubilization solution, absorption of the produced formazan was measured at 570 nm.

Data Analysis

All graphs and statistical calculations were made using GraphPad Prism 5.0 (GraphPad Software, Inc.) Statistical differences between groups were determined using a two- or one-tailed Student’s t test. Values are expressed as means ± SD and p < 0.05 was considered statistically significant.

Results

Repeated injections with BTA for up to 6 months did not change the macroscopic appearance, morphology, or size of keloid scars in 4 patients (fig. 1a). Objective measurements using optical profilometry confirmed the visual impression and confirmed no significant change in keloid volume and height after BTA therapy (fig. 1b–d).

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**Fig. 1.** BTA shows no effect on keloid appearance in vivo. BTA (Azzalure, 125 Speywood units diluted in 0.5 ml saline) was injected directly into the keloid tissue every 2 months using a 24-gauge needle for a maximum of 6 months. The clinical course was monitored (a) and keloids were analyzed by 3-D optical profiling. Color-coded surface topography images of one representative keloid at baseline and 2 months after the last injection, as well as differences in scar height, are displayed in b and c. The changes in volume ($V_{eth}$) of 4 patients are summarized in d (mean ± SD).
Fig. 2. BTA shows no effect on keloid-derived fibroblasts. Isolated primary fibroblasts from keloid patients (n = 3) were cultured and treated with BTA in increasing concentrations. mRNA abundance of TGF-β1–3, COL1A1, COL1A2, COL3A1, fibronectin 1, laminin-β2, α-SMA, and PCNA was determined by real-time qPCR after 24 h (a–d). Cell proliferation was analyzed using a BrdU assay and cell metabolism was measured with an MTT assay (e, f). Values are expressed as means ± SD from representative experiments performed in triplicate.
In cultured fibroblasts isolated from keloid scars BTA treatment did not change the expression of TGF-β1–3, COL1A1, COL1A2, or COL3A1 (fig. 2a, b). Also, the transcriptional activity of genes involved in the synthesis of ECM proteins which are excessively activated in keloid scars such as fibronectin 1, laminin-β2, and α-SMA was unchanged after BTA treatment (fig. 2c).

Consistent with the macroscopic impression that the cell proliferation of keloid fibroblasts was unchanged after BTA treatment, the expression of the proliferation marker PCNA remained unchanged after BTA therapy (fig. 2d).

These observations were confirmed in BrdU and MTA assays demonstrating that keloid fibroblasts treated with BTA show similar proliferative and metabolic activity when compared to control treated cells (fig. 2e, f).

Discussion

Currently, successful treatment of keloids remains difficult. As a consequence, both clinicians and their patients value even the smallest improvements in the appearance of those pathologic scars after treatment. In an effort to successfully treat keloids, various novel approaches have been introduced in recent years including various laser techniques, intralesional 5-FU, interferon injections, or topically applied imiquimod (to name a few) [10]. Very recently, the use of BTA was suggested to extend the spectrum of treatment for excessive scars. Xiao et al. [11] studied 19 patients suffering from HS who received intralesional injections of BTA (2.5 units/ml at 1-month intervals, exact substance unknown) for a total of 3 months. At a 6-month follow up, all patients presented with clinical improvement of their scars resulting in high subjective satisfaction. Clinical symptoms such as erythema, pruritus, and a pliability score decreased significantly after BTA injections when compared to baseline [11]. In another prospective, uncontrolled study, Zhibo et al. [8] demonstrated that BTA injected into 12 keloids (70–140 units per session every 3 months, exact substance unknown) resulted in significant improvement of the treated scar at a 1-year follow up. In this study none of the patients showed failure of therapy.

At present, the effect of many therapies used for excessive scars has never been tested in clinical trials. Only a few therapeutic modalities have been shown to be effective in well-designed prospective studies with adequate control groups. As the reported results for BTA treatment for excessive scars are very promising, we attempted to elucidate the potential beneficial effects of BTA on scar appearance in an objective manner. A three-dimensional optical profiling system was employed to measure decreases in scar height and volume after BTA treatment. Measurements were taken at baseline and 3 months after the final injection. The Primos 3D imaging system projects light onto a specific surface of the skin and records the image with a CCD camera. Skin surface micro-topography is reconstructed using temporal phase shift algorithms to generate 3-D images [12]. Subsequently, an intraindividual comparison between baseline and later time points is made and any subjective influences on scar evaluation can be avoided. Nevertheless, objective evaluation of BTA-treated keloids using optical profilometry confirmed the macroscopic assessment and no significant changes after BTA therapy compared to baseline could be detected in our patients. Although we treated a relatively small number of patients and used different doses of BTA, at least a slight improvement in scar height or volume should have been noted if BTA is as effective in reducing scar height as previously described [8, 11]. Thus, based on those observations, the suggested clinical efficiency of intra-lesional BTA for the treatment of existent keloids remains uncertain.

Doubtless, due to poor understanding of the complex molecular mechanisms underlying the process of excessive scarring, most therapeutic approaches remain unsatisfactory [1]. Excessive scar tissue formation results from increased deposition of large amounts of ECM in the dermis [13]. Pathologic scars are densely populated by fibroblasts and inflammatory cells which maintain a fibrogenic micro milieu by secreting TGF-β and other cytokines [14]. Increased production of collagen, proteoglycans, and fibronectin, deficient ECM degradation, and remodeling are the consequences [2]. Current research in keloid pathophysiology further suggests that alterations of the fibroblast phenotype by several growth factors and downstream signaling pathways are involved in the pathogenesis of excessive scar formation [15]. In particular, TGF-β cytokines and downstream smad pathways are key regulators in early stages of normal scar and/or keloid formation. While TGF-β1 and TGF-β2 represent the most important stimulators of collagen and proteoglycan synthesis, administration of TGF-β3 reduces connective tissue deposition [16–18]. Effects on those signaling mediators could be responsible for the proposed effect of BTA on excessive scars. Indeed, a recent in vitro study by Xiao et al. [9] reported that BTA may exert its beneficial effects on excessive scar appearance by inhibiting the proliferation of fibroblasts and by decreasing the levels of...
TGF-β1. In order to investigate the potential underlying molecular mechanisms of BTA treatment on keloid tissue, we incubated keloid-derived fibroblasts with different concentrations of BTA and the expression of various factors known to be involved in excessive scar formation were determined. However, no differences in expression ECM markers such as fibronectin-1, laminin-β2, or α-SMA, markers for collagen synthesis or TGF-β1, TGF-β2, and TGF-β3 proteins could be observed in keloid-derived fibroblasts after BTA treatment. Also, no differences in the proliferative and metabolic activity of keloid-derived fibroblasts after BTA incubation were detected when compared to nontreated cells.

Thus, while reduction of the tensile force by prophylactic BTA injections into the musculature adjacent to the respective wound during the course of wound healing and scar formation might represent a comprehensible mechanism of action for aesthetic improvement of postsurgical scars, the proposed mechanisms of action of BTA on existing keloids as suggested elsewhere remain unclear [9]. Certainly, more in-depth studies on the in vitro and in vivo effects of BTA on pathologic scars and/or mature keloids are needed before a comparatively expensive therapy for this particular indication can be postulated. Also, distinct factors such as genetic background, race, the phase of keloid development, and the location of the pathologic scar could have an impact on the success of this proposed treatment method.

References