The Effects of Botulinum Toxin A on Collagen Synthesis, Expression of MMP (matrix metalloproteinases)-1,2,9 and TIMP (tissue inhibitors of metalloproteinase)-1 in the Keloid Fibroblasts

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Keloids are characterized by excessive extracellular matrix (ECM) deposition such as collagen, fibronectin, elastin, and proteoglycans in the dermis. Recently, the use of botulinum toxin A (BTXA) in the treatment of keloids have had good results. To investigate the therapeutic effect of BTXA on the keloids, we evaluated the mRNA expression of collagen type I, type III, MMP (matrix metalloproteinases)-1, and TIMP (tissue inhibitor of metalloproteinases)-1 on keloid fibroblasts (KFs, n=5) after administration of BTXA. We also evaluated the enzymatic activity of MMP-2 and 9 by using zymography with BTXA. The same process was repeated after administration of TGF-β in addition to BTXA. Type III collagen mRNA expression was decreased significantly when BTXA was administrated on KFs regardless of the presence or absence of TGF-β. MMP-1 mRNA expression in KFs was increased according to the BTXA concentration increment, however, not increased with TGF-β. Moreover, MMP-2 enzymatic activity in KFs was increased when BTXA administrated regardless of the presence or absence of TGF-β. These results suggest that the down regulation of collagen III expression, the up regulation of MMP-1, and increased MMP-2 enzymatic activity on KFs after BTXA administration are able to decrease the excess collagen deposition in keloids.

Key Words: Keloid, Botulinum toxins, Collagen type III, Matrix metalloproteinases, Tissue inhibitor of metalloproteinases


I. INTRODUCTION

Keloids are pathologic conditions resulting from excessive extracellular matrix (ECM) deposition in the dermis. They are often associated with clinical symptoms such as pruritus, pain and hyperesthesia. They can cause functional impairment such as limited joint motion in addition to their poor aesthetic appearance. Recently, the need of the keloid treatment is increasing by these reasons.

Keloid fibroblasts (KFs) are not different from human normal dermal fibroblasts in size and shape, however, KFs can proliferate more than normal fibroblasts and produce high level of ECM such as collagen, fibronectin, elastin, and proteoglycans. Keloids are also known as an abnormal balance between proliferation and apoptosis, which can lead to failure of homeostasis in the wound healing process.

Elevated levels of Transforming growth factor (TGF)-β and platelet derived growth factor (PDGF) have been found in keloid tissue along with aberrant levels of their activity. The elevated growth-factor activity is assumed that caused by increased expression of their respective receptors. TGF-β stimulates fibroblasts to produce and deposit collagen and...
ECM factors. TGF-β also induces production of PDGF, which controls the rate of granulation tissue formation and stimulates collagen production during the later stages of wound healing. Furthermore, in the previous studies, the excessive ECM production in keloids is related to an increase or decrease of matrix metalloproteinases (MMP-1, -2, -3, -9, -13) and an increase of tissue inhibitor of metalloproteinases (TIMPs). However, the exact mechanism of keloids have not been known yet.

Symptomatic keloids have been treated in a variety ways, such as pressure dressings, antihistamine therapy, steroid injections, radiation therapy, chemotherapy (5-flurouracil, imiquimod, interferon-alpha, verapamil etc) and surgical excision. Lasers, silicone, and topical retinoids also have been used. Besides the existing treatment options, botulinum toxin A (BTXA) has been used in the treatment of keloids, and a good result of it have been reported. Clinical observations indicate that BTXA can improve the eventual appearance of keloids, as inhibit the growth of keloids as well. However, the mechanism of BTXA is unknown, aparted from the viewpoints that BTXA improves keloids by decreasing tensile force of muscle and skin near keloid tissue.

MMP-2, -9 are known as gelatinases, which degrade denatured collagen (types I and III) fibrils, type IV collagen, fibronectin and elastin. In addition, these enzymes can potentiates the degradation of ECM components byactivating collagenase-3 (MMP-13) and neutrophil collagenase (MMP-8). Therefore, gelatinases have an important role in physiologic soft tissue remodeling and pathologic wound healing. MMP-9 activity is crucial in the epithelization process, whereas MMP-2 activity is important during the prolonged remodeling phase. The MMP-2, -9 act on cleaved collagen more effectively than other MMPs. MMP-1 is known for the capacity of reducing previously formed ECM. For these reason, we hypothesized that BTXA administration can affect molecular mechanisms of keloid such as collagen synthesis and enzymatic activity of MMP or TIMP. In this study, we investigated the mRNA expression of type I, III collagen synthesis, MMP-1 and TIMP-1 in KFs after administration of various amounts of BTXA with or without TGF-β. We also evaluated enzymatic activity of MMP-2, 9 in KFs with zymography after administration of various amounts of BTXA with or without TGF-β as well.

II. MATERIALS AND METHODS

A. Isolation and culture keloid–derived fibroblast culture
Keloid-derived fibroblasts (KFs, n=5) were obtained from the central dermal layer of keloid within 12 months of onset. Informed consent, which was approved by the Yonsei University College of Medicine Institutional Review Board, was acquired from all patients. Keloids were diagnosed by plastic surgeons and confirmed by pathologists. The Waymouth method was used for the tissue culture. The obtained tissue was laid on petridish, and then washed twice with phosphate-buffered saline (PBS). A 7 ml of 0.25% trypsin solution was equilibrated at 37°C. This solution added in the tissue, and then cells were isolated from it by Pasteur pipet. After 10 minutes, the separated cells were cultured in 100 ml Dulbecco’s modified Eagle’s medium (DMEM; GIBCO, Grand Island, NY, USA) supplemented with heat-inactivated 10% fetal bovine serum (FBS) and penicillin (30 U/ml), streptomycin (300 mg/ml), and actinomysin, and then cen trifugation by 3500RPM for 15 seconds. After the precipitates were removed, re-centrifugation was done for 20 minutes to collect the next precipitates. The collected precipitates were suspended in 5ml of the culture medium, and incubated in a humidified incubator at 37°C and 5% CO2. The culture medium was changed in 2–3 day intervals. When primary cell culture reached confluent state, the culture medium was removed and washed with PBS. The cells on the bottom of culture container were isolated completely with 2ml of 0.025% trypsin. Three to four times subcultured fibroblasts were used in this study. The experimental group was added at various concentration of botulinum toxin A (BTXA) (Allergan Corp., Irvine, CA, USA), and control group was not.

B. Reverse transcriptase–polymerase chain reaction (RT–PCR)
Total RNA of KFs was isolated by a Trizol reagent (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer’s protocol. One microgram of total RNA was converted into the complementary DNA using a First Strand cDNA Synthesis kit (Promega Corp., Madison, WI, USA) with random primers. A polymerase chain reaction was performed using 2 × Taq Premix 2 (Solgent Co., Ltd., Seoul, Korea) and the synthetic gene-specific primers for collagen types I and III, MMP-1, and TIMP-1. Agarose gel electrophoresis was performed to separate samples of each reaction product, which was visualized by ethidium bromide staining, and photographed with 290-nm ultraviolet illumination. The density of each band was measured by Image J.

C. MMP zymography
Zymography was performed to evaluation of enzymatic activity of MMP-2 and MMP-9. Culture supernatants were collected and analyzed by gel substrate zymography. 10 mL of non-reducing sample buffer (125 mM Tris–HCl [pH 6.8], 10% [v/v] glycerol, 0.1% [w/v] BPB) was added to each sample.
III. RESULTS

A. The effect of BTXA on the mRNA expression of collagen type I and III in the KFs

The effect of BTXA on the collagen type I and III in the KFs was analyzed by RT-PCR. Type I collagen mRNA expression was not affected by BTXA administration, as well as by BTXA and TGF-β simultaneous administration. However, type III collagen mRNA expression was decreased significantly (p<0.05) after administration of BTXA 0.5 unit/10^5 cells compared with control group. The type III collagen mRNA expression was decreased significantly after TGF-β simultaneous administration in addition to BTXA 0.5 unit/10^5 cells (Fig. 1).

B. The effect of BTXA on the mRNA expression of MMP-1 and TIMP-1 in the KFs

The effect of BTXA on MMP-1 and TIMP-1 mRNA expression was analyzed by RT-PCR. BTXA increased mRNA expression of MMP-1 significantly compared with control group (p<0.05). The expression degree of MMP-1 increased more as BTXA concentration increased. (p<0.05) However, there was not significantly increased expression of MMP-1 mRNA after administration of TGF-β in addition to BTX. The mRNA expression of TIMP-1 was not affected by BTXA administration regardless of TGF-β administration (Fig. 2).

C. The effect of BTXA on MMP-2 and 9 enzymatic activity in the KFs

The MMP-2 and 9 were known as gelatinase A and B respectively, which break down ECM like other MMP as well. The enzymatic activity of MMP-2 and 9 were analyzed with zymography. MMP-2 activity was significantly increased after BTXA administration (p<0.05) whether TGF-β was administrated or not. (p<0.05) However, MMP-9 activity increased after BTXA administration in comparison with the control group, but not statistically significant (Fig. 3).

D. Statistics

Statistical analysis between experimental and control group were made using paired t-tests. Values were expressed as mean ± standard deviation (SD), with statistical significance set at p <0.05. Data analysis relied on standard software PASW Statistics 10.0 (SPSS, IBM, Inc, Chicago, IL, USA).

IV. DISCUSSION

Most therapeutic approaches for keloids remain clinically unsatisfactory, probably due to poor knowledge of the complex mechanisms underlying the process of excessive scarring. Hence, alternatives are needed.19,20,23,24 Recently, the use of BTXA was suggested to extend the spectrum of treatment for keloids.25 Xiao et al., report that a flattening of the lesion and a significant decrease in size was observed after BTXA injection in keloid.25 Uyesugi et al., declare that BTXA successfully treats the neuropathic symptoms associated with keloid scars.2 How-
ever, most studies included a small number cases and lacked the experimental basis at the molecular or genetic level. They cannot ascertain the detailed mechanism between BTXA and keloids as well.

We investigated the potential underlying molecular mecha-

Fig. 1. The effect of Botulinum toxin A on the mRNA expression of collagen type I and III in the keloid fibroblasts. (A): The effect of BTXA with or without TGF-β on the collagen type I and III in the KFs was analyzed by RT-PCR. Agarose gel electrophoresis was performed, and the density of each band stood for mRNA expression of collagen. (B): Type I collagen expression was not affected by BTXA and TGF-β. (C): Type III collagen mRNA expression significantly decrease in the keloid fibroblasts with BTXA 0.5 unit/10⁵ cells. (p<0.05) After administration of BTXA 0.5 unit/10⁵ cells and TGF-β simultaneously, type III collagen mRNA expression decreased significantly as well (col1: collagen type I, col3: collagen type III, BTXA: botulinum toxin A).

Fig. 2. The effect of Botulinum toxin A on the mRNA expression of MMP-1 and TIMP-1 in the keloid fibroblasts. (A): The effect of BTXA with or without TGF-β on the MMP-1 and TIMP-1 in the KFs was analyzed by RT-PCR. Agarose gel electrophoresis was performed, and the density of each band stood for mRNA expression of MMP-1 and TIMP-1. (B): MMP-1 mRNA expression increased by BTXA significantly. However, this increase was disappeared after administration of TGF-β in addition to BTXA. (C): TIMP-1 was not affected by administration of BTXA with TGF-β or not (BTXA: botulinum toxin A).
Administration of BTXA to keloid fibroblasts, the enzymatic activity of MMP-2 and 9 were examined by zymography. MMP-2 activity increased significantly after administration of BTXA \((p<0.05)\) and even after simultaneous administration of BTXA and TGF-β. MMP-9 activity was not affected by BTXA and TGF-β.

Regardless of the presence or absence of TGF-β, Type I and III collagens are accumulated high in keloid. It is the meaningful result that BTXA reduced type III collagen mRNA expression with or without TGF-β in this study, even though did not effect on type I collagen mRNA expression. However, contrary to our hypothesis, type I and III collagen mRNA expression was more increased with administration of BTXA 1.0 unit/10^5 cells than 0.5 unit/10^5 cells. For this reason, further evaluation for the therapeutic range of BTXA concentration has been needed.

MMP-1 mRNA expression in KFs was increased according to the BTXA concentration increment, however, not increased with TGF-β in addition to BTXA. Moreover, MMP-2 enzymatic activity in KFs was increased when BTXA administered regardless of the presence or absence of TGF-β. MMP-1 and MMP-2 are the fibroblast collagenase and gelatinase A respectively, and both break down ECM.

In addition, we expected that TIMP mRNA expression decreases after administration of BTXA, but no significant change was noted. We also expected that enzymatic activity of gelatinases (MMP-2, 9) increases with BTXA, but only MMP-2 activity increased according to the BTXA concentration. MMP-9 activity was not affected by BTXA and TGF-β. These may be explained that BTXA selectively affect MMP-1 and 2 or BTXA concentration of this study was not an effective range for TIMP and MMP-9. However, more work is needed to explain these results clearly.

Moreover, some previous studies have shown that BTXA decreases mRNA expression of TGF-β in KFs. In this study, after administration of BTXA with exogenous TGF-β, mRNA expression of type III collagen decreased and enzymatic activity of MMP-2 increased. These results may suggest that BTXA can block an effect of TGF-β on KFs as well as a gene expression TGF-β in KFs.

As for study limitations, there is conflicting research with this study. Gauglitz et al, declared that BTXA injection did not result in keloid regression, and no differences in ECM marker expression, collagen synthesis, or TGF was observed after BTXA administration on KFs. However, they did not assess the change of MMP activity in KFs, and there are a lot of enzymes for ECM degradation and ECM markers involving keloid growth and proliferation, so further experimental research was needed.

A better understanding of the pathophysiology of keloid scarring hold great promise for developing novel therapeutic strategies. In this regard, keloids are a result of excessive accumulation of ECM which has be partly caused by increased tensile force during the scar formation process. Additionally, the unbalance of cellular dynamics caused by the overabundance of cellular proliferation, and the lack of ECM degradation and cellular apoptosis plays a crucial role in the formation and growth of keloid. A novel management for keloid treatment should target these findings. We can assume that such a therapeutic effect (reduction of the tensile force during around the keloid wound, as well as effective regulation of the balance between cellular proliferation and apoptosis) could be reached by BTXA. There are two possible hypotheses evidences supporting the potential usefulness of BTXA for keloid treatment. First, BTXA prevents contraction of muscle and skin near keloid tissue, which decrease tensile force during the course of cicatrization. Second, some research showed that BTXA can influence on cellular dynamics and ECM degradation.

We, therefore, can assume that this study presents experimental data for the therapeutic effect of BTXA on keloid besides clinical studies.

**V. CONCLUSION**

In this study, we could provide an experimental base of BTXA for keloid management, and assume BTXA intralesional injection will be a good alternative for keloid management as well.

**REFERENCES**

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