Effects of Cucurbitin B on Proliferation, Migration and Apoptosis in Human Bladder Cancer T24 Cells

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Abstract

Background: Cucurbitin B is a tetracyclic triterpene compound extracted from the Cucurbitaceae plant. In recent years, it has been found that it can inhibit the growth of various tumor cells such as breast cancer, laryngeal cancer, pancreatic cancer and colon cancer. Studies on its effect on bladder cancer have not been reported. In this study, we will describe the role of cucurbitin B in human bladder cancer T24 cells. Methods: T24 cells were cultured in vitro, and the effect of cucurbitin B on cell proliferation was observed by CCK8 assay. The effect of cell migration was observed by Transwell assay. Flow cytometry was used to detect the apoptosis rate and cell cycle distribution, and Western blot was used to detect the protein expression of Caspase-3 and Caspase-9. Results: Cucurbitin B inhibited the proliferation of T24 cells in a does- and time-dependent manner. Transwell results suggested that cucurbitacin B can inhibit the migration of T24 cells. Flow cytometry analysis showed that cucurbitin B caused G2/M phase arrest of T24 cells and induced T24 cell apoptosis in a dose-dependent manner. Western blot results showed that the expression of Caspase-3 and Caspase-9 was up-regulated after the treatment of Cucurbitacin B. Conclusion: Cucurbitin B inhibits the proliferation, migration and induces apoptosis of human bladder cancer T24 in a does- and time- manner.

Keywords

Cucurbitacin B, Bladder cancer, T24 cells, Apoptasis.

1. Introduction

Bladder cancer is the sixth most common cancer in the United States. It is estimated that there will be 80,470 new cases of bladder cancer in the United States in 2019 and about 17,670 deaths [1]. Bladder cancer occurs mainly in men and is closely related to smoking. With the advancement of medicine, many treatment options for bladder cancer have appeared, including surgery and postoperative chemotherapy and radiotherapy, but the mortality rate of bladder cancer is still high. At the same time, postoperative chemoradiotherapy has large side effects, which is often difficult for patients to tolerate[2].

Cucurbitin B is derived from cucurbitaceous plants and is a highly oxidized tetracyclic triterpene containing 32 carbons. It has various biological activities such as anti-tumor and improving immunity[3, 4]. Recent researches showed that low concentrations of cucurbitin B can inhibit a variety of tumors, such as leukemia, lung cancer, breast cancer[5-7]. And the mechanism of cucurbitin B in different tumor cells is not exactly the same. The effect of cucurbitin B on bladder cancer and its mechanism have not been reported. In this study, we will drscribe the effect of cucurbitin B on the proliferation, migration and apoptosis of human bladder cancer cell T24 and the related mechanism.

2. Materials and Methods

2.1 Materials and reagents

The human bladder cancer cell lines T24 was purchased from Shanghai institutes for Biological Sciences, CAS. Cucurbitacin B, RPMI 1640 medium, fetal bovine serum (FBS), penicillin, streptomycin and 0.25% trypsin were purchased from Sigma-Aldrich. All primary and secondary

antibodies were purchased from Abcam. All other reagents used in the study were purchased from Sigma-Aldrich.

2.2 Cell culture

The cells was cultured with RPMI 1640 medium supplemented with 10% FBS, 2mM L-glutamine, 50 U/mL penicillin and 50 U/mL streptomycin at 37℃ under a humidified 5% CO2 and 95% air at one atmosphere. The medium was changed every 2 days.

2.3 Mearsurement of cell viability.

5,000 cells/well suspension cells were seeded in 100 μL culture medium in 96-well plates. After 24h, the cells were treated with the different concentrations of cucurbitacin B (400nM, 800nM and 1000nM) . The control group and all experimental groups were set up with 5 duplicate wells, and cells were then incubated at 37℃ for 24 hours, 48 hours, and 72 hours. After incubated with CCK8 for 2 to 4 hours, it was placed in a multifunctional microplate reader, and the absorbance was detected at 450 nm.

2.4 Transwell assay

The transwell migration assay was conducted in 24-well plate transwell chamber system with 8.0 μm pore size. T24 cells were suspended in serum-free medium and adjust the cell density to 2X105 per 100 μL and seeded in the upper chamber insert,while the lower chamber was filled with 10% FBS medium. Cucurbitin B was added at a concentration gradient of 400nM, 800 nM and 1000 nM. After incubation for 24 hour at 37℃, PBS was used to wash the chamber, and gently wipe off the cells in the upper chamber with a cotton swab. The lower chamber was fixed in 4% paraformaldehyde for 30 minutes and crystal violet was stained for 30 min. Then the chambers were placed under an inverted phase contrast microscpe to observe the migrated cells.

2.5 Analysis of apoptosis by Annexin V assay

T24 cells were seeded into 6-well culture plates at 5×105 cells/well and incubated overnight. The cells were treated with Cucurbitin B with final concentrations of 400nM, 800nM, and 1000nM for 48 hours. Then cells were digested with 0.25% trypsin, washed twice with ice-cold PBS and collected by centrifugation at 1000 r/min for 5 minutes at 4℃. 500μL binding buffer was added to suspend the cells, adjust the cell concentration to 1×106 / mL, 5μL Annexin V-FITC and 5μL PI were added and mixed gently. Then the cells were incubated for 15 minutes at room temperature in dark. The apoptosis rate was analyzed using a flow cytometer.

2.6 Cell cycle distribution and phase determination

The cells in logarithmic growth phase were seeded in 6-well culture plates with 5×105 cells/well and incubated overnight. After treated with cucurbitin B with final concentrations of 400nM, 800nM and 1000nM for 48 hours, cells were collected and centrifuged. Then the cells were washed twice with PBS (1000 r/min, 5min), fixed with 70% ice-cold ethanol overnight at 4℃. Cells were resuspended in PBS containing 50mg/L RNase and 25mg/L propidium iodide before detection on the machine. After 15 minutes incubation at room temperature in the dark, the cells were analyzed by flow cytometer. 1×104 cells were collected from each sample, and the results were analyzed using ModFit software.

2.7 Western-blot Analysis

The cells were collected and washed twice at 4℃ with ice-cold PBS and centrifuged at 1000 r/min for 10 minutes. Ice-cold lysis buffer was used to lyse cells for 30 minutes on ice. Then cells were centrifuged at 12000 r/min for 5 minutes and the supernatants were collected and assayed for protein content by Bradford protein assay using Bovine serum albumin (BSA) as standard. Total protein was separated using 10% SDS-PAGE and transferred to PVDF membrane. The membrane was blocked with Western blocking solution and incubated at room temperature for 1 hour. They were then incubated overnight at 4 ℃ with primary antibody followed by incubation at room temperature for 1

hour with secondary antibody. The immunoreactive bands were detected using an enhanced chemiluminescence (ECL) kit. The gray value of each band was analysed by Image-J software.

2.8 Statistical analysis

All experiments were repeated 3 times with consistent trends. SPSS 22.0 statistical software was used for analysis and GraphPad Prism 8.0 software was used to plot the experimental data. Comparisons within groups were compared using paired t test, and comparisons between groups were analyzed using single factor analysis of variance. $P<0.05$ indicated that the differences were statistically significant.

3. Results

3.1 Cucurbitacin B inhibits T24 cell proliferation in a dose- and time-dependent manner.

The examined concentrations of cucurbitacin B inhibited cell growth and induced cytotoxicity of T24 cells. The inhibitation rate in each group was determined by CCK8 assay. T24 cells treated with different concentrations of cucurbitacin B (400, 800, 1000 nM) exhibited reduced cell proliferation in a does- and time-dependent manner and the results are presented in Fig.1. The inhibitation rate at 72h of T24 cells treated with cucurbitacin B at 400, 800, 1000 nM was 21.9%, 45.7%, 62.5% and the half maximal inhibitory concentration (IC50) was 887.1 ±2.95 nM after a 72h treatment.

3.2 Cucurbitacin B inhibits T24 cell migration.

The influence of cucurbitacin B on the cell migration of T24 cells was assessed by transwell. Following incubation with cucurbitacin B (400, 800, 1000nM) for 24h, the number of cells at the lower chamber was counted. The number of cells that were not treated with cucurbitacin B was significantly more when compared with T24 cells that were exposed to cucurbitacin B in a does-depent manner ($P<0.05$) as shown in Fig.2. Our data suggested that the growth and movement of the cells were markedly inhibited when treated with cucurbitacin B.

3.3 Detection of apoptotic rate in T24 cells.

Annexin V/PI dual labeling flow cytometry was used to detect the apoptosis rate. The results showed that the percentage of apoptotic cells was higher with cucurbitacin B treatment compared to control group (Fig.3), the percentage of apoptotic cells were increased from 5% to 14.23%, 15.2% and 30.75%, respectively.

3.4 Detection of cell cycle distribution in T24 cells

Cell cycle distribution of T24 cells after treatment with cucurbitacin B (400, 800, 1000nM) for 24h was measured by flow cytometry. We found that after treatment with cucurbitin B at different concentrations, the percentage of T24 cells in the G1 phase decreased, the percentage of G2/M cells increased. The number of cells in G2/M phase was increased from 9.51% to 16.95%, 22.01% and 45.24% after concentration of 400, 800, 1000nM treatments, respectively. It indicates that cucurbitacin B has a cycle blocking effect on bladder cancer cells, and this effect is a does-dependent manner (Fig.4).

3.5 Cucurbitacin B up-regulated the expression of Caspase-3 and Caspase-9 in T24 cells

To investigate if cucurbitacin B induces apoptosis in T24 cells through caspases-dependent pathway, cells were exposed to cucurbitacin B for 48h treatment and then extracted protein. Western-blot results indicate that the expression of cleaved form of Caspase-3 and Caspase-9 increased after cucurbitin B was applied to T24 cells for 48 hours (Fig.5). This result suggests that cucurbitacin B induces apoptosis of T24 cells via caspase-dependent pathway.

4. Dicussion

As the most common malignant tumor of the urinary system, bladder cancer is mainly treated by surgical removal of the primary lesion and postoperative chemotherapy. However, due to the insidious onset of bladder cancer, newly discovered bladder cancer is often accompanied by muscular

infiltration and even distant metastasis[8, 9]. The high metastasis rate and recurrence rate are the main reasons for the high mortality rate of bladder cancer[10, 11]. Therefore, research on finding new anticancer drugs for patients with metastatic bladder cancer is particularly important.

This study investigated the effect of cucurbitin B on bladder cancer from the perspective of cell proliferation, migration, and apoptosis. After treating human bladder cancer T24 cells with different concentrations of cucurbitin B for a certain period of time, the cell proliferation was detected by the CCK8 assay. The results showed that cucurbitatin B had a significant inhibitory effect on the proliferation of T24 cells in a dose- and time-dependent manner. The cells were treated with the same concentration and Transwell results showed that cucurbitin B also inhibited the migration capacity of T24 cells.

Many anticancer drugs inhibit tumor cell growth by causing cell cycle arrest and apoptosis. Caspase is a family of cysteine proteases, which are widely expressed in normal human tissues and various tumor tissues. They are a class of proteolytic enzymes that mediate apoptosis. The cascade of reactions they initiate is the process of apoptosis signal transduction. It can regulate the apoptosis by interacting with many protein factors. After the cell receives the apoptotic signal, cytochrome C is released from the mitochondria into the cytoplasm, which activates downstream Caspase-9, which in turn activates the enzymatic cascade of Caspase proteases, including Caspase-2, 3, 6, 7, 8, 10 etc. Caspase-3 is the most important apoptotic effector. It plays final pivotal role in the apoptotic program initiated by various factors. It is related to chromosome aggregation, DNA breakage and the formation of apoptotic bodies[12]. Most stimulants cause cell apoptosis through the cascade activation response of Caspase. Studies have shown that Caspase-3 is the most important effector protein lyase in apoptosis. Most of the factors that trigger apoptosis ultimately require Caspase-3-mediated signal transduction pathways to cause apoptosis[13, 14]. Flow cytometry results showed that with the increase of does concentration, the apoptosis rate increased significantly. In order to further study the specific molecular mechanism of cucurbitin B on bladder cancer cell apoptosis, we examined the expression of apoptosis-related proteins. The WB results showed that the expression of Caspase-3 and Caspase-9 protein increased in T24 cells treated with cucurbitacin B.

In this study, flow cytometry was used to detect changes in cell cycle distribution caused by cucurbitacin B at different concentrations on T24 cells. It was found that as the concentration of cucurbitacin B increased, a significant G2/M phase block appeared. Cells cannot finish the complete mitosis process, cell proliferation is inhibited, and combined with increased apoptosis rate, tumor growth is inhibited. This is consistent with recent reports that cucurbitacin B inhibits the growth of other tumors. Tannin-Spitz et al[15] reported that alone or combined with cucurbitacin and E can cause G2/M phase arrest and apoptosis in breast cancer cells.

5. Conclusion

In summary, cucurbitin B has a growth and migration inhibitory effect on T24 cells. And cucurbitin B has a cycle blocking effect on bladder cancer cells, and this effect is a dose-dependent manner. Cucurbitacin B significantly upregulates Caspase signalling and induces apoptosis in T24 cells. Therefore, these findings indicate that cucurbitacin B is a potential potent candidate for bladder cancer therapy in the future. Further experiments under in vivo conditions will be required to confirm the effects of cucurbitacin B.

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