Effects of Botulinum Toxin A on Cytokine Synthesis in a Cell Culture Model of Cutaneous Scarring

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Objective: To evaluate possible botulinum toxin A effects in a cell culture model.

Methods: In a cell culture model with dermal fibroblasts and microvascular endothelial cells, possible botulinum toxin A effects were evaluated. Cell proliferation and cytokine expression were analyzed using viability assays and enzyme-linked immunosorbent assay techniques.

Results: Neither cell proliferation nor cytokines and growth factors (interleukin 6, monocyte chemoattractant protein 2, fibroblast growth factor, macrophage colony-stimulating factor, and vascular endothelial growth factor) were affected by botulinum toxin A incubation.

Conclusions: The present data do not add evidence to suggest a significant therapeutic role of botulinum toxin A injections for cutaneous wound healing beyond chemoinmobilization. Further studies that include patient-specific cells of hypertrophic scars are required to better understand what role botulinum toxin A can play in the treatment of mature scar tissue.


Since its first description in 2000,1 chemoimmobilization of cutaneous wounds has become an accepted treatment, especially for traumatic and iatrogenic wounds of the face and neck. Targeted injection of botulinum toxin A into the musculature surrounding a wound or incision site minimizes mechanical distortion. As a secondary effect, inflammatory influences during the early healing phase are reduced. A broad body of literature2-5 has followed the first reported case and includes basic laboratory research, animal studies, clinical reviews, and prospective human trials. The underlying mechanism of action of this treatment concept is widely accepted: chemodenervation causes mechanical immobilization of the cutaneous wound. As a result, chronic inflammatory stimuli are minimized and their associated negative effects on scar formation are reduced. Less noticeable scars develop if treatment is initiated during the early phases of wound healing and continues for the typical duration of action of 2 to 3 months. In clinical practice, treatment is performed early: immediately after traumatic injury or before an elective operation.

More recently, Xiao et al6,7 reported the injection of botulinum toxin A into hypertrophic scars in an attempt to ameliorate various chronic scar-related symptoms. Erythema, pliability, and itching scores improved in this noncontrolled study in 19 patients, with an average follow-up of 6 months. The same authors showed shifts in cell cycle distribution in fibroblasts, which were explanted from hypertrophic scars and incubated with botulinum toxin A. Suppression of transforming growth factor β; and the inhibition of fibroblast growth was also reported.8 These authors suggest that the changes observed in vitro serve as an explanation for the clinical effects described herein.

Injecting mature cutaneous scars with botulinum toxin A represents a concept that differs fundamentally from the therapeutic approach to immobilize wounds in the early healing phase. Scar tissue, rather than surrounding musculature, becomes the therapeutic target; primary trophic and metabolic changes within the scar tissue, rather than secondary reduction of inflammation through immobilization, are intended; and the timing of treatment is late in the wound healing process rather than during the early phase.

The available body of in vitro data is small and does not conclusively show fundamentally relevant structural or metabolic effects of botulinum toxin A in cutaneous scar tissue on a cellular level. The data by Xiao et al8 derived from 8 scar tissue specimens are suggestive of an anti-
proliferative effect of botulinum toxin A on scar-derived fibroblasts. Both enzyme-linked immunosorbent assay (ELISA)–based measurement of transforming growth factor β1 and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay–based fibroblast proliferation were inhibited. Incubation was performed with a botulinum toxin A product less widely used to date in basic research.

The present study was designed to test whether effects of botulinum toxin A suggestive of cutaneous scar tissue modulation can be detected in a broader, multicellular model in vitro. Fibroblasts and endothelial cells mediate 2 central aspects of scar tissue: fibrosis and metabolism. Therefore, these 2 key cell types were selected for study in the present model.

A broad screening approach was intended to mirror the breadth and possible relevance of changes on the cellular level. Different cytokines and growth factors were analyzed with respect to the 3 phases of wound healing: interleukin 6 (IL-6) and monocyte chemoattractant protein 2 (MCP-2) represent markers of the inflammatory phase. Fibroblast growth factor (FGF), macrophage colony-stimulating factor (M-CSF), and vascular endothelial growth factor (VEGF) represent factors of proliferation and remodeling.

### METHODS

#### CELL CULTURE

Normal human fibroblasts (NHFs; Bio Whittaker Europe, Verviers, Belgium) were maintained in Dulbecco modified Eagle medium F-12 (Sigma-Aldrich, Munich, Germany) supplemented with 10% fetal bovine serum (Invitrogen, Darmstadt, Germany) and penicillin/streptomycin, 1% (Sigma-Aldrich) and were used for experiments at passages 5 through 9. Human dermal microvascular endothelial cells (HDMECs, adult donor, catalog number C-12212; PromoCell, Heidelberg, Germany) were maintained in endothelial cell growth medium MV (PromoCell, catalog number C-22020) and used for experiments at passages 5 through 6. The culture incubator was set at 37°C with 5% carbon dioxide.

#### BOTULINUM TOXIN A ASSAY

Botulinum toxin A (Botox; Allergan Inc, Irvine, California) was dissolved in phosphate-buffered saline at a concentration of 10.0 IU/mL. The storage temperature was −20°C.

#### CELL PROLIFERATION ASSAY

Normal human fibroblasts or HDMECs (3 × 10^4 cells in 200 µL of the respective culture medium per well) were seeded in triplicate into 96-well plates. After 24 hours, the medium was replaced by a medium containing predetermined concentrations of botulinum toxin A or the appropriate amount of phosphate-buffered saline, which served as a control. Four doses were analyzed: 1.0 IU per 4 mL (1.83 pg/mL), 2.5 IU per 4 mL (4.56 pg/mL), 5.0 IU per 4 mL (9.13 pg/mL), and 10.0 IU per 4 mL (18.25 pg/mL). After 48 hours and 72 hours, resazurin, (4.56 pg/mL), 5.0 IU per 4 mL (9.13 pg/mL), and 10.0 IU per 4 mL (18.25 pg/mL) botulinum toxin A. Culture medium containing the appropriate amount of phosphate-buffered saline was added to the control cells. After 48 hours or 72 hours, the supernatant was used for protein determination.

#### ENZYME-LINKED IMMUNOSORBENT ASSAY

Soluble cytokine production in the supernatants of cultured NHFs and HDMECs was tested by ELISA (DuoSet ELISA Development Systems; R&D Systems). Prior to use, the cell culture supernatants were centrifuged at 3000 rpm for 5 minutes. The DuoSet kits human FGF basic (catalog No. DY233), human IL-6 (DY206), human CCL-8/MCP-2 (DY281), human M-CSF (DY216), and human VEGF (DY293B) were used according to the manufacturer’s instructions. Measurements were obtained in triplicate.

### RESULTS

To analyze the effect of botulinum toxin A on proliferation of NHFs and HDMECs, the cell proliferation assay as described herein was carried out using the concentrations of botulinum toxin A listed in the “Cell Proliferation Assay” subsection of the “Methods” section. The results show that the toxin neither stimulates nor inhibits proliferation of NHFs and HDMECs at any of the concentrations used (Figure 1).

#### EXPRESSION OF CYTOKINES AND GROWTH FACTORS

After incubation with 1 IU of botulinum toxin A per 4 mL of culture medium, there were no statistically significant differences compared with controls in the expression of VEGF (P = .07), M-CSF (P = .30), and IL-6 (P = .18) in NHFs (Figure 2). The protein levels of FGF and MCP-2 were below the detection level of 5 pg/mL. Incubation of NHFs in a higher concentration of botulinum toxin A (2.5 IU) per 4 mL of culture medium for a longer period (72 hours) did not significantly change the expression of VEGF (P = .14), M-CSF (P = .74), and IL-6 (P = .22) in NHFs (Figure 3). The protein levels of FGF and MCP-2 remained below the detection level. Concerning HDMECs, we showed that botulinum toxin A at a concentration of 1 IU per 4 mL has no significant effect (P > .05) on the expression of FGF (P = .30).
Aspects of botulinum toxin A effects on wound healing other than mechanical chemoinmobilization are part of the present discussion.

Cutaneous wound healing is a complex process that may be categorized into 3 phases: inflammation, proliferation, and remodeling. The exact cellular mechanisms that occur during these phases of the wound healing process are not fully understood. Under physiologic conditions, a complex and coordinated interaction of multiple cytokines and growth factors with various cell types leads to the formation of a mature scar. Twelve months or later after the initial injury, this mature scar is typically fibrous, bradytrophic, and of reduced mechanical strength. Aberrations at various times during the wound healing process can lead to pathologic healing. Early effects include mechanical tension on the healing wound, which leads to prolonged inflammation and more visible scar hypertrophy. Effects shown in vitro after application of...
cyclic stretching on the dermal fibroblast correlate with this clinical observation, including rounding up of the cell bodies, disruption of fibroblast cytoplasmic extensions, and subsequently an increased tissue water content. In turn, absence of these effects in the unstretched control groups likely correlates with the indirect clinical effect of immobilization during the early healing phase.

In contrast, botulinum toxin A injections into mature scar tissue late in the healing process are suggested to induce changes on the cellular level in representative cell types. Clinical effects, such as decreased erythema and enhanced pliability, are signs of reduced metabolism and proliferative activity. To measure these effects, the proliferation assay was performed for both NHFs and HDMECs. After incubation with rising concentrations of botulinum toxin A (1.0-10.0 IU) and fluorescence measurement after resazurin incubation, no significant differences in proliferation rate were observed between the experimental and control groups. This is in contrast to a study with prostate cancer cells that revealed an inhibition of cell growth by incubation with 1 IU of botulinum toxin A. The present results seem relevant with respect to the key role of fibroblasts and endothelial cells in cutaneous wound healing. The absence of antiproliferative effects of botulinum toxin A suggests that it does not interfere with important cell proliferation during early wound healing processes. However, the absence of antiproliferative effects does not support the clinical observation of reduced thickening of hypertrophic scars after injection of botulinum toxin A.

With respect to the inflammatory phase and the key role of IL-6 in inflammatory tissue reactions, we investigated IL-6 expression after botulinum toxin A incubation. It is known that IL-6 modulates immune responses and is essential for timely wound healing. In IL-6-deficient mice, wound healing is delayed as a result of impaired re-epithelialization, angiogenesis, and macrophage infiltration. However, overexpression of IL-6 leads to increased activity of keratinocytes and may be a factor that favors scarring. Modulation of IL-6 expression may be useful in treating wounds, but in our study, botulinum toxin A had no effect on IL-6 synthesis on fibroblasts or on endothelial cells. Synthesis of IL-6 is regulated via the intracellular nuclear factor kB pathway, and an immediate effect of botulinum toxin A on IL-6 expression has not been conclusively demonstrated.

Endothelial damage and impaired microcirculation are important problems in healing wounds. Moreover, hypertrophic scars often show erythemalike lesions, which are associated with increased vascular permeability. In addition, the increased metabolic requirements during wound healing require neovascularization. Endothelial cells are therefore key for physiologic healing of cutaneous wounds. A recent animal study suggested that intradermal injection of botulinum toxin A could favor microcirculation by increased expression of VEGF and platelet/endothelial cell adhesion molecule. The detailed mechanism of this observation remains unknown. The authors of that study suggested that botulinum toxin A affects the autonomic sympathetic nervous system of the skin through the suppression of sympathetic neurons. This may lead to vasodilation. An increase in the density of the vessel walls has also been observed. One possible mechanism may be activation of endothelial cells.

The predominant aspect of the proliferation phase is neovascularization. Vascular endothelial growth factor is a typical proangiogenic factor, and extensive research has evaluated its role in angiogenesis during cutaneous wound healing. Kim et al documented a higher survival rate of cutaneous flaps after intradermal botulinum toxin A injections. The authors suggested an increased expression of VEGF messenger RNA for this effect. In our cell culture model, we found no effect of botulinum toxin A on VEGF protein synthesis in fibroblasts, with the amount of VEGF in the supernatants of endothelial cells below the very low detection level of the assay used (5 pg/mL). Expression of VEGF is complex and mediated through hypoxia inducible factor 1, a master transcription factor activated by environmental stresses, such as hypoxia and acidic pH, as well as by several growth factors and cytokines. Whether botulinum toxin A is able to interact with this mechanism remains unclear.

Macrophage colony-stimulating factor is known to facilitate monocyte survival, monocyte-to-macrophage conversion, and macrophage proliferation. It is suggested that upregulation of M-CSF plays an active role in wound healing and formation of scar tissues. This effect may be mediated by regulating endothelial cell chemokine expression, especially of proinflammatory factors such as monocyte chemotactic protein 1.

Several more cytokines and growth factors affect epithelialization and have the potential to be used for therapeutic modulation of cutaneous wound healing. In a rat model, topically applied M-CSF and basic FGF accelerated wound closure. Because these factors could mediate possible therapeutic effects of botulinum toxin A, we included them in the present experiment. In our cell culture model, no effect on the synthesis of FGF was found after incubation with botulinum toxin A in HDMECs. In NHFs, measurements of FGF were below the detection limit. Macrophage colony-stimulating factor was detectable in NHFs, but its expression level did not change after incubation with botulinum toxin A. Whether there are immediate effects of botulinum toxin A on fibroblasts is still subject to debate. In a rabbit model, Wang and colleagues studied the use of botulinum toxin A to treat hypertrophic scars. The authors showed a significant decrease of fibroblast activity and suppressed expression of collagen I and III in hypertrophic scars by botulinum toxin A. In addition, Xiao et al observed effects of botulinum toxin A on the expression of transforming growth factor β in fibroblasts of hypertrophic scars. An indirect effect of botulinum toxin A on immobilized wounds was documented in a rat study. Lee et al showed an early reduction of infiltration of inflammatory cells and less fibrosis in the wounds of animals injected with botulinum toxin A. The possible mechanisms through which botulinum toxin A modulates the expression of these cytokines are incompletely understood, and further research is required to elucidate whether botulinum toxin A affects pathways other than the well-researched interference with intracellular cytoskeletal proteins.

The situation regarding endothelial cells is similar: Madalinski and Kalinowski suggested pharmacologic sphincterotomy with botulinum toxin A in patients with anal fissure and showed that botulinum toxin A not only has an effect on the motor end plate but also influences
nitric oxide synthase of endothelial cells. This new biochemical pathway of botulinum toxin A influence might be regulated by decreased activity of the RhoA/Rho kinase signaling. This aspect is promising not only for patients undergoing sphincterotomy but also in those with wound healing difficulties. Further investigations are necessary for a better understanding of this mechanism.

The endothelial cytoskeleton is believed to play an important role in the regulation of endothelial permeability. In a cell culture model, botulinum toxin C2 induced interendothelial gaps. A further effect of botulinum toxin A on endothelial cells was observed by Aepfelbacher et al., who found that botulinum toxin A blocked migration of human umbilical vein endothelial cells in an in vitro wound repair model. In our study, we could not identify any effect of botulinum toxin A on endothelial cells with respect to synthesis of the tested cytokines and growth factors. The results of the present study do not consistently confirm some of the published clinical and basic data regarding the effects of botulinum toxin A on mature scar tissue.

There are some limitations to our study. We used a commercial cell line of dermal fibroblasts and microvascular endothelial cells and did not examine patient-specific scar tissue. Further studies that include a patient-specific cell culture model will be necessary to rule out the effects of pharmacologic modulators on healing wounds.

**CONCLUSIONS**

The present study was conducted to elucidate a potential role of botulinum toxin A on mature cutaneous scar tissue. A range of chemokines representative of important clinical effects was measured in a multicellular model. No relevant effects of botulinum toxin A on any of the investigated effects were observed. The present data do not add evidence to suggest a significant therapeutic role of botulinum toxin A injections concerning cellular effects on fibroblasts and endothelial cells. This is in contrast to the well-established mechanism of immobilizing scars during the early phases of wound healing. Further studies that include patient-specific cells of hypertrophic scars are required to better understand what role botulinum toxin A can play in the treatment of mature scar tissue and which mechanisms can explain possible therapeutic effects on a cellular level.

**REFERENCES**