Apoptosis-inducing effects of lentinan on the proliferation of human bladder cancer T24 cells

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Abstract: The aim of this study was to explore the effects of lentinan on the proliferation of human bladder cancer T24 cells and the mechanism regarding the inhibition of cell growth. When gene regulation technique was used to build pcDNA3-TRPM8 expression plasmid, TRPM8 channel activator-lentinan was used for intervention to observe the proliferation of T24 cells. Flow cytometry cell screening method was used to observe the cell ratio of each cell cycle of T24 cells and the ratio of apoptotic and dying cells under the intervention of different concentrations of lentinan using PI single-staining and Annexin V-FITC/PI double-staining. JC-1 and DCFH-DA fluorescence probes were used to observe the influence of different concentrations of lentinan on the mitochondrial membrane potential of T24 cells and intracellular reactive oxygen species (ROS) by confocal microscope. pcDNA-TRPM8 plasmid was successfully constructed, and lentinan could inhibit the growth of T24 cells in a dose-dependent pattern. Lentinan played its biological effect through TRPM8 channel to further inhibit the growth of T24 cells, reduced the mitochondrial membrane potential of bladder cancer T24 cell line, and increased the generation of ROS in human bladder cancer T24 cell line. Lentinan led to mitochondrial depolarization or activation of non-mitochondrial pathway to induce intracellular ROS generation, thus eventually inducing T24 cell death and growth inhibition.

Keywords: Lentinan, bladder cancer, T24 cell, apoptosis.

INTRODUCTION

Bladder cancer is a common malignant lesion of the urinary system with the incidence and the mortality rate ranking fourth and eighth respectively among male malignant tumors. Although the majority of new patients can be treated with transurethral resection of bladder tumor (TUR-Bt), it has high postoperative tumor recurrence rate (Hamasaki *et al.*, 2010). Therefore, how to reduce the rate of tumor recurrence in patients with bladder cancer is the focus of research. Recent studies have shown that postoperative intravesical instillation chemotherapy and immunotherapy can reduce the recurrence rate in patients, but the effect of existing drugs is limited in relieving tumor recurrence rate, and some of the side effects are intolerable. Therefore, it is urgent to seek safe and efficient drugs to solve the problem of high recurrence in patients with bladder cancer (Matsushima *et al.*, 2010).

Polysaccharides and unsaturated fatty acids are the main ingredients of *Lentinus edodes* which also contains a variety of ergosterol and fungisterol that can be transformed into vitamin D. Lentinan is the effective active constitute extracted from high-quality *L. edodes* fruiting bodies, which can regulate the activity of T lymphocytes with immune function in human body, and reduce the capability of methylcholanthrene in tumor induction (Higashi *et al.*, 2012). *L. edodes* has a strong inhibitory effect on cancer cells, with the inhibition rate of 97.5% on mouse sarcoma 180 and 80% on Ehrlich carcinoma. Although lentinan cannot directly kill tumor cells in vivo, it can play its antitumor activity by improving immune function (Harada *et al.*, 2010). Lentinan can enhance the activity of splenetic and peritoneal NK cells in vivo as well as induce interferon dose-dependently, and its activity has synergistic effects on interleukin or interferon inducers (Nakai *et al.*, 2012). In addition, it has been proven that lentinan can enhance the anti-HIV activity of deoxythymidine in vitro (Hao *et al.*, 2012). The active ingredient of lentinan is β-(1-3)-D-glucan with branches, whose main chain consists of glucosyl group linked by β-(1-3), glucosyl group linked by β-(1-6) randomly distributed along the main chain in a comb structure. Studies have shown that the active polysaccharide of β-(1-3)-D-glucan is effective for inhibiting heterologous, homologous and even hereditary tumors (Sreenivasulu *et al.*, 2010).

This study aims to explore the impact of lentinan on the proliferation of bladder cancer cells and its mechanism, aiming to lay theoretical foundation for the clinical treatment of bladder cancer and new drug development regarding lentinan.

MATERIALS AND METHODS

Materials

The main reagents and apparatus included: lentinan, Hangzhou Johncan Biotech Co., Ltd.; human bladder

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cancer T24 cells, Sigma (the United States); Annexin V-FITC kit, Amresco (the United States); JC-1 mitochondrial membrane potential detection kit, Shanghai Ruiqi Biological Technology Co., Ltd.; DCFH-DA fluorescent probe, Fermentas (the United States); CO_2 incubator, Zhengzhou Nanbei Instrument & Equipment Co., Ltd.; laser scanning confocal microscope (DCM-3D), Labnet (the United States); FACSCalibur flow cytometry instrument, Thermo (the United States).

Construction of pcDNA3-TRPM8 expression plasmid

TRPM8 primer sequences were synthesized by Beijing Institute of Biological Products: its upstream primer was 5'-ATAGGATCCATGTCCTGAGGGAGCCAG-3', its downstream primer was 5'-CGCCTCGAGTGTTA ACCCATAGCAA-3', with the length of 1625 bp. BamH I and Xho I restriction cleavage sites were added to forward and reverse primers respectively. Part of the enzyme-digested products were detected by agarose gel electrophoresis, and the remaining products were purified using TaKaRa PCR purification kit for connection with plasmid, and the purified enzyme-digested products were recovered. The products were linked with plasmid by T4DNA ligase at 22°C for 5 min, DH5a competent cells were transformed and cultured selectively with ampicillin, and finally the plasmid was extracted after positive clone amplification for double digestion and DNA sequencing.

Detection of cell proliferation under the prevention of lentinan

Culture solution (100μl) containing 1×10^5 T24 target cells to each well of 96-well culture plates, and cultured in incubator containing 5% CO2 and saturated water vapor at 37°C for 6 h for cell adhesion. The lentinan was dissolved in DMSO to form a stock solution to a final concentration of 100mM. McCoy's 5A culture solution was used to successively prepare 100μl of working fluids containing 0, 10, 100, 500, 1000 and 2000μM lentinan. Three reduplicate wells were set for each group of concentration. The above lentinan working fluid of each concentration was added for routine culture in CO_2 incubator for 72h and then 20μl of CCK-8 solution was added to each well and continued to be incubated in cell incubator. After 2h, the culture solution was removed completely to measure the absorbance at 450 nm and to record data for analysis.

Cell apoptosis detection

After being treated with lentinan for 72h, the cells were digested with 0.25% trypsinization for cell collection. The collected cells were washed twice with cold PBS, added 500 μl of binding buffer suspension cells to prepare to single cell suspension, added 5μl of Annexin V-FITC, mixed evenly, added 5μl of PI, and mixed evenly for reaction for 10 min at room temperature in dark. Flow cytometry was used for detection, with the excitation wavelength of 488 nm and emission wavelength of 530 nm. Green and red fluorescences were observed through FLL and FL3 pathways respectively.

Detection of mitochondrial membrane potential

T24 cells were incubated in 35mm glass-bottomed culture dish for routine culture. After cell adhesion, working solution containing final concentrations of 0, 10, 100, 500, 1000 and 2000μM of lentinan was successively added for continuous culture in incubator for 72h. Then the culture solution was removed, and 1ml of JC-1 staining working solution was added, mixed with ultra-pure water to fully dissolve, added 5 times volume of JC-1 staining buffer to a JC-1 working solution, fully mixed and incubated in cell incubator at 37°C for 20min. During the incubation, appropriate amount of JC-1 staining buffer was prepared by adding 4 ml of distilled water into 1ml of JC-1 staining buffer, and placed in ice bath. After the incubation at 37°C, the supernatant was sucked out, washed with JC-1 staining buffer twice, and then added 2ml of cell culture solution to observe by a confocal microscope. The excitation light and emission light were set to 490 nm and 530nm respectively for JC-1 monomer detection, and 525nm and 590 nm respectively for JC-1 polymer detection to record the fluorescence ratio.

Detection of active oxygen

T24 cells were incubated in 35mm glass-bottomed culture dish for routine culture. After cell adhesion, working solution containing final concentrations of 0, 10, 100, 500, 1000 and 2000μM of lentinan was successively added for continuous culture in incubator for 72 h. Then the culture solution was removed, 1ml of DCFH-DA working solution was added, and incubated in incubator at 37°C for 20 min. Then the cells were washed with serum-free cell culture solution three times to sufficiently remove the DCFH-DA not entering the cells. Laser scanning confocal microscope was used for direct observation with the excitation wavelength of 488 nm and emission wavelength of 525 nm. The fluorescence value was recorded for analysis (Aranda *et al.*, 2013).

STATISTICAL ANALYSIS

All data were analyzed by SPSS 15.0 and expressed as ($\bar{x}$ ±s). The data were subjected to one-way ANOVA using P<0.05 as statistically significant difference.

RESULTS

Construction of pcDNA-TRPM8 plasmid

After double digestion with Xho I and BamH I, the pcDNA3-TRPM8 recombinant could be cut into two fragments of 3.8 kb and 6.0 kb, which are consistent with the theoretical sizes in zymogram analysis by DNASTAR SepMan software, prompting that PCR amplification product was successfully linked with the vector (fig. 1).

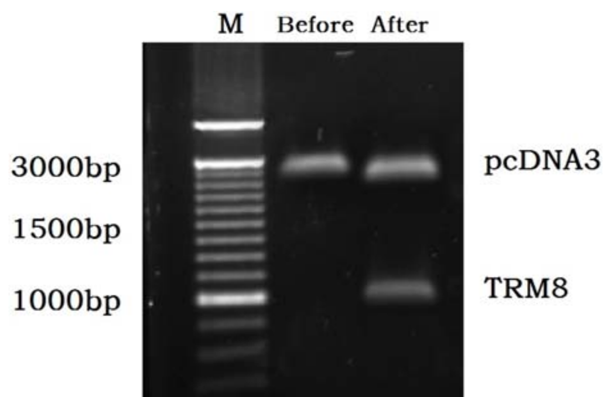

Fig. 1: Identification of pcDNA3-TRPM8 recombinant

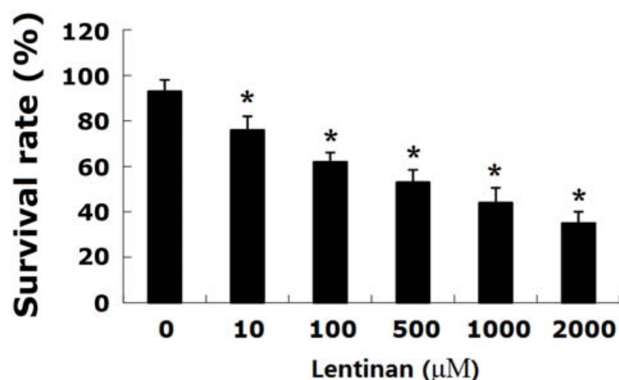

Fig. 2: Survival rates of T24 cells under the intervention of lentinan. Compared with the control group, * P<0.05.

Inhibitory effects of lentinan on the growth of T24 cells in a dose-dependent pattern

To explore the effect of TRPM8 channel on T24 cells, this study further used lentinan to affect the cells and to observe their growth. As shown in table 1, the OD values of the 0, 10, 100, 500, 1000 and 2000μM of lentinan intervention groups decreased dose-dependently, and the difference was statistically significant compared with that of the control group (lentinan= 0 μM) (P<0.05), indicating that lentinan can inhibit the growth of T24 cells in a dose-dependent paradigm. In case that the cell survival rate of the control group was 100%, as the lentinan concentration reached 10 μM, the cell survival rate was decreased, and declined further with rising lentinan concentration. The apparent distribution is described in fig. 2.

Inhibitory effects of lentinan on the growth of T24 cells via TRPM8 pathway

The inhibitory effect of lentinan on T24 cells were observed by the way that TRPM8 expression was pre-silenced in the cells using siRNA interference technique. As shown in table 2, under the intervention of 1000μM lentinan, the OD values of the control group (0μM), siRNA + lentinan group and lentinan group were significantly different (P<0.05). Inter-group comparison found that the control group was not significantly different from the siRNA + lentinan group (P>0.05), but was significantly different from the lentinan group (P<0.05), indicating that there is a significant difference only between the lentinan group and the other groups. In case that the cell survival rate of the control group was 100%, under the intervention of 1000μM lentinan, the cell survival rate was lower, but after the TRPM8 was silenced, the cell survival rate was increased significantly. The results suggest that lentinan exerts its biological effects through the TRPM8 channel, thus further suppressing the growth of T24 cells (table 2, fig. 3).

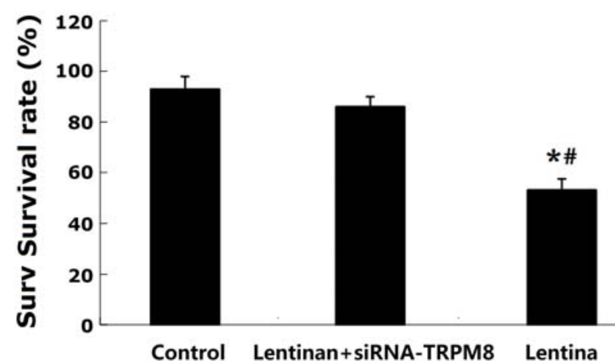

Fig. 3: Survival rates of T24 cells under the intervention of lentinan and siRNA. Compared with the control group, *P<0.05; compared with the siRNA + lentinan group, #P<0.05.

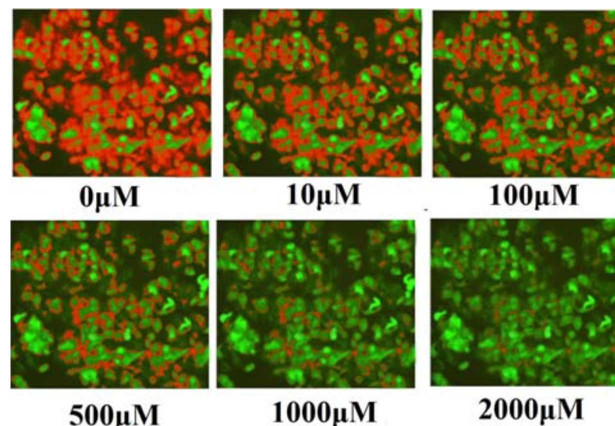

Fig. 4: Mitochondrial membrane potential of T24 cells under the intervention of lentinan.

Apoptosis-inducing effects of lentinan on T24 cells

In this study, the effect of lentinan on T24 cell apoptosis was observed using Annexin V-FITC/PI double staining. We found that after the intervention of different concentrations of lentinan for 72 h, no T24 cell apoptosis appeared, and the cell mortality rate was gradually increased with elevating dose of lentinan (table 3). Statistical analysis showed that no statistically significant difference was found in the apoptosis rate of T24 cells in overall samples (P>0.05), but there was a significant difference in the cell mortality rate (P<0.05). Compared with the control group, the differences were statistically significant among the 10, 100, 500, 1000 and 2000μM of

lentinan groups (P<0.05). The results suggest that lentinan can dose-dependently induce the death rather than the apoptosis of T24 cells, so as to further inhibit their growth.

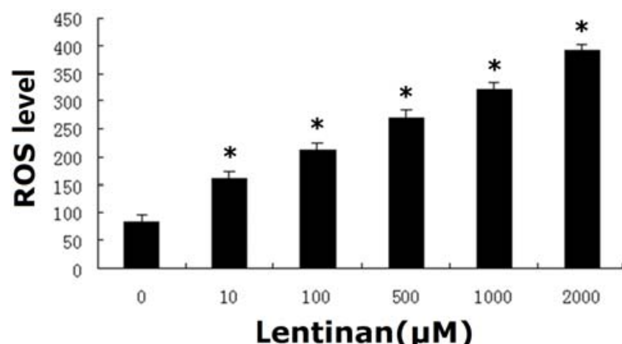

Fig. 5: ROS contents in T24 cells under the intervention of lentinan. Compared with the control group, * P<0.05.

Table 1: OD values of T24 cells under the intervention of lentinan

Group	OD value
0 μM (control)	0.942±0.031
10 μM	0.827±0.096*
100 μM	0.751±0.084*
500 μM	0.638±0.071*
1000 μM	0.512±0.058*
2000 μM	0.429±0.051*

Compared with the control group, * P<0.05.

Table 2: OD values of T24 cells under the intervention of lentinan and siRNA-TRPM8

Group	OD value
Control	0.869v0.038
Lentinan + siRNA-TRPM8 (1mM)	0.813±0.041
Lentinan (1mM)	0.526±0.037*#

Compared with the control group, *P<0.05; compared with the siRNA + lentinan group, #P<0.05.

Decrease of mitochondrial membrane potential by lentinan

The mitochondrial membrane potential was measured using the JC-1 fluorescent dye technique. After T24 cells were intervened with lentinan for 72h and loaded JC-1 fluorescence probe, confocal fluorescence microscope was used to observe the fluorescence excited by the T24 cells, and the red/green fluorescence ratio was recorded (the ratio and was proportional to the mitochondrial membrane potential, so the former was used to represent the latter in this study). The results showed that when the lentinan concentration reached 100μM, the ratio of red/green fluorescence excited by T24 cells was significantly decreased, i.e. the mitochondrial membrane potential was reduced dose-dependently. Statistical analysis showed that there were significant differences among the overall samples (P<0.05), and inter-group comparison showed that the differences were statistically significant among all groups (P<0.05) except that between the control group and the 10μM of lentinan group (P>0.05). The results reveal that in this study, only when the lentinan concentration reached 100μM could it significantly induce the mitochondrial membrane depolarization of T24 cells (table 4, fig. 4).

Table 3: Apoptosis-inducing effects of lentinan on T24 cells

Group	OD value
0μM (control)	0.814±0.051
10μM	0.728±0.047*
100μM	0.638±0.063*
500μM	0.572±0.060*
1000μM	0.467±0.049*
2000μM	0.382±0.075*

Compared with the control group, *P<0.05

Table 4: Ratio of red/green fluorescence under the intervention of lentinan

Group	Ratio of red/green fluorescence
0 μM (control)	0.913±0.068
10 μM	0.845±0.066
100 μM	0.782±0.054*
500 μM	0.675±0.052*
1000 μM	0.542±0.046*
2000 μM	0.471±0.044*

Compared with the control group, * P<0.05.

Increase of ROS by lentinan

In this study, DCFH-DA fluorescent probe was also used for the detection of ROS level in T24 cells under the intervention of lentinan. The result showed that when the lentinan concentration reached 10μM, the red fluorescence of T24 cells was gradually increased with the dose increase of lentinan. Analysis of variance showed that there were significant differences among the overall samples (P<0.05) and compared with the control group, there were statistically significant differences in the 10, 100, 500, 1000 and 2000μM of lentinan groups (P<0.05). In case that the ROS level of the control group was 100%, after the intervention with 10, 100, 500, 1000 and 2000μM of lentinan for 72h, the ROS level in T24 cells was successively increased in a dose-dependent way. The result indicates that lentinan can dose-dependently increase the ROS level in T24 cells (table 5, fig. 5).

DISCUSSION

TRPM8 (transient receptor potential melastatin 8) is a kind of coding Ca^{2+} permeability cation pathway, which can be activated when stimulated by low temperature and drugs to participate in the adjustment of Ca^{2+}

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concentration in cells (Zhu *et al.*, 2011). Early researches believed that in the normal tissues of mammals, the expression of TRPM8 was almost limited to sensory nerve and prostate, so it was regarded as the specific gene of some cancers (Valero *et al.*, 2012). However, nowadays, more and more researches have shown that TRPM8 has different levels of expressions both in vascular smooth muscle and urogenital system, but the bioactivity in these tissues still requires studies (Gkika *et al.*, 2010). The expression of TRPM8 is closely related with the tumor grading, so some scholars think that TRPM8 can be taken as the diagnosis indicator of cancer (Bai *et al.*, 2010). In addition, it is also found that in addition to bladder cancer, TRPM8 mRNA also has significant different expression in normal and malignant tissues such as mammary gland, colon and skin (Chodon *et al.*, 2010). Therefore, some conjecture that the expression of TRPM8 might be related with cancer biology (Li *et al.*, 2009). TRPM8 is a Ca^{2+} pathway, while Ca^{2+} activity has been proved to play an important role in regulating the biological behavior of tumor (Knowlton and McKemy, 2011).

Table 5: ROS fluorescence under the intervention of lentinan

Group	ROS fluorescence
0μM (control)	168.33±24.25
10μM	291.26±33.57*
100μM	360.67±35.54*
500μM	492.28±40.62*
1000μM	618.87±51.65*
2000μM	709.26±59.28*

Compared with the control group, * P<0.05.

Programmed cell death (PCD) is the active cell death controlled by gene, which does not occur in physiological conditions, but also occurs by the induction of cytokines like tumor necrosis factor α (TNF-α) and chemical drugs (Omori *et al.*, 2012). Since the end of the last century, people have always treated PCD equivalent to cell apoptosis (caspase-mediated cell death). In recent years, numerous research results show that cell apoptosis is just one of those early discovered in many PCD mechanisms and it has also been researched clearly (Itani *et al.*, 2012). The damages of some organelles, including mitochondria, endoplasmic reticulum and lysosome will all increase the calcium ion, generate reactive oxygen species (ROS) and release some effect proteins, finally leading to the occurrence of non-caspase dependent PCD. The mechanism of PCD is complex and diversified (Hu *et al.*, 2012).

In this study, we detected the possible way of lentinan to induce T24 cells. The results show that after 72 h of lentinan intervention, there was no change in cell cycle or occurrence of apoptosis to cause direct cell death, and with the dose increase of lentinan, the action is strengthened (Wang *et al.*, 2012). This non-apoptotic cell death is the same as that discovered by Ina, et al. in human gastric carcinoma PC3 cells. Their results show that lentinan can directly induce the death of PC3 cells, and there is also no classic apoptosis phenomenon observed (Ina *et al.*, 2011). Sreenivasulu et al. also found that lentinan directly induced the necrosis-like PCD of human leukemic HL-60 cell line, which was also a non-classical pathway of apoptosis (Sreenivasulu *et al.*, 2010).

Reactive oxygen species (ROS) is a single-electron reduzate of oxygen in the body, including superoxide ion, hydrogen peroxide (H_2O_2), hydroxyl radical and nitric oxide, etc. ROS can destroy organelles, like endoplasmic reticulum, mitochondrial membrane and cell membrane, causing the redistribution of intracellular Ca^{2+}, inward flowing of extra cellular Ca^{2+}, resulting in Ca^{2+} level changes with a complicated relation among Ca^{2+}-mitochondria-ROS (Chen *et al.*, 2013). In addition to the classic apoptotic caspase system, ROS also participates in the regulation of two important signal pathways: Ras-Raf-MEKl/2-ERKl/2 and p38 mitogen-activated protein kinases (MAPK). The early views generally think that Ras-Raf-MEKl/2-ERKl/2 was related with the promotion of tumor growth, so it was a tumor factor, while p38 MAPK related with tumor cell death, so it was an anti-tumor factor (Achour *et al.*, 2013). The results of this study show that lentinan can increase the level of ROS in T24 cells, down-regulate the expression level of Ras protein, and fully inhabit the phosphorylation state of Ras-Raf-MEKl/2-ERKl/2 pathway protein, but has no effect on the expression level and phosphorylation state of p38 and JNK.

In summary, the study results indicate that by increasing Ca^{2+} in T24 cells of human bladder cancer cell line, lentinan can further reduce the mitochondrial membrane potential, lead to the mitochondrial depolarization, so as to induce the generation of ROS in T24 cells and further inhibit Ras-Raf-MEKl/2-ERKl/2 signal pathway, finally causing T24 cell death and growth inhibition. This lays foundation for further exploring the effect of lentinan and TRPM8 channel on human bladder cancer.

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