

RESEARCH ARTICLE

Effect of Botulinum Toxin A on Proliferation and Apoptosis in the T47D Breast Cancer Cell Line

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Abstract

The present study was performed to assess the activity of the botulinum toxin A on breast cancer cells. The T47D cell line was exposed to diverse concentrations of the botulinum toxin A and cell viability and apoptosis were estimated using MTT and propidium iodine/annexin V methods, respectively. Botulinum toxin A exerted greater cytotoxic activity in T47D cells in comparison with MCF10A normal cells; this appeared to be via apoptotic processes caspase-3 and -7. In conclusion, botulinum toxin A induces caspase-3 and -7 dependent apoptotic processes in the T47D breast cancer cell line.

Keywords: Breast cancer - botulinum toxin A - apoptosis - caspases

Asian Pacific J Cancer Prev, 14 (2), 891-894

Introduction

Breast cancer in women is a major health problem worldwide, with approximately 1.3 million women was estimated to be diagnosed with breast cancer in 2008 (Smith et al., 2012; Bray et al., 2013). Breast cancer is a heterogeneous disease in terms of gene expression, morphology, clinical course, and response to treatment. Therapeutic options for this breast cancer include surgery, adjuvant chemotherapy, radiotherapy, hormonal therapy or targeted therapy (Kim et al., 2012; Lee et al., 2012; Sendur et al., 2012; Truin et al., 2012; Zhou et al., 2012; Edge, 2013).

The most widely known action of botulinum toxin A (BtxA) is its relaxing effect on skeletal muscles. BtxA is therefore widely used for the symptomatic relief of spasticity, and other movement disorders. Recently there has been great interest in the use of botulinum toxin type A for prostate cancer. This interest stems from the fact that BtxA inhibits the growth of LNCaP human prostate cancer cells *in vitro* and *in vivo* and induces apoptosis in a dose-dependent manner (Mazo et al., 2008; Karsenty et al., 2009; Proietti et al., 2012). Moreover, BtxA has been shown for up-regulate 167 genes and down-regulate 60 genes relevant to focal adhesion, cell adhesion molecules, adherents and gap junction related pathways in carcinoma cell lines (Thirunavukkarasus et al., 2011; Gorgal et al., 2012; Nam et al., 2012; Tegenge et al., 2012). The

objective of this study was to test the activity of BtxA against breast cancer cell proliferation underlying this phenomenon as a trial for developing a novel functional anticancer drug. Thus, we investigated the effect of BtxA on cell proliferation and apoptosis using T47D human breast cancer cell line. The results showed that treatment allowed for equivalent loss of cell viability in cancerous cells when compared to a high dose of BtxA.

Materials and Methods

Cell culture

MCF10A cells (CRL-10317, ATCC), a spontaneously immortalized but non-tumoral cell line, were cultured in DMEM/F-12 medium (PAA, Carlsbad, CA) supplemented with 10 µg/mL of human insulin, 20 ng/mL of epidermal growth factor, 0.5 µg/mL of hydrocortisone (Sigma, St. Louis, MO), 5% horse serum (Invitrogen), 100 U/mL of penicillin and 100 µg/mL of streptomycin (PAA). T47D cells (HTB-133, ATCC), a triple positive breast cancer cell line, were grown in RPMI-1640 medium (Sigma, St. Louis, MO), supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 µg/mL of streptomycin.

Botulinum toxin A

Botox[®], injectable therapeutic agent, contains 4.8 ng of BtxA per vial of 100 units /Allergan, Inc., Markham,

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Ontario, Canada). It was used as freshly reconstituted (less than 2 h, stored at 4 °C) solution in sterile 0.9% saline.

Determination of cell viability

The effect of BtxA on T47D cells viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) assay. Briefly, 1×10^4 cells/well were treated with different concentrations of BtxA (0-15 U). After 24 h incubation the cells were washed twice with phosphate buffered saline (PBS) and MTT (0.5 mg/mL PBS) was added to each well and incubated at 37°C for 30 min. The formazan crystals that formed were dissolved by adding dimethyl sulfoxide (DMSO, 100 μ L/well), and the absorbance was read at 570 nm using a microplate reader (Model 3550; BIO-RAD, Richmond, USA). The reduction in cell viability after BtxA treatment was expressed in terms of control (nonBtxA treated) cells. Percentages of cell survival were calculated as follows: %cell survival=(absorbance of treated cells/absorbance of cells with vehicle solvent)X100. The half inhibitory concentration (IC_{50}) was calculated from the dose-response curve obtained by plotting the percentage of cell survival versus the concentration of BtxA.

Morphological studies

Morphological changes consistent with cell death were observed by using a normal inverted microscope. Cells were treated for 24 h with BtxA (0-15 U). Untreated cells were used for a negative control. The morphological changes of the cells were observed 24 h post-treatment.

FACS analysis, PI and PI/Annexin V staining

In order to determine the effect of BtxA on T47D cells, FACS analysis was carried out. For propidium iodide (PI) staining, cells were seeded in 6-well plates at a density of 1×10^4 cells/mL. After 24 h of attachment, cancer cells were treated with indicated BtxA concentrations for different time intervals. Floating and attached cells were harvested, washed in PBS, fixed in ice-cold ethanol (70% v/v) and stored at -20°C. For analysis, cells were washed in PBS and suspended in PI (25 mg/mL) in PBS with RNase A (200 μ g/mL). For PI/Annexin V double staining, treated cells were harvested and suspended in binding buffer (HEPES pH 7.4, $CaCl_2$ 2.5 mM, NaCl 140 mM). Aliquots of cells were incubated for 15 min with Annexin V FITC and PI (5 μ g/mL) (Invitrogen). During all FACS analyses, 10^4 events for each sample were analyzed. Flow cytometry analyses were carried out on a FACScalibur system (BD Biosciences) followed by analysis using CellQuest Pro software (BD Biosciences).

Caspase-Glo 3/7 assay

The influence of BtxA on caspase 3/7 activity in T47D cell line was detected using Caspase-Glo 3/7 Assay kit (Promega). Cells cultured in PRMI were seeded in 96-well plates and treated with the BtxA IC_{50} or DMSO (solvent control). After 24 h treatment, 100 μ l of caspase 3/7 reagent were added to each well, mixed and incubated for 1 h at room temperature. Luminescence was measured using well Infinite M2000 Pro™ instrument (Tecan). Caspase 3/7 activity was expressed as percentage of the untreated

control.

Statistics

All experiments were repeated at least three times. The data are presented as means \pm standard errors. The statistical analysis was performed using paired Student's t-test and a value of $p < 0.05$ was considered statistically significant.

Results

BtxA exhibit cytotoxic properties in T47D cells

In order to evaluate the effect of the BtxA, an MTT assay was monitored on T47D cells. After 24 h of exposure to BtxA, significant inhibitory effects on proliferation were observed. The BtxA exhibited cytotoxic activity toward cancer cells tested, displaying reduced IC_{50} (5.3 U BtxA) (Figure 1). Shorter (3-, 6-, 12-hour) exposures to BtxA also induced cytotoxicity (data not shown). These results suggest a specific cytotoxic effect against cancer cells.

Morphological evaluation

The effect of BtxA on cell adhesion was examined by detaching the treated cells from the cultured. Upon treatment with BtxA for 24 h, T47D cells displayed significant morphological changes. Observations showed that cells were detached from the monolayer and were rounded up. Furthermore, some of the cells had become granulated and vacuolated, possessed condensed chromatin, and displayed membrane shrinkage. First, major phenotypic changes were noticed when cancer cells were incubated in the presence of BtxA (Figure 2). Treated

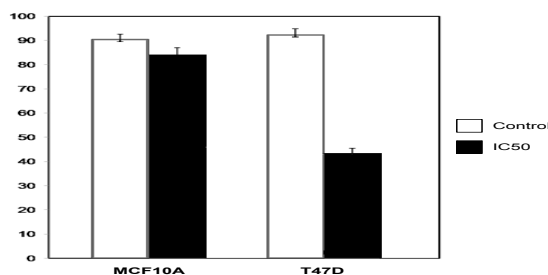


Figure 1. The BtxA-induced Inhibitory Effect on Proliferation of T47D Cells. MCF10A and T47D cells were incubated during 24 h in presence or absence of IC_{50} concentration (5.3 U) of BtxA. MTT assay was performed as detailed in materials and methods. The treatment results are shown as a percentage of the un-induced vehicle control (+/-SE)

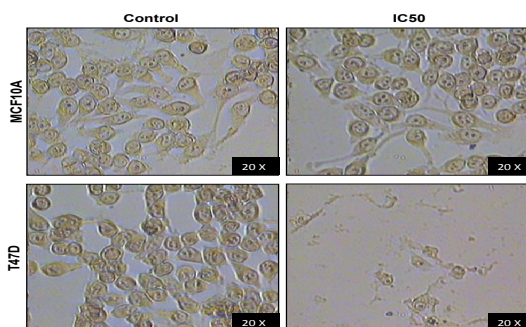


Figure 2. Cytotoxic Effects of BtxA towards T47D Cells. MCF10A and T47D cells were incubated during 24 h in presence or absence of IC_{50} concentration (5.3 U) of BtxA. Cells were photographed using an inverted microscope

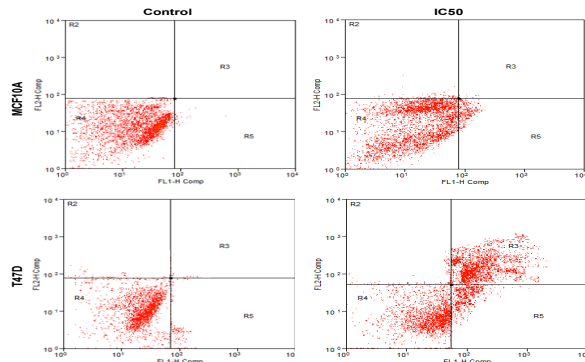


Figure 3. BtxA Induces Apoptosis in T47D cells. Untreated and treated MCF10A and T47D cells with IC50 concentration (5.3 U) of BtxA during 24 h, as evidenced by PI/Annexin V double staining and FACS analysis

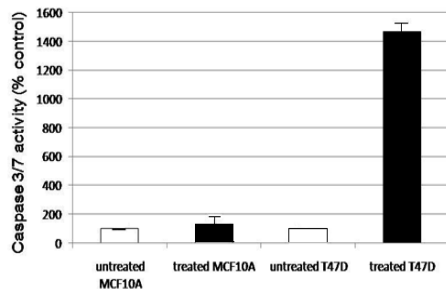


Figure 4. BtxA Induces Apoptosis via Caspases-3 and -7 in T47D Cells. Untreated and treated MCF10A and T47D cells with IC50 concentration (5.3 U) of BtxA during 24 h. Caspase 3/7 activity was expressed as percentage of the untreated control (+/-SE)

T47D cells exhibited a reduced round-shape cellular form before complete detachment from cell culture dish.

BtxA induces apoptosis via caspase-3 in T47D cells

We have next investigated the role of BtxA in cell death. To this end, cells were collected after 24 h treatment with respective IC₅₀ (5.3 U BtxA), double-stained with PI and Annexin V-FITC and analyzed by FACS (Figure 3). The kinetic of cell interaction with Annexin V revealed that BtxA acts very fast (not shown). Interestingly, with T47D we revealed a preapoptotic sub-population of 19.8% (PI-/Annexin V+ quadrant), an apoptotic sub-population of 52.1% (PI+/Annexin V+ quadrant), and a necrotic sub-population of 2.3% (PI+/Annexin V- quadrant). On the other hand, the kinetic for MCF10A non-tumoral cells revealed a preapoptotic sub-population of 10.3% (PI-/Annexin V+ quadrant), an apoptotic sub-population of 1.1% (PI+/Annexin V+ quadrant), and a necrotic sub-population of 2.3% (PI+/Annexin V- quadrant). These findings suggested that the process of death induced by BtxA in T47D cells is apoptosis pathway. Consistent with this observation, the caspase-3 activity in T47D cells treated with BtxA was fourteen fold higher than in those MCF10A cells treated with BtxA (Figure 4).

Discussion

Woman breast cancer is the most important cause of mortality in the world (Haghighat et al., 2012; Yang et al., 2012). Nowadays, some cytotoxic agents are used

for its treatment including doxorubicin, daunorubicin, bleomycin, and cisplatin. However, they are costly and known to induce several side effects such as myelosuppression, anemia, and most importantly the generation of cellular resistance. For this, it is important to find alternative therapies or drugs to overcome these drawbacks (Hamedeyzdan et al., 2012; Hassan et al., 2012; Saraydin et al., 2012).

The present study was conducted to analyze the effects of BtxA on human breast cancer cell line, T47D. The results clearly indicate BtxA is highly cytotoxic to the cells in a dose and time dependent manner. No effect of low concentrations of BtxA (≤ 0.1 U) on cell viability was observed. However, at high BtxA concentrations (≥ 5 U), cell viability decreased significantly to approximately 50% of control levels.

Therefore, we investigated the apoptotic changes in mammary cancer cells induced by BtxA, using dual apoptosis assay kit. The morphological changes (cells with a reduced round-shape cellular) occurred early, after 3 h treatment. These specific shapes are well known as the apoptotic volume decrease (AVD) due to massive efflux of K⁺ and Cl⁻ through their specific channels, leading to water escape from the cytoplasm, the latter being considered as a major hallmark of apoptotic cells (Gorgal et al., 2012; Hasegawa et al., 2012; Yurinskaya et al., 2012). We found BtxA induced cytotoxicity and apoptosis in T47D cells.

Inhibiting apoptosis is a possible mechanism of tumor development, and many chemopreventive agents act through the induction of apoptosis to inhibit the carcinogenic process. Therefore, the induction of apoptosis in breast cancer cells may be one of the mechanisms of the anticancer effect of BtxA. Here, we found that BtxA induced apoptotic cell death in T47D cells. This finding was similar to another study showing that BtxA inhibits the growth of LNCaP human prostate cancer cells *in vitro* and *in vivo* (Karsenty et al., 2009). Moreover, BtxA has been shown for induces apoptosis in cells transformed by PLC- γ I overexpression (Nam et al., 2012).

Caspases, comprised of 12 proteins, are a family of cysteinyl aspartate-specific proteases involved in apoptosis and are subdivided into initiator (caspases-8, -9 and -10) and executioner (caspases-3, -6 and -7) caspases. The intrinsic and extrinsic pathways converge at caspase-3. Active caspases and 8 of the intrinsic and extrinsic pathways, respectively, have been shown to directly cleave and activate the effector protease caspase-3 (Kominami et al., 2012). In the present study, we have shown that caspases-3 and -7 are activated during BtxA-mediated apoptosis.

Therefore, it can be suggested that BtxA treatment may be used as an alternative treatment against breast cancer. However, the mechanism and pathways by which BtxA induced cytotoxic activity on T47D human breast cancer cell line need further investigation.

Acknowledgements

C Bandala was recipient of a fellowship from Mexico's National Council of Science and Technology (CONACyT)

and from Mexican Institute of Social Security (IMSS) during the performance of this work.

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