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Anti-photoaging potential of Botulinum Toxin Type A in UVB-induced premature senescence of human dermal fibroblasts *in vitro* through decreasing senescence-related proteins

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ABSTRACT

This study was aimed to evaluate the anti-photoaging effects of Botulinum Toxin Type A (BoNTA) in Ultraviolet B-induced premature senescence (UVB-SIPS) of human dermal fibroblasts (HDFs) *in vitro* and the underlying mechanism. We established a stress-induced premature senescence model by repeated subcytotoxic exposures to Ultraviolet B (UVB) irradiation. The aging condition was determined by cytochemical staining of senescence-associated β-galactosidase (SA-β-gal). The tumor suppressor and senescence-associated protein levels of $p16^{INK-4a}$, $p21^{WAF-1}$, and p53 were estimated by Western blotting. The G1 phase cell growth arrest was analyzed by flow cytometry. The mRNA expressions of p16, p21, p53, COL1a1, COL3a1, MMP1, and MMP3 were determined by real-time PCR. The level of Col-1, Col-3, MMP-1, and MMP-3 were determined by ELISA. Compared with the UVB-irradiated group, we found that the irradiated fibroblasts additionally treated with BoNTA demonstrated a decrease in the expression of SA-β-gal, a decrease in the level of tumor suppressor and senescence-associated proteins, a decrease in the G1 phase cell proportion, an increase in the production of Col-1 and Col-3, and a decrease in the secretion of MMP-1 and MMP-3, in a dose-dependent manner. Taken together, these results indicate that BoNTA significantly antagonizes premature senescence induced by UVB in HDFs *in vitro*, therefore potential of intradermal BoNTA injection as anti-photoaging treatment still remains a question.

1. Introduction

Skin changes are the most obvious signs of aging. Skin aging can be divided into intrinsic or chronologic aging, which affects all body organs, and extrinsic aging, which occurs due to exposure to environmental factors. One of the most important extrinsic aging factors is sunlight, which causes skin photoaging [1–3]. UVB interacts with cellular chromatophores and photosensitizers, resulting in the generation of reactive oxygen species, DNA damage, and the activation of signaling pathways related to senescence and connective tissue degradation [4]. Premature senescence of human dermal fibroblasts (HDFs) can be induced by exposures to a variety of oxidative stress and DNA damaging agents. A model of stress-induced premature senescence of HDFs by using UVB (UVB-SIPS) represents an alternative *in vitro* model in photoaging research for investigating photoaging-related mechanisms [5–8].

Fibroblast is the main cellular component in dermis that produces procollagen type I (proCol-1) and type III (proCol-3), and secretes Matrix metalloproteinases (MMPs). ProCol-1 and proCol-3 are the precursors of collagen type I (Col-1) and type III (Col-3), respectively. The dermis contains predominantly Col-1 (85–90%) with lesser amounts of Col-3 (10-15%). Collagen is the most abundant basic element of fibrous components in the dermis and is responsible for maintaining the structural integrity of the skin by joining cells together and to the extracellular matrix (ECM). MMPs are one family of structurally related enzymes that have the collective ability to degrade nearly all ECM components, including collagens [9,10].

Wrinkle formation in the skin is closely associated with the degradation of ECM. HDFs in UVB-SIPS are known to secrete more matrix metalloproteinase-1 (MMP-1) and matrix metalloproteinase-3 (MMP-3) [11–13] and synthesize less Col-1 and Col-3 [14–16], contributing to wrinkle formation. To date, there are several agents that reduce the secretions of MMP-1 and MMP-3 [17,18], and increase the expressions of Col-1 and Col-3 [10,15,16] on photoaged HDFs have been proposed as possible anti-photoaging agents.

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Botulinum Toxin Type A (BoNTA) is one of seven neurotoxins produced by *Clostridium botulinum*. BoNTA induces chemodenervation through its action on presynaptic neurons, preventing the release of acetylcholine and leading to functional denervation of striated muscle for 2–6 months after injection. This results in muscle fiber atrophy and subsequent clinical flaccid paralysis [19]. Consequently, its cosmetic use in treating wrinkles induced by muscle hyperactivity is widespread [20,21].

Some physicians have observed a face-lifting effect after intradermal injection of BoNTA to the mid and lower face [22,23]. It has been claimed that greater collagen synthesis, lower sebum production, and smaller facial pore size cause the effect [24,25]. However, it has been reported that needle pricks themselves without BoNTA can make the skin become smoother [26]. Therefore the face-lifting effects of intradermal BoNTA injection were controversial. Nevertheless, Sang-Ha Oh, et al. demonstrated that BoNTA significantly increased overall collagen levels and decreased collagen degradation of non-aged HDFs *in vitro* [9]. This showed the positive effects of BoNTA on HDFs for remodeling skin. However, it has not been demonstrated yet whether BoNTA has any anti-photoaging effect on the HDFs.

In this study, we incubated UVB-stressed HDFs with BoNTA at three different concentrations (1, 2.5 or 5 U/10^6 cells) for 48 h. We evaluated the effects of BoNTA on collagen production, collagen degradation, and senescence-related markers in photoaged HDFs at 96 h after the last stress. We aim to evaluate the anti-photoaging effects of BoNTA on UVB-SIPS model of HDFs and the underlying mechanism.

2. Materials and methods

2.1. Cell culture

Normal human skin samples were obtained from circumcisions in accordance with the ethical committee approval process of Jiangsu Provincial People's Hospital (Nanjing, China). Specimens were sterilized in 70% ethanol, minced, and incubated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin in an atmosphere of 5% CO_2 at 37 °C. Dermal HDFs normally grew from the explants after 5–7 days. The cells from passages 8–11 were used in this study.

2.2. Ultraviolet B (UVB) irradiation

UVB-stressed cells were irradiated at a subcytotoxic dose of 10 mJ/cm^2 once a day for 5 days [5]. Before UVB irradiation, the medium was removed and covered with phosphate buffered saline (PBS). UVB irradiation was delivered by using a Philips TL 20W/12 (Eindhoven, The Netherlands), a fluorescent bulb emitting 280–320 nm wavelength with a peak at 313 nm. Irradiation output was monitored by using a Waldmann UV-meter (Waldmann, Villigen-Schwenningen, Germany).

2.3. Group divisions and Botulinum Toxin Type A (BoNTA) treatment

The cells were divided into six groups: (1) control group: kept in the same culture condition for 5 days, then serum-starved for 4 days, without receiving UVB irradiation or BoNTA treatment; (2) UVB-SIPS group: the cells received UVB irradiation for 5 days, then serum-starved for 4 days, without receiving BoNTA treatment; (3) BoNTA group: kept in the same culture condition for 5 days, serum-starved for 2 days, then were incubated in serum-free DMEM with BoNTA at a dose of 5 U/10^6 cells for 2 days, without receiving UVB irradiation; (4), (5), (6) UVB-SIPS + BoNTA groups: the cells received UVB irradiation for 5 days, serum-starved for 2 days, and then were incubated in serum-free DMEM with BoNTA at three different doses (1, 2.5 or 5 U/10^6 cells, respectively) for 2 days. All groups were washed with PBS and the medium was changed once everyday, except during BoNTA treatment. While the other groups were incubated with BoNTA, the medium of Control and UVB groups were not changed. All groups were studied at 24 h after the last treatment. Each method of detection consists of these six groups of cell culture, containing about 1×10^7 cells in each group. There were at least 3 samples that were statistically studied in each group. The experiment was repeated thrice independently. The BoNTA used in this study was manufactured by Lanzhou Institute of Biological Products Co., Ltd., Lanzhou, China.

2.4. ß-galactosidase staining for detection of senescent cells

To measure one of the biomarkers of senescence, senescence-associated ß-galactosidase (SA-ß-gal) staining was performed. The cells were fixed in 2% formaldehyde/0.2% glutaraldehyde, rinsed with PBS and incubated overnight at 37 °C with fresh SA-ß-gal stain solution, which is composed of 1 mg of 5-bromo-4-chloro-3-indolyl ß-D galactoside (X-Gal) per mL (stock = 20 mg of dimethylformamide per mL), 40 mM citric acid, sodium phosphate(pH 6.0), 5 mM potassium ferrocyanide, 150 mM NaCl, and 2 mM MgCl2. The SA-β-gal activity was assessed by using the method originally described by Oh, et al. [27]: All of the blue-stained cells that were found in ten fields (5×10^5 cells) were counted under a microscope with × 100 magnification and expressed as the percentage of positive cells. To avoid cell staining that is caused by cell confluence rather than by proliferative senescence, the assay was performed in subconfluent cultures displaying comparable cell density.

2.5. Flow cytometry for detection of G1 phase-cell percentage

To determine whether UVB-stressed HDFs exhibit cell growth arrest, cell-cycle analysis with flow cytometry was performed. HDFs were fixed with 70% alcohol, washed twice with PBS, digested with RNase and stained with propidium iodide (PI). A flow cytometer (FAC-Scan, BD, NJ, USA) was used to gather data and images, to analyze the cell cycle, and to calculate the percentage of cells in the G1 phase.

2.6. Western blotting for detection of $p16^{INK-4a}$, $p21^{WAF-1}$, and p53

To investigate the level of senescence-related proteins ($p16^{INK-4a}$, $p21^{WAF-1}$, and p53 proteins), Western Blotting analysis was performed. The cells were lyzed in 62.5 mM Tris–HCl (pH 6.8) containing 2% w/v SDS, and the protein concentration was determined by the Pierce BCA assay (Thermo Fisher Scientific, Rockford, IL, USA). Mercaptoethanol and bromophenol blue were added to make the final composition equivalent to the LaemmLi sample buffer. Samples were fractionated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto Immobilon-P membrane (Millipore, Billerica, MA, USA). Rabbit anti-mouse HRP (1:1000 dilution) and goat anti-rabbit HRP (1:1000 dilution) were used as secondary antibodies (Biotime, Haimen, China). Antibody binding was visualized via Pierce ECL reagents (Thermo Fisher Scientific). We used mouse monoclonal antibodies against p53 and $p21^{WAF-1}$ (Cell Signaling Technology, California, USA), rabbit monoclonal antibody against $p16^{INK-4a}$ (sc-601) (Bethyl Laboratories, USA), and monoclonal anti-actin antibody (Beyotime, China) as a control. Quantification of protein bands was established by Band-Scan software (PROZYME, San Leandro®, California).