RESEARCH ARTICLE

Inactivated Sendai Virus Strain Tianjin Induces Apoptosis in Human Breast Cancer MDA-MB-231 Cells

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Abstract

Sendai virus strain Tianjin is a novel genotype. Here, we investigate the antitumor and proapoptotic effects of ultraviolet-inactivated Sendai virus strain Tianjin (UV-Tianjin) on human breast cancer MDA-MB-231 cells *in vitro*, as well as the involvement of the apoptotic pathway in the mechanism of UV-Tianjin-induced antitumor effects. MTT assays showed that treatment with UV-Tianjin dose-dependently inhibited the proliferation of MDA-MB-231 cells but not normal MCF 10A breast epithelium cells. Hoechst staining and flow cytometric analysis revealed that UV-Tianjin induced apoptosis of MDA-MB-231 cells in a dose-dependent manner. Moreover, UV-Tianjin treatment resulted in reduction in the mitochondria membrane potential (MMP) and release of cytochrome complex (cyt c) via regulation of Bax and Bcl-2, as well as activation of caspase-9, caspase-3, Fas, FasL and caspase-8 in MDA-MB-231 cells. In summary, our study suggests that UV-Tianjin exhibits anticancer activity in human breast cancer MDA-MB-231 cells through inducing apoptosis, which may involve both the endogenous mitochondrial and exogenous death receptor pathways.

Keywords: Sendai virus strain Tianjin - human breast cancer MDA-MB-231 cells - apoptosis - caspase

Asian Pac J Cancer Prev, **15 (12)**, 5023-5028

Introduction

Breast cancer is a life-threatening disease among women worldwide with the rate of reported incidence and mortality increasing annually (Jemal et al., 2011; Ibrahim et al., 2013). Although chemotherapy is effective against breast cancer, it is accompanied by varied side effects including vasomotor syndrome, nausea and vomiting, postmastectomy edema, hair loss and psychological stress (Gonzalez-Angulo et al., 2007; Kado et al., 2012; Wang et al., 2012; Love et al., 2013). Furthermore, a recent study has reported that Trastuzumab, an HER2-targeting agent with efficacy in metastatic HER2-positive breast cancer patients, showed more adverse events, especially cardiac toxicity, in combination with chemotherapy or hormone therapy in HER2-positive metastatic breast cancer patients (Zhu et al., 2013). Therefore, there is an urgent need to develop new and more effective therapeutic agents with lower side effects for the treatment of breast cancer.

Oncolytic virotherapy is a promising treatment modality that uses replication-competent viruses to destroy cancers. A variety of viruses have been developed as oncolytic therapeutics, including adenovirus, vaccinia virus, measles virus, herpes simplex virus, vesicular stomatitis virus, Newcastle disease virus, and reovirus (Russell et al., 2012; He et al., 2014; Ilkow et al., 2014; Schenk et al., 2014). However, very few studies have been conducted on the antitumor effect of inactivated replication-defective viral particles that lack oncolytic ability. Sendai virus, also known as murine parainfluenza virus type 1 or hemagglutinating virus of Japan, is a member of the genus Respirovirus within the family Paramyxoviridae, members of which primarily infect mammals. Ultraviolet-inactivated, replication-defective Sendai virus [hemagglutinating virus of Japan envelope (HVJ-E)] is considered to be a safe and efficient nonviral vector for drug delivery because it can incorporate DNA, RNA, proteins, or drugs and deliver them into cells (Kaneda, 2003; Ito et al., 2005; Kaneda et al., 2005; Zhang et al., 2008). In addition, HVJ-E has been extensively used in anticancer vaccination strategies (Hiraoka et al., 2004; Yamano et al., 2006). Recent studies have shown that HVJ-E induces apoptosis in castration-resistant human prostate cancer cells and human glioblastomas in a dose-dependent manner, without any toxic effects on nonmalignant prostate epithelial cells and primary human astrocytes (Kawaguchi et al., 2009; Tanaka et al., 2010). However, the molecular mechanism of HVJ-E-induced apoptosis in cancer cells remains to be further elucidated.

Sendai virus strain Tianjin was isolated from the lungs of a marmoset in 1999 and proved to be a novel genotype of Sendai virus (Shi et al., 2008). In our previous study, ultraviolet-inactivated Tianjin strain (UV-Tianjin) suppressed the growth of colon carcinomas in mice by inducing an antitumor immune response and tumor-cell apoptosis (Shi et al., 2013). Furthermore, UV-Tianjin induces apoptosis of rat glioma cells both *in vitro* and *in vivo* (Han et al., 2013). This study was designed to

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investigate the effect of UV-Tianjin on the proliferation and apoptosis of the human breast cancer cell line MDA-MB-231 *in vitro*, and the possible mechanism.

Materials and Methods

Antibodies

Anti-caspase-7 (9492), anti-caspase-8 (9746) and anti-caspase-9 (9502) antibodies were obtained from Cell Signaling Technology (Shanghai, China). Anti-Bcl-2 (sc-509), anti-Bax (sc-20067), anti-cyt c (sc-13561), anti-Fas (sc-1023), anti-FasL (sc-6237) and anti-β-actin (sc-8432) antibodies were purchased from Santa Cruz Biotechnology (Beijing, China).

Cell culture and virus

Human breast cancer cell line MDA-MB-231 and human breast epithelial cell line MCF 10A were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). MDA-MB-231 cells were maintained in Leibovitz's L-15 media containing 10% (v/v) fetal bovine serum (Hyclone, Tianjin, China) at 37°C in non-CO_2 conditions. MCF 10A cells were maintained in Dulbecco's modified Eagle's medium/nutrient mixture F-12 (Invitrogen, Beijing, China) supplemented with 5% (v/v) horse serum (Invitrogen, Beijing, China), 20 ng/ml human recombinant EGF (R&D, Beijing, China), 0.5 μg/ml hydrocortisone (Sigma, Beijing, China), 100 ng/ml cholera toxin (Sigma, Beijing, China), 10 μg/ml bovine insulin (Sigma, Beijing, China) at 37°C in a 5% CO_2 incubator. Sendai virus strain Tianjin (GenBank: EF679198) was propagated in the chorioallantoic fluid from 9 to 11 day-old chicken eggs, after which it was purified by centrifugation and inactivated by UV irradiation (99 mJ/cm^2), as previously described (Kaneda et al., 2002). Inactivated virus could not replicate, but its capacity for viral fusion remains intact.

Cell viability assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma-Aldrich, Shanghai, China) assay was carried out as described previously (Li et al., 2013). In brief, MDA-MB-231 or MCF 10A cells were seeded at a density of 2.0×10^4 cells/well in 96-well culture plates. After 24 h incubation, UV-Tianjin [multiplicity of infection (MOI): 10-400] was added to the monolayer and the culture continued for an additional 24 h. Then 10 μl of 5 mg/ml MTT was added to each well and re-incubated for an additional 4 h at 37°C. After removal of the MTT-containing medium, 100 μl of DMSO was added to dissolve the water insoluble formazan. Finally, the optical density (OD) value of each well was measured using a microplate reader (Bio-Rad, Hercules, CA, USA) at 490 nm. The following formula was used to calculate cell viability.

Viability (%)=(OD490sample-OD490blank) / (OD490control-OD490 blank)×100 %.

Hoechst staining

MDA-MB-231 cells were plated at a density of 2×10^5 cells/well onto cover slips in 6-well plates. After incubation for 24 h, the cells were treated with UV-Tianjin (MOI: 40 or 80) for 24 h. Then the cells were washed twice with phosphate buffer saline (PBS) and incubated with 10 mg/ml Hoechst 33342 (KeyGen Biotech, Nanjing, China) for 20 min in the dark. After rinsing with PBS, the cells were examined using a fluorescent microscope (Nikon Eclipse E600, Tokyo, Japan). Apoptotic cells were identified by the characteristic bright blue fluorescence of nuclei that appears due to condensed or fragmented chromatin.

Flow cytometric analysis of apoptosis

Apoptotic cells were examined using an annexin V-FITC/Propidium iodide (PI) apoptosis detection kit (Biovision, Wuhan, China) according to the manufacturer's instructions. In brief, 5×10^5 cells were harvested by trypsinization after treatment with various doses of UV-Tianjin (MOI: 20, 40 or 80) for 24 h. Then the cells were washed twice with PBS and resuspended in 500 μl of binding buffer. Cell suspensions were incubated with 5 μl of annexin V-FITC and 5 μl of PI for 10 min at room temperature in darkness and then evaluated by flow cytometry (Becton & Dickinson, Franklin Lakes, NJ, USA). For caspase inhibitor assays, cells were pretreated with 20 μM pan caspase inhibitor (Z-VAD-FMK) (Sigma-Aldrich, Shanghai, China) for 2 h and then treated with UV-Tianjin (MOI 80) for an additional 24 h. The extent of apoptosis was then determined using flow cytometry, as described above.

Caspase activity assay

For caspase activity detection, the caspase-3, -8, -9 colorimetric assay kits (KeyGen Biotech, Nanjing, China) were used according to the manufacturer's instructions. Briefly, MDA-MB-231 cells were cultured in 6-well plates overnight and then treated with UV-Tianjin (MOI: 20, 40 or 80) for 24 h. The cells were harvested and lysed in 100 μl of lysis buffer on ice for 30 min. After centrifugation, the supernatant containing 150 μg protein was incubated with 200 μM caspase-3 (Ac-DEVD-pNA), caspase-8 (Ac-IETD-pNA) or caspase-9 (Ac-LEHD-pNA) substrates in reaction buffer at 37°C for 4 h in the dark. The OD value of released pNA at 405 nm was measured using a microplate reader. The fold-increase in caspase-3, -8, -9 activities was determined by direct comparison to the level of the un-induced control, which was considered as 1.

Flow cytometric analysis of MMP

Changes in the MMP were assayed using the JC-10 MMP assay kit (Beijing Fanbo, Beijing, China). Briefly, MDA-MB-231 cells were plated in 6-well plates. After incubation overnight, the cells were treated with UV-Tianjin (MOI: 5, 20 or 80) for 24 h. The cell density was adjusted to 1×10^6 cells/ml and then incubated for 30 min at 37°C with JC-10 dye. After rinsing twice with JC-10 staining buffer, the red and green fluorescences were monitored with flow cytometry. A decline of the ratio of red to green fluorescence indicates the loss of MMP. Quantitative analysis was conducted using the CellQuest analysis software.

Western blot analysis

Western blot analysis was performed as described (Zou et al., 2012), with modifications. MDA-MB-231 cells were treated with UV-Tianjin (MOI: 20 or 80) for 24 h and then harvested using a cell scraper. Cell pellets were resuspended in 100 μl of RIPA buffer (1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM sodium phosphate, pH 7.2, 2 mM EDTA, 50 mM sodium fluoride, 0.2 mM sodium vanadate) containing protease inhibitor cocktail (Solarbio, Beijing, China) and lysed for 30 min on ice. Following BCA assay, 40 μg of total protein from each lysate was separated by SDS-PAGE and then transferred to a PVDF membrane. Membranes were blocked overnight at 4°C with 5% nonfat dry milk in Tris-buffered saline Tween (TBST, 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% Tween-20), incubated with a primary antibody for 1 h at room temperature in 5% nonfat dry milk/TBST, washed three times with TBST, and incubated with HRP-linked secondary antibodies in 5% nonfat dry milk/TBST for 1 h at room temperature. After washing with TBST, protein bands were visualized by chemiluminescence on X-ray films.

Statistical analysis

Each experiment was replicated at least three times. Data were presented as the mean±standard deviation (SD), and statistical comparisons were made using the student's t test or one-way ANOVA test. A p value <0.05 was considered statistically significant.

Results

UV-Tianjin inhibited the proliferation of MDA-MB-231 cells

When we treated human breast cancer cell line MDA-MB-231 or non-tumorigenic human breast epithelial cell line MCF 10A with UV-Tianjin at various MOI for 24 h, as shown in Figure 1A, cell viability was dose-dependently suppressed in MDA-MB-231 cells, but not in MCF 10A cells, indicating the selective inhibitory effect of UV-Tianjin towards cancer cells. Microscopic examination showed that UV-Tianjin induced extensive cell fusion in MDA-MB-231 cells, but not in normal breast epithelium cell (Figure 1B).

UV-Tianjin induced apoptosis of MDA-MB-231 cells

To determine whether UV-Tianjin-mediated growth

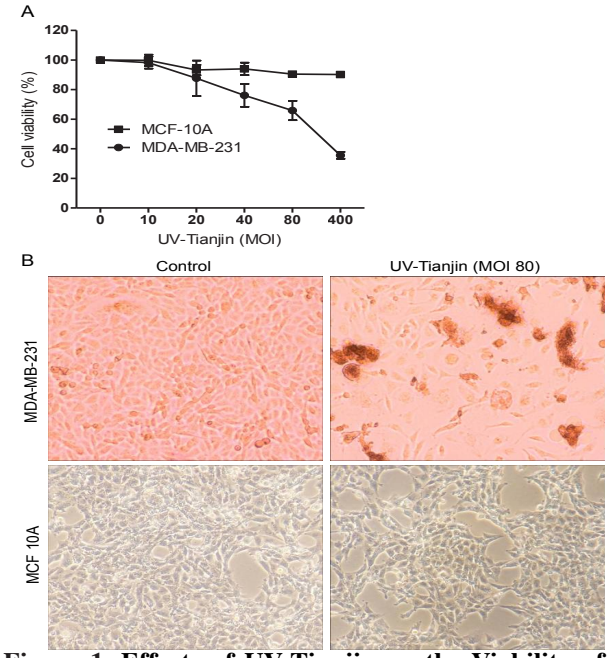

Figure 1. Effects of UV-Tianjin on the Viability of MDA-MB-231 and MCF 10A Cells. A) Cell viability of MDA-MB-231 and MCF 10A cells was determined by MTT assay after UV-Tianjin treatment for 24 h. The results were expressed as mean±SD of three experiments in quadruplicate. **B)** Representative photographs of MDA-MB-231 and MCF 10A cells under microscope after UV-Tianjin treatment (MOI 80) for 24 h (× 100)

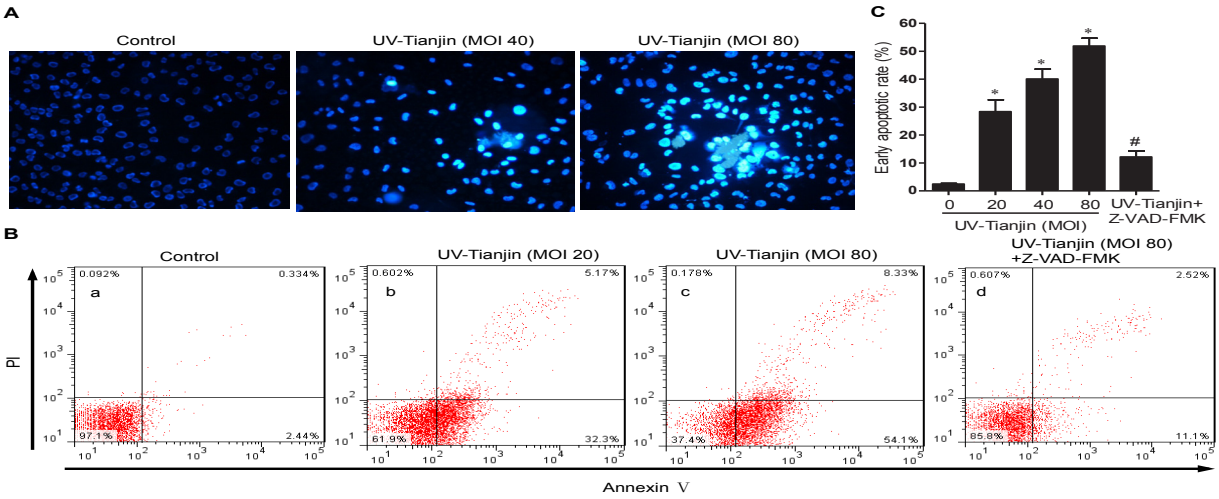

Figure 2. UV-Tianjin-induced apoptosis in MDA-MB-231 cells after 24 h treatment. A) Representative photographs of MDA-MB-231 cells under fluorescence microscope after Hoechst 33342 staining (× 200). **B)** (a-c) Annexin V-FITC / PI double staining was performed to determine the apoptotic rate following treatment with different doses of UV-Tianjin for 24 h. The lower right quadrant (annexin V+ / PI-) represents early apoptosis, and the upper right quadrant (annexin V+ / PI+) represents late apoptosis and necrosis. (d) Flow cytometry was used to assess the effect of pan-caspase inhibitor on UV-Tianjin-induced apoptosis of MDA-MB-231 cells. **C)** The histogram showed the apoptotic rate (%) of MDA-MB-231 cells by flow cytometry. *p<0.01 compared with control. #p<0.01 compared with UV-Tianjin (MOI 80)

inhibition resulted from apoptosis, we stained MDA-MB-231 cells with Hoechst 33342 dye. The results showed that after UV-Tianjin treatment, some cells were stained bright blue, which indicated apoptosis. Moreover, a dose-dependent increase in apoptosis was observed in MDA-MB-231 cells following UV-Tianjin treatment (Figure 2A).

We subsequently estimated the number of apoptotic cells by flow cytometry. The results demonstrated that the early apoptotic cells accounted for (28.4±4.2) %, (40.1±3.5) % and (51.9±2.8) % of the cells with UV-Tianjin treatment for 24 h at MOI 20, 40 and 80, respectively. A significant difference in the number of apoptotic cells was observed between treated and untreated MDA-MB-231 cells [$p<0.01$, Figure 2B(a-c), C]. Taken together, our results showed that UV-Tianjin could effectively initiate apoptosis in MDA-MB-231 cells.

UV-Tianjin-induced apoptosis in MDA-MB-231 cells is caspase dependent

To investigate the possible mechanism underlying UV-Tianjin-induced apoptosis in MDA-MB-231 cells, caspase-3, -8 and -9 activities were assayed using a colorimetric method. The results showed that the caspase-3, -8 and -9 activities increased in a dose-dependent manner following UV-Tianjin treatment for 24 h (Figure 3C). Meanwhile, when we pretreated the cells with pan-caspase inhibitor, Z-VAD-FMK for 2 h, UV-Tianjin-induced apoptosis (MOI 80) had been partially suppressed [Figure 2B(d)]. It is well known that caspase-8 and caspase-9 are essential proteases of extrinsic and intrinsic apoptotic pathways, while caspase-3 acts as a downstream effector of these two pathways. Thus, our data suggested that UV-Tianjin-induced apoptosis is caspase dependent and may involve in both the endogenous mitochondrial pathway and the exogenous death receptor pathway.

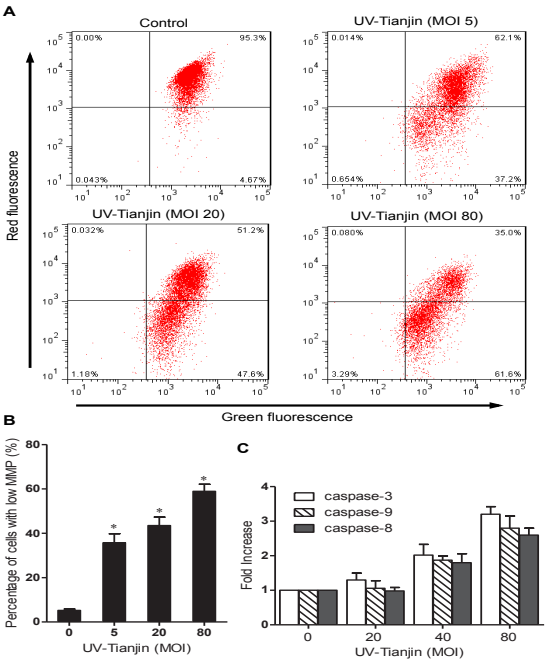

Figure 3. Effects of UV-Tianjin on the MMP and Caspase Activities. A) JC-10 dye was used to analyze the change in MMP by flow cytometry following the treatment of MDA-MB-231 cells with different doses of UV-Tianjin for 24 h. The reduced MMP indicated by a decrease in red fluorescence. **B)** The histogram represents the percentage of cells with low MMP, which expressed as mean±SD of three experiments. *$p <$ 0.01 compared with control. **C)** Activation of caspase-3, -8 and -9 proteins in MDA-MB-231 cells after UV-Tianjin treatment using a colorimetric method

Both mitochondrial pathway and death receptor pathway play a role in UV-Tianjin-induced apoptosis in MDA-MB-231 cells

To investigate the involvement of mitochondrial pathway in UV-Tianjin-induced apoptosis, JC-10 coloration was used to examine the changes in MMP in MDA-MB-231 cells. As shown in Figure 3A and B, flow cytometric data suggested the red fluorescence intensity was significantly decreased after UV-Tianjin treatment for 24 h, indicating the disruption of MMP. From these data, we could conclude that UV-Tianjin downregulated MMP in a dose-dependent manner, indicating the important role of mitochondria in UV-Tianjin induced apoptosis.

Following this, we examined the expression levels of Bax, Bcl-2, cyt c, caspase-9 and -3 inside the cytoplasm by western blotting. It was found that when the doses of UV-Tianjin were increased, the expression of Bcl-2 protein was downregulated, while the level of Bax, cyt c, cleaved caspase-9 and -3 was upregulated (Figure 4A and B). Our data demonstrated that UV-Tianjin changed MMP to promote a change in the levels of Bax and Bcl-2 as well as the release of cyt c, which then activated caspase-9, followed by an increase in caspase-3 activation. Caspase-3 eventually resulted in the apoptosis of MDA-MB-231 cells.

To confirm the involvement of the death receptor pathway in UV-Tianjin-induced apoptosis, we next examined the effect of UV-Tianjin on the expression levels of Fas, FasL and downstream caspase-8. The results demonstrated that a visible increase in Fas, FasL, and cleaved caspase-8 was observed after UV-Tianjin treatment at MOI 80, as compared with control cells and cells treated with 20 MOI of UV-Tianjin (Figure 4B).

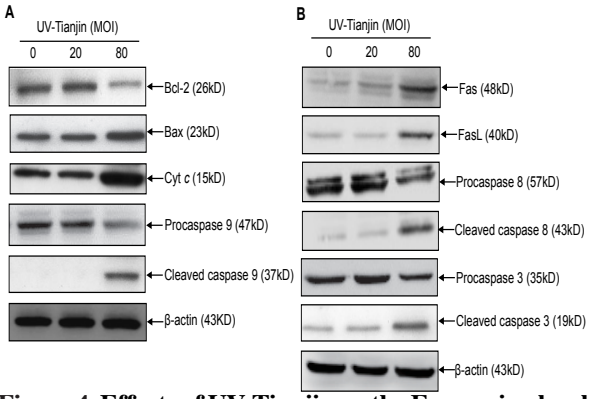

Figure 4. Effects of UV-Tianjin on the Expression levels of Apoptosis associated Proteins. (A) Western blot analysis was used to determine the expression levels of Bax, Bcl-2, cyt c and caspase-9 proteins after UV-Tianjin treatment for 24 h. (B) Western blot analysis was used to analyze the expression of Fas, FasL, caspase-8 and -3 proteins after UV-Tianjin treatment for 24 h. Results are representative of three independent experiments

Discussion

Apoptosis is a process of programmed cell death that may occur in multicellular organisms. Deregulation of apoptosis can disrupt the delicate balance between cell proliferation and cell death and can lead to diseases such as cancer (Thompson, 1995; Danial and Korsmeyer, 2004). Targeting apoptosis is a promising strategy for cancer drug discovery. In the present study, we demonstrated that UV-Tianjin significantly inhibited the proliferation of human breast cancer MDA-MB-231 cells in a dose-dependent manner. Moreover, Hoechst staining and flow cytometry analysis revealed that UV-Tianjin induced the apoptosis of MDA-MB-231 cells in a dose-dependent manner *in vitro*.

Apoptosis occurs through two main pathways: the intrinsic (mitochondrial-mediated) and the extrinsic (death receptor-mediated) pathway. The mitochondrial pathway is regulated by the Bcl-2 family proteins which includes the anti-apoptotic proteins (Bcl-2, etc.), the proapoptotic proteins (Bax and Bak) (Bi et al., 2013). Accumulation of proapoptotic proteins on the mitochondrial outer membrane results in increased mitochondrial membrane permeability, causing the release of cyt c into the cytoplasm (Strasser et al., 2011). Cyt c binds to the cytosolic protein Apaf-1 to facilitate the formation of apoptosomes, which can then recruit and activate the inactive procaspase-9. Once activated, caspase-9 can directly activate caspase-3 or -7 and trigger a cascade of events leading to apoptosis (Li et al., 1997; Srinivasula et al., 1998). To clarify whether the mitochondrial pathway is involved in UV-Tianjin-induced apoptosis, we examined the changes of MMP using JC-10 coloration and the activation of some apoptosis-associated proteins by Western blotting. Our data showed that after UV-Tianjin treatment, the JC-10 red fluorescence intensity was significantly decreased in a dose-dependent manner, which meant the loss of MMP and the initiation of the apoptosis process. Furthermore, Bax and cyt c expression increased, while Bcl-2 expression decreased when the amounts of UV-Tianjin were increased. Both procaspase-9 and procaspase-3 were cleaved in MDA-MB-231 cells following 80 MOI of UV-Tianjin treatment. These results suggested that the UV-Tianjin-induced apoptosis might be through the intrinsic pathway.

The death receptor pathway occurs due to the activation of various cell death surface receptors following binding to the relevant ligands. The Fas-FasL system is the best characterized member of the extrinsic pathway family in apoptosis. When FasL binds to Fas on target cells, caspases-8 and then caspases-3/-7 are activated and the cells die through programmed cell death (Gu et al., 2014). Our results showed that the levels of Fas, FasL, active caspase-8 and -3 increased following 80 MOI of UV-Tianjin treatment. Based on these results, we can conclude that UV-Tianjin induced apoptosis of MDA-MB-231 cells may involve both the death receptor dependent extrinsic and mitochondria dependent intrinsic apoptotic pathways. More research is needed to confirm whether the extrinsic or intrinsic pathway play a role in the UV-Tianjin-induced apoptosis.

In summary, our study has demonstrated that UV-Tianjin induced the apoptosis of human breast cancer MDA-MB-231 cells through promoting the release of cyt c, activation of caspase family and expression of Fas and FasL, which may involve both the endogenous mitochondrial pathway and the exogenous death receptor pathway. Therefore, UV-Tianjin may offer important contributions for the development of a novel drug to prevent and cure breast cancer in the future.

Acknowledgements

This work was supported by National Natural Science Foundation of China (No. 81172168).

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