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Original Research

Ent-Dihydrotucumanoic acid promotes apoptosis in PC-3 human prostate cancer cells

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Abstract: Prostate cancer (PC) has become a disease that pose a serious threat to men's health and life. In recent years, due to the changes of environment, lifestyle and other factors, the incidence of PC has been increasing rapidly in recent years, which is a serious threat to men's health. Ent-Dihydrotucumanoic Acid (DTA) is a compound isolated from Asteraceae of gymnosperms, which has many pharmacological effects. The effect of DTA on the growth of tumor cell line was studied by CCK-8 method, mitochondrial membrane potential and apoptosis were detected by flow cytometry, apoptosis-related genes were detected by Western blot assay, and the absorptivity of Caspase-3 and Caspase-9 was measured by spectrophotometer. It was found that DTA induces apoptosis of human prostate cancer cell line PC3 through mitochondrial pathway, thus preventing the development of prostate cancer. It lays the experimental foundation for the further development of DTA.

Key words: Ent-Dihydrotucumanoic Acid; PC-3; Apoptosis; Prostate cancer.

Introduction

Prostate cancer (PC) is a common malignant tumor in male reproductive system (1). In North America and Europe, prostate cancer is the second most common malignant tumor in men and one of the leading causes of cancer mortality in the United States (2). In China, the incidence of prostate cancer is much lower than that in western countries, but in recent years, with the prolongation of life expectancy, the change of diet habits, and the improvement and popularization of screening technology, the incidence of PC in Chinese males has been increasing year by year (3). The early symptoms of PC are often not obvious, and most of them progress to the late stage. PC is a androgen-dependent tumor, which may be treated with surgery at the early stage, and is usually treated with surgery, radiotherapy, hormone therapy and chemotherapy, or a combination of several methods at the late stages of the disease (4). However, these traditional methods have a lot of drawbacks. Hormone blocking therapy with the extension of treatment time, the tumor often turns into steroid-resistant prostate cancer, often accompanied by distant metastasis, especially bone metastasis. The effect of radiotherapy and chemotherapy is not good, the prognosis is very poor, and the existing chemotherapy will have significant side effects, such as urinary incontinence (5). Therefore, it is particularly important to find new therapeutic drugs or other treatments.

Antitumor components extracted from natural plants play an increasingly important role in the treatment of malignant tumors. It has become a hot topic and main direction of the research and development of new anti-tumor drugs to search for natural active ingredients from plants and screen a large number of new compounds with high efficiency in order to search for anti-tumor drugs with high efficiency and specificity (6). So far, 6.17 million compounds with anti-tumor activity have been isolated. The main antitumor drugs include paclitaxel, camptothecin, vinblastine, resveratrol, podophyllotoxin, artemisinin, ginseng and so on (7). They have become the main melody in the botanical drugs market of anti-tumor drugs because of their strong specificity and small side effects in tumor therapy (8). The search for anticancer drugs with small side effects has attracted increasing attention (9).

Gymnosperma glutinosum (Spreng) Less (Asteraceae) is a viscous shrub from the southern United States to Central America (10) and Ent-Dihydrotucumanoic Acid (DTA, Fig.1) is a compound isolated from Asteraceae of gymnosperms (11, 12). DTA has a variety of biological activities and can be used in the treatment of pain, fever, diarrhea, cancer, rheumatism, wounds, infections and other diseases (13). However, the me-

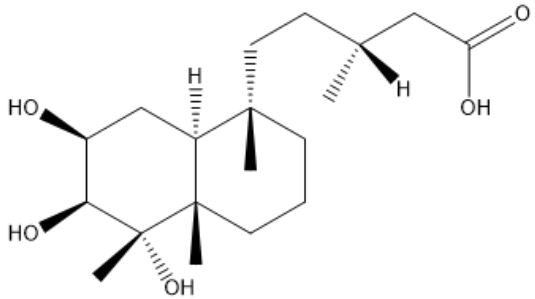

Figure 1. Chemical structure of Ent-Dihydrotucumanoic Acid.

chanism of PC-3 cell apoptosis induced by DTA has not been reported. In this study, apoptosis of prostate cancer PC-3 cells induced by DTA via mitochondrial dependent pathway and the effect of DTA on the expression of apoptosis-related protein Bcl-2, Bax, Cytochrome C, Caspase-3 and Caspase-9 was investigated.

Materials and Methods

Cell lines, drugs and major reagents

Human Prostate Cancer PC-3 cells (purchased from the Cell Bank of the Shanghai Academy of Sciences). The cell lines were cultured in a RPMI 1640 medium containing 10% calf serum, 100U·mL^{-1} penicillin and 100 μg·mL^{-1} streptomycin and placed in a cell incubator containing 5% CO_2 saturated humidity at 37 ℃. The cells in logarithmic growth phase were used in the experiment. DTA, caspase-3 and caspase-9 activity detection kit, rabbit anti-caspase-3 and rabbit anti-caspase-9 antibodies were purchased from Sigma; RPMI-1640 culture medium and serum were purchased from Gibco; CCK-8 activity test kit was purchased from Nanjing Enjing Biotechnology Co., Ltd.; Annexin V-PE/7-AAD cell apoptosis double staining kit was purchased from Kaiji Company; Anti-Bcl-2 and anti-Bax antibodies were purchased from Cell Signaling Technology; anti-β-actin mouse mAb (HPR Conjugate) and anti-mouse IgG labeled by HPR were purchased from CST; HPR labeled sheep anti-rabbit IgG was purchased from Proteintech and other reagents were imported packing or domestic analytical purity.

Detection of cell activity by CCK-8 method

PC3 cells were inoculated in 96-well plate with 5,000 cells per well and 100 μL of culture medium were added to each well. After culturing at 37 °C with 5% CO_2 for 48 h, 100 μL of culture medium was added to the culture plate, and different concentrations of DTA (0, 5, 10, 20 and 40 μM) were stimulated for 48 h or 20 μM DTA was stimulated for different times (0, 24, 48 and 72 h). The blank group was added cell-free medium with 3 multiple wells in each group. After reaching the corresponding time point at 37 °C and 5% CO_2, the original culture medium was removed, and the cells were washed with the new medium for 2 times. New medium and 10μL CCK-8 were added and cultured at the incubator for 2h, the absorbance (A) at 450nm wavelength was determined by enzyme labeling instrument.

Detection of apoptosis rate of prostate cancer PC-3 cells by flow cytometry

The PC-3 cells containing 1×10^6 cells/mL were inoculated into the 6-well cell culture plate. After culturing for 24 h, 0, 10, 20 and 40 μM DTA were added to each well, and inoculated for 48 h. The culture medium was removed, the cells was rinsed with PBS for 2 times and digested by 25% trypsin. PC-3 cells were collected by 1000×g centrifugation and rinsed with PBS for 2 times. The cells were suspended with 400μL of 1 × binding buffer and the concentration was about 1×10^6 cells/mL. 5μL of Annexin V-PE was added to cell suspension, mixed gently and incubated at 28 °C for 15min. After adding 10 μL of 7-AAD, the mixture was mixed gently and incubated for 5 min at 2 °C to 8 °C avoiding light. The apoptosis rate of PC-3 cells was detected by flow cytometry or fluorescence microscope within 1 h.

Detection of mitochondrial membrane potential (Δψ m) by flow cytometry

The PC-3 cells containing 1×10^6 cells/mL were inoculated into the 6-well cell culture plate. After culturing for 24 h, each well was incubated with 0, 10, 20 and 40 μM DTA for 48 h. The culture medium was removed, the cells was rinsed with PBS for 2 times and digested by 25% trypsin. PC-3 cells were collected by 1000×g centrifugation and rinsed with PBS for 2 times. Cells were suspended in 1 mL of Rh123 staining solution with the final concentration of Rh123 was 10 μg/mL, then incubated at 37 °C avoiding light for 30 min. PC-3 cells were detected by flow cytometry with the excitation wavelength was 488 nm, and the emission wavelength was 510 nm. There were 3 parallel experiments in each group.

Detection of protein expression by Western blot assay

PC3 cells at logarithmic growth phase were selected and inoculated into 6-well plates with 1×10^6 per well. After culturing at 37 °C with 5% CO_2 for 48 h, the culture medium was changed, and then incubated again for 48 h with 0, 10, 20 and 40 μM DTA, respectively. Each group of cells were collected, rinsed with DPBS for 2 times, 150 μL of RIPA lytic solution was added, and placed on ice for 30 min. After centrifuging at 13000×g for 10 to 15 min, the supernatant was quantified by BCA protein, 5 × sample buffer solution was added, denatured at 98 °C for 5 min, and 12 % SDS-polyacrylamide gel was used for electrophoresis, followed by electrical transfer to PVDF membrane. The PVDF membrane was sealed with 5% skim milk powder solution for 2 h, the primary antibody was added and incubated overnight at 4 °C. After rinsing the membrane with TBST for 3 times, the second antibody labeled by horseradish peroxidase was added and incubated at room temperature for 1 h. The membrane was rinsed with TBST for 3 times, colored by chemiluminescence substrate SuperSignal West Pico, and Kodak IS4000R image workstation was used for chemiluminescence detection. The grayscale value of protein band was measured by Gelpro software after image scanning.

Detection of Caspase-3 and Caspase-9 protein activity

The PC-3 cells containing 1×10^6 cells/mL were inoculated into the 6-well cell culture plate. After culturing for 24 h, each well was incubated with 0, 10, 20 and 40 μM DTA for 48 h. The cells were digested with 0.25% trypsin and 150 μL protein lysate was added. Then 50 μL of 2 × Reaction Buffer was added, 5 μL enzyme substrates of Caspase-3 and Caspase-9 were added and incubated at 37 °C avoiding light for 4 h. The absorptivity was measured by spectrophotometer (100 μL colorimetric dish) at λ = 405 nm or 400 nm. OD inducer / OD negative control ratio was calculated to determine the activation degree of Caspase-3 and Caspase-9 in apoptotic inducer group.

Statistical method

The GraphpadPrism7.0 statistical software was used for statistical analysis of the research data. The data was expressed as mean ± standard deviation (x ± SD). Single factor analysis of variance (one-way ANOVA) was used for multi-group comparison, and Student-Newman-Keuls test (SNK-q) was performed for pairwise comparison. $P < 0.05$ represents significant difference in statistics.

Results

Effect of DTA on cell activity

The results of CCK8 assay showed that the proliferation of PC-3 cells was obviously inhibited by different concentrations of DTA. As shown in figure 2, the proliferation of PC-3 cells in the experimental group was significantly inhibited with the increase of DTA concentration and the prolongation of the action time (Fig. 2) compared with the control group. DTA can induce the decrease of PC3 cell activity and inhibit cell growth in a dose and time dependent manner.

Effect of PXD on apoptosis of PC3 cells

PC-3 cells were incubated with different concentrations of DTA for 48 h, and the apoptosis rate was measured by flow cytometry. The results showed that with 10, 20 and 40 μM DTA treatment of PC-3 cells, the apoptosis rate was markedly increased with the increase of DTA concentration, and was dose-dependent (Fig. 3) ($P < 0.05$).

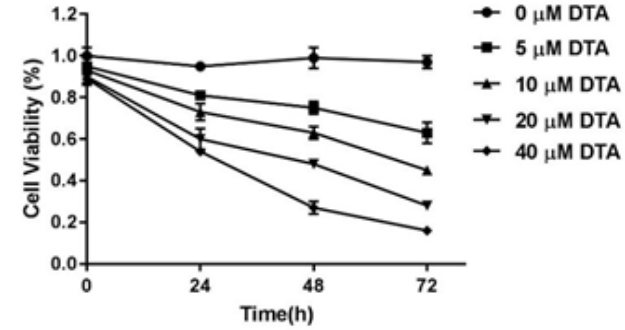

Figure 2. The effect of DTA on PC3 cell viability detected by CCK-8 assay. PC-3 cells are treated with various concentrations of DTA for 24, 48, or 72 h. Data are shown as mean ± SD from three independent experiments.

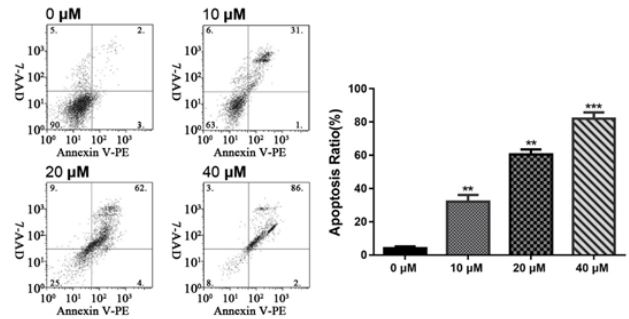

Figure 3. Effects of DTA on the apoptosis of PC-3 cells. Data represent the mean ± SD of at least three independent experiments. The statistical significance (*, $P < 0.05$,**, $P < 0.01$,***, $P < 0.001$) of results were determined through Student's t test vs. 0 μM DTA group.

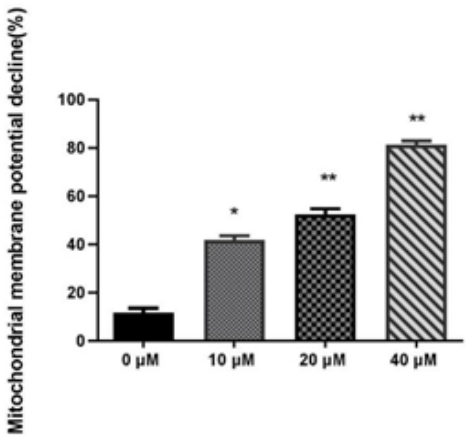

Figure 4. The effect of DTA on the mitochondrial membrane potential of PC3 cells. Mean ± SD. n =3. *, $P < 0.05$, **, $P < 0.01$, vs 0 M DTA group.

Effect of DTA on mitochondrial membrane potential of PC3 cells

The effect of DTA on mitochondrial membrane potential of PC-3 cells was detected by flow cytometry. After treatment of PC-3 cells with DTA, the mitochondrial membrane potential also showed a decreasing trend in a concentration-dependent manner with the increase of DTA concentration from 0 μM to 40 μM ($P < 0.05$) (Fig. 4).

Apoptosis of PC3 cells mediated by DTA through mitochondrial pathway

In order to further study the molecular mechanism of apoptosis in PC3 cells induced by DTA, a series of signal molecules involved in mitochondria mediated apoptosis pathway were detected by Western blot. The cytoplasmic and mitochondrial proteins of PC3 cells were extracted to detect the expression of cytochrome C (Cyt C). The results indicated that after 48 h of DTA stimulation, the content of Bcl-2 protein decreased significantly, Bax protein levels increased, Cy-tochrome C in cytoplasm increased obviously, Cytochrome C in mitochondria decreased significantly, mitochondrial Cyt C protein was released into cytoplasm from mitochondria. The difference was statistically significant ($P < 0.05$) (Fig. 5).

Changes of Caspase-3 and Caspase-9 activity in PC3 cells induced by DTA

When PC-3 cells were treated with different concen-

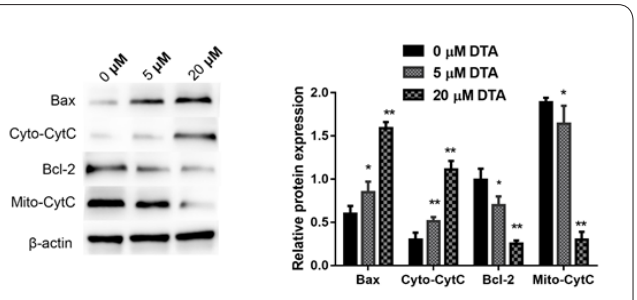

Figure 5. The expression of mitochondrial apoptosis pathway-related proteins Bcl-2, Bax, cytoplasmic cytochrome C (cyto-Cyt C) and andmitochondrial cytochrome C (mito-Cyt C) were determined by Western blot. Mean ± SD. n =3. *, $P < 0.05$, **, $P < 0.01$, vs 0 μM DTA group.

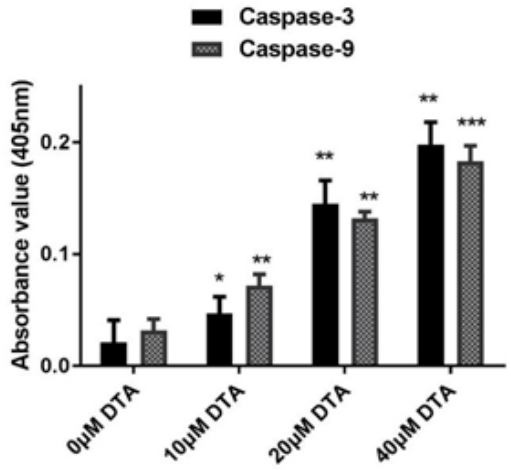

Figure 6. Effects of different concentrations of DTA on the activity of caspase-3 and caspase-9 in PC-3 cells. Mean ± SD. n =3. *, P < 0.05, **, P < 0.01, ***, P < 0.001 vs 0 μM DTA group.

trations of DTA, pro-Caspase-3 was activated to produce active-Caspase-3. The activation of Caspase-3 increased in a concentration-dependent manner with the change of drug concentration. After DTA treatment, pro-Caspase-9 in PC-3 cells was also activated, showing a concentration-dependent manner (Fig. 6) and the difference was statistically significant ($P < 0.05$).

Discussion

Prostate cancer is an important male tumor disease in Europe and America. Epidemiological research shows that the occurrence of prostate cancer is mainly related to age, race, family genetic background, geographical location and diet (14). It is generally believed that the development from normal prostate tissue to prostate cancer involves four main stages: prostatic intraepithelial neoplasia, local prostate cancer, invasive prostate cancer, and metastatic prostate cancer. Each step is accompanied by genetic changes and abnormalities in various genes (15, 16).

In order to investigate the effect of DTA on apoptosis of prostate cancer cells, human prostate cancer cell line PC3 was selected to detect the effect of DTA on the activity of PC3 cells at different concentrations and different treatment time by CCK-8 assay (17). The results showed that DTA could significantly reduce the activity of PC3 cells in a dose and time dependent manner. To further investigate whether the decrease of PC3 cell activity induced by DTA is related to apoptosis, the results of apoptosis staining by flow cytometry analysis showed that DTA could induce apoptosis of PC3 cells significantly compared with the control group. This indicates that DTA inhibits the cell proliferation activity and is related to the induction of apoptosis.

Mitochondrial membrane potential (MMP) is one of the important parameters to reflect the functional state of mitochondria in cells. Rhodamine 123 is a kind of fluorescent dye that can selectively stain mitochondria of living cells through cell membrane, showing yellowish green fluorescence (18). It is widely used to detect mitochondrial membrane potential and also to detect apoptosis. When apoptosis occurs, mitochondrial transport capacity and fluorescence intensity decrease, according to which mitochondrial membrane potential can be measured. The results of mitochondrial membrane potential showed that the mitochondrial membrane potential decreased in a concentration-dependent manner with the increase of DTA concentration in PC-3 cells treated with DTA.

Apoptosis, also known as programmed death, is an important mechanism by which genes regulate cell senescence and maintain the normal functioning of the body, and are precisely regulated by a series of intracellular and extracellular signaling pathways. A variety of apoptotic signals are transmitted to cells through signal transduction pathways to activate target genes and induce apoptosis (19). Therefore, the imbalance of apoptosis often leads to tumorigenesis. Previous studies have shown that there are three major signal transduction pathways in apoptosis: 1. The transduction pathway mediated by death receptor and the main transduction objects are exogenous signals. Death receptor is also a protein group, all of which are transmembrane, and its effector substances are also varied, among which caspase 8 is more important. 2. Mitochondrial mediated transduction pathway and the transduction object is endogenous signal. The effector substance is Cytochrome C, which activates caspase 9. 3. Endoplasmic reticulum mediated transduction pathway and the transduction object is still endogenous signal. The effector substance is Ca^{2+}, which in turn activates caspase 12. Although the starting positions and effectors of the three pathways are different, they can be interacted by complex chains such as Ca^{2+} and Cytochrome C, caspase 8 and Ca^{2+}, caspase 8 and Cytochrome C, etc. All the three pathways need to eventually activate downstream caspase 3 to enter a common apoptotic pathway and lead to apoptosis (20). Bcl- 2, one of the earliest oncogenes, has no direct effect on cell proliferation, but it can inhibit apoptosis induced by various factors (21). High expression of Bcl-2 has become one of the characteristics of many malignant tumors. The mechanism of inhibiting apoptosis may be that the mitochondria release Cytochrome C into the cytoplasm, while Cytochrome C activates the caspase protein family. When cytochrome C is reduced, the activation of the caspase protein family is also reduced, and the cell apoptosis is also inhibited. Bax and Bcl-2 belong to the same family and Bax has strong antagonistic effect on Bcl-2, which can directly promote cell apoptosis and co-regulate the tendency of cell proliferation and death together with Bcl-2 (22-23). Cytochrome C is a large transmembrane protein complex existed in bacteria or mitochondria. Studies on apoptosis have shown that, Cytochrome C is associated with apoptosis, and Cytochrome C leaked from mitochondria can induce apoptosis (24). Cytochrome C is located in the space between the outer membrane and intima of mitochondria. Apoptosis inducers can induce Cytochrome C release from mitochondria to cytoplasm and bind to Apaf-1 (25). Cytochrome C/Apaf-1 complex activates Caspase-9, which in turn activates Caspase-3 and other downstream Caspases. The release of Cytochrome C occurs before the activation of cysteine protease and the DNA breaking, which can be regarded as the initial marker of apoptosis.The possible mechanism is to activate the caspase protein family, especially the key protein caspase3, by reducing the expression of Bcl- 2

and increasing the expression of Bax, that is, Bcl- 2/ Bax decreased and the expression of Cox- 2 reduced in PC- 3 cells. Finally, caspase cascade reaction was activated to induce apoptosis. The western blot results of this study indicate that DTA has a good tumor inhibition effect, can inhibit the proliferation of human prostate cancer cell PC-3, and induce its apoptosis. The showed that DTA could release Cytochrome C from mitochondria to cytoplasm in PC-3 cells. Then followed activation of pro-caspase-3 and pro-caspase-9 significantly increased. These experimental results provide a strong theoretical basis for the further research and development of new drugs and have potential application value.

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Author's contribution
All work was done by the author named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Min Zhou; Min Zhou, Weibing Li collected and analysed the data; Min Zhou wrote the text and all authors have read and approved the text prior to publication.

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