RESEARCH ARTICLE

Inotodiol Inhabits Proliferation and Induces Apoptosis through Modulating Expression of cyclinE, p27, bcl-2, and bax in Human Cervical Cancer HeLa Cells

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Abstract

***Inonotus obliquus* is a medicinal mushroom that has been used as an effective agent to treat various diseases such as diabetes, tuberculosis and cancer. Inotodiol, an included triterpenoid shows significant anti-tumor effect. However, the mechanisms have not been well documented. In this study, we aimed to explore the effect of inotodiol on proliferation and apoptosis in human cervical cancer HeLa cells and investigated the underlying molecular mechanisms. HeLa cells were treated with different concentrations of inotodiol. The MTT assay was used to evaluate cell proliferating ability, flow cytometry (FCM) was employed for cell cycle analysis and cell apoptosis, while expression of cyclinE, p27, bcl-2 and bax was detected by immunocytochemistry. Proliferation of HeLa cells was inhibited by inotodiolin a dose-dependent manner at 24h (r=0.9999, $p<0.01$). A sub-G_1 peak (apoptotic cells) of HeLa cells was detected after treatment and the apoptosis rate with the concentration and longer incubation time (r=1.0, $p<0.01$), while the percentage of cells in S phase and G_2/M phase decreased significantly. Immunocytochemistry assay showed that the expression of cyclin E and bcl-2 in the treated cells significantly decreased, while the expression of p27 and bax obviously increased, compared with the control group ($p<0.05$). The results of our research indicate that inotodiol isolated from *Inonotus obliquus* inhibited the proliferation of HeLa cells and induced apoptosis *in vitro*. The mechanisms may be related to promoting apoptosis through increasing the expression of bax and cutting bcl-2 and affecting the cell cycle by down-regulation the expression of cyclin E and up-regulation of p27. The results further indicate the potential value of inotodiol for treatment of human cervical cancer.**

Keywords: Cervical cancer - inotodiol - cell cycle - cyclinE - p27 - bcl-2 - bax

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Introduction

Cervical cancer is the third most prevalent malignancy among women worldwide (Ferlay et al., 2010), with an estimated approximately 530000 new cases and 275, 000 women death each year. Patients with advanced-stage or relapsed cervical cancer are often given priority to combination radiation treatment with chemotherapy treatment due to the lost opportunity to operation, but long-term and high-dose chemotherapy usually cause drug resistance and adverse reaction (van et al., 2013). So it is a new direction of cervical cancer therapy to seeking for anti-tumor drugs of high efficiency and low toxicity from natural products in recent years.

Natural product-oriented synthetic derivatives have played an important role in anti-tumor drug discovery (Koehn et al., 2005; Mishra et al., 2011). Inonotus obliquus (*I. obliquus*) is a medicinal mushroom that has been used as an effective agent to treat various diseases such as diabetes, tuberculosis and cancer (Zhong et al., 2009; Song et al., 2013). Inotodiol, a lanostane triterpenoid extract of *I. obliquus* have been identified to have various biological effects, including anti-tumor (KahlosK, 1987; Nakata et al., 2007), anti-oxidant (Zhao et al., 2012), anti mutation (Ham et al., 2009). Recent reports showed that Inotodiol could inhibit proliferation and induce the apoptosis of A549 cells *in vitro* (Zhong et al., 2011), moreover, Inotodiol have low cytotoxicity against normal cells (Mi et al., 2010). These data suggest that Inotodiol could be a potential natural product for the treatment of cancer. However, Inotodiol on other types of cancer and the precise molecular mechanisms of the compound have not been well documented. In the present investigation, we explored the anti-tumor effects of Inotodiol against cervical cancer HeLa cells, and attempted to further elucidate the possible mechanisms of action, providing a basis for future development of this agent as human cervical cancer therapy.

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Materials and Methods

Cell and cultivation

Human cervical cancer cell line HeLa were cultured in RPMI1640 (GIBCO, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA), sodium bicarbonate 2 g, 100 u/ml penicillin and 100 μg/ml streptomycin, pH 7.0~7.2 and maintained at 37°C in a humid atmosphere containing 5% CO_2. Passage of the cells was achieved every 2~3 days through the digestion of 0.25% trypsin (GIBCO, USA). Cells in the logarithmic growth phase were seeded according to acquired concentrations.

Cell proliferation assay

The cytotoxic potential of Inotodiol was evaluated by the MTT assay (Chung et al., 2005). Inotodiol separated from *I. obliquus* were extracted and identified by Dr. Zhao Fenqin (Zhao et al., 2006). The purity of Inotodiol was 99%. Inotodiol were dissolved in DMSO (Thermo, USA), and the stock solutions were diluted with RPMI 1640 medium (DMSO concentration<0.01%). HeLa cells were plated onto 96 wells at a density of 1×10^5 cells/ml and incubated with Inotodiol (0, 12.5, 25, 50, and 100 μg/ml) for 24 and 48h. The control was physiological saline only. MTT (Sigma , USA) (5 mg/ml) was added to each well , and then incubated for an additional 4h at 37°C. The blue formazan product was dissolved in 150 μL DMSO with shaking for 10 min. The extent of the reduction of MTT was quantified by measuring the absorbance at 570 nm using a spectrophotometric plate reader (Bio-Rad, Tokyo, Japan). The experiments were performed in triplicate (n=3). Inhibitory%=[100- (ODt/ODs)×100]%. ODt and ODs are the optical density of the test substance and solvent control, respectively (Ramachandran et al., 2008) .

Cell cycle analysis

To determine cell cycle distribution analysis, HeLa cells were treated with 50, 100 μg/ml Inotodiol (with IC_{50} values obtained from MTT assay) for 24h and 48h. After treatment, the cells were collected and by trypsinization. Cells were centrifuged at 1000rpm for 5min and washed twice with ice-cold PBS, then centrifuged at 1000rpm for 4min. Cells were suspended with ice-cold PBS and separated into single cells, fixed with 70% ethanol ice-cold, incubated with 0.1 mg/ml RNase for 30 min at 37°C, and stained with 50 μg/ml PI (Sigma, USA) (Darzynkiewicz et al., 2001). Cell cycle was detected by flow cytometry (BD, USA) with standard procedures.

Immunocytochemical staining analysis

HeLa human cervical cancer cell lines were treated with 50 μg/ml Inotodiol for 48h. After 48h, cells for each group were harvested and fixed with a freshly prepared acetone solution for 10min at 4°C. The assay was performed according to the recommendations of the instruction manual of UltraSensitiveTM SP kit (Maxim, China). The primary antibodys mouse anti-cyclinE, p27, bcl-2 and bax (Santa Cruz, USA) diluted at 1:100. Immunocytochemistry judge standard positive reaction of cyclinE and p27 were both brown color situated in nucleolus. Positive reaction of bcl-2 was brown color situated in cytoplasm, and that of bax was brown color situated in cytoplasm and /or cytomembrane. For the negative controls, the primary antibodies were replaced with phosphate-buffered saline (PBS). Positive cells and cell population were counted as follows: ten areas were selected at random, then positive cells and cell population were counted and recorded respectively at high power field (HPF, ×400 magnification). Cell index (%)=positive cells/cells population×100%.

Statistical analysis

All experiments were performed in triplicate. Data were expressed as mean±SD. Statistical analyses were performed using GraphPad Prism 5.0 software package (GraphPad Software, United States). Statistical analysis was determined by two-way ANOVA and Bonferroni correction. A p value less than 0.05 was considered statistically significant.

Results

Effect of inotodiol on proliferation of HeLa cells

Inotodiol (0, 12.5, 25, 50, 100 μg/ml) was evaluated for its effects on cervical cancer HeLa cells proliferation *in vitro* by MTT assay for 24 and 48 h. Cell inhibation was raised up with higher concentration and longer incubation time and the effect was in a dose-dependent manner (r=0.9999, p<0.01) (Figure 1A). The inhibitory rate of Inotodiol in HeLa cells was 7.67%, 13.00%, 24.77% and 48.50 for 12.5, 25, 50, and 100 μg/ml at 24h; 5.60%, 20.33%, 49.17% and 61.83% for 12.5, 25, 50, and 100 μg/ml at 48h, respectively. Significant anti-proliferative effects were seen in HeLa cells with Inotodiol at a concentration of 100 μg/ml. IC_{50} was 116.5 μg/ml at 24h, and 62.9 μg/ml at 48h. Inotodiol also induced morphological changes seen as cell shrinkage and rounding. Besides, a significant decrease in cell density was observed when compared to the control cells (Figure 1 B, C and D).

Effect of inotodiol on cell cycle distribution and apoptosis in HeLa cells

Apoptosis of the HeLa cells was performed after 24h

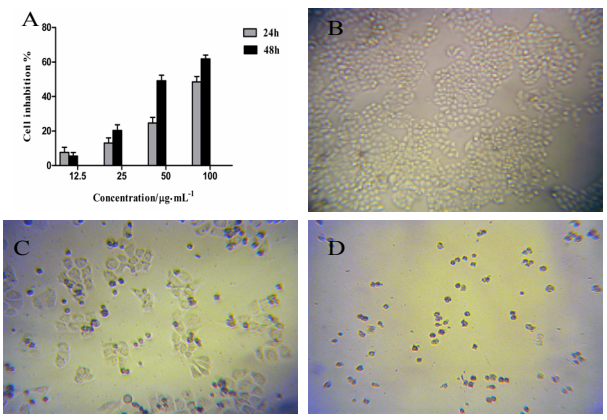

Figure 1. Effects of Inotodiol on Proliferation Inhibitory Rate of HeLa Cells. A) Cell growth inhabition of inotodiol. **B)** Control **C)** 50 μg/ml. **D)**100 μg/ml (×200)

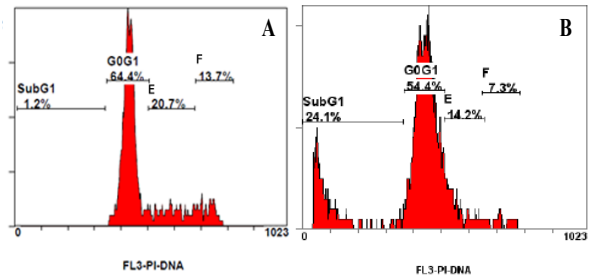

Figure 2. Assay Graphs of Flow Cytometry after Inotodiol Treatment for 24h. A) Control; **B)** 100 μg/ml

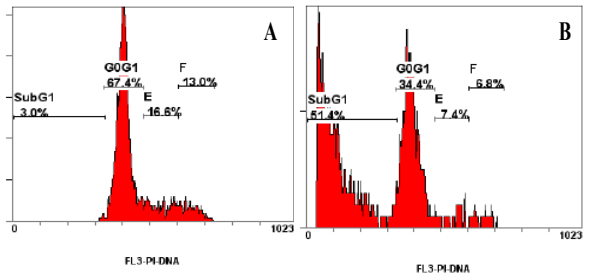

Figure 3. Assay Graphs of Flow Cytometry after Inotodiol Treatment for 48h. A) Control; **B)** 100 μg/ml

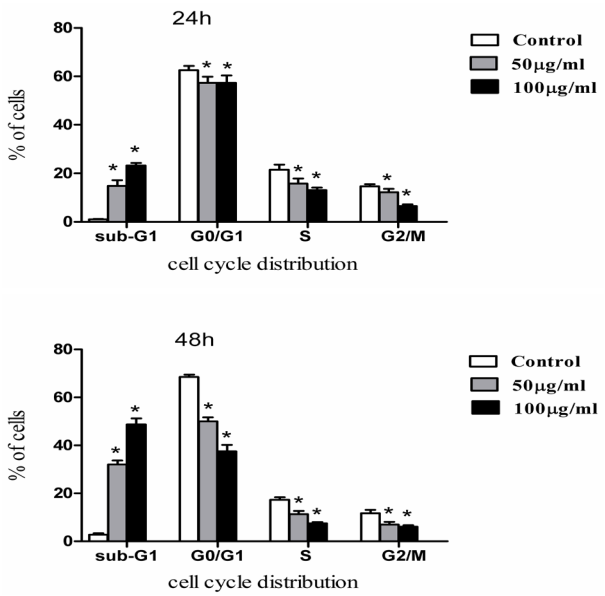

Figure 4. Comparison of Percentage of Cells in Different Phases of the Cell Cycle (n=3, $\bar{x}\pm s$) on treatment with inotodiol (50, 100 μg/ml) for A) 24h **B)** and 48h. *p<0.05, compared with the control group

and 48h of incubation with Inotodiol (50, 100 μg/ml) respectively. After the incubation of 100 μg/ml Inotodiol for 24h, apoptosis sub-peak appears in flow cytometry detection, more apoptosis cells were detected with higher concentration of Inotodiol or longer incubation time. Especially, the apoptosis index can be up to 51.4% after 100 μg/ml Inotodiol treatment for 48h (Figure 2, Figure 3). In addition, with increasing of Inotodiol concentration, cell cycle changed markedly including the percentage of S phase and G_2/M phase decreased significantly (p<0.05), together with G_0/G_1 phase down is obvious at 48h (p<0.01), but no significant difference in G_0/G_1 phase between 50 μg/ml and 100 μg/ml Inotodiol group at 24h (Figure 4).

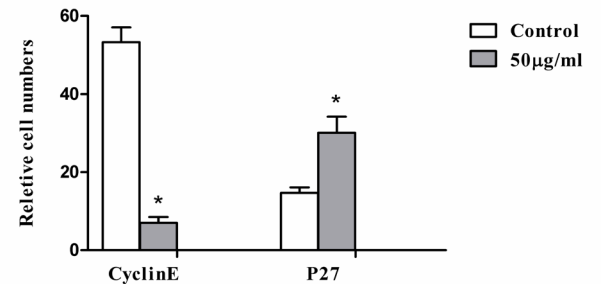

Figure 5. Immunocytochemical Staining Analysis of Differential Expression of CyclinE and P27 of HeLa Cells on Treatment with Inotodiol (50 μg/ml) for 48h. *p<0.05

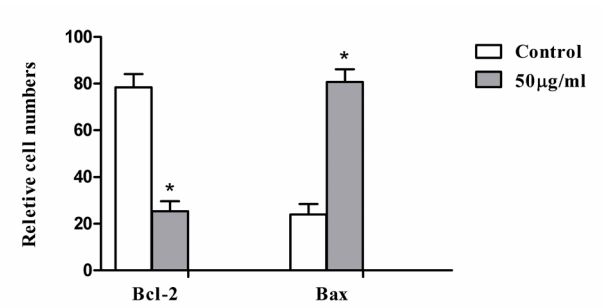

Figure 6. Immunocytochemical Staining Analysis of Differential Expression of Bcl-2 and Bax of HeLa cells on Treatment with Inotodiol (50 μg/ml) for 48h. *p<0.05

Effects of inotodiol on expression of cyclinE and p27 in HeLa cells

After treated with Inotodiol at concentration of 50 μg/ml for 48h, the expression of cyclinE decreased markedly, while the expression of p27 increased markedly in Inotodiol group. There were statistic significance in the two groups compared with the control group (p<0.05, Figure 5).

Effects of inotodiol on expression of bcl-2 and bax in HeLa cells

After treated with Inotodiol at concentration of 50 μg/ml for 48h, the expression of bcl-2 decreased markedly, while the expression of bax increased markedly in Inotodiol group. There were statistic significance in the two groups compared with the control group (p<0.05, Figure 6).

Discussion

Recent report showed that six main constituents were isolated from *I. obliquus* and they were identified as lanosterol (1), 3β-hydroxy-8, 24-dien-21-al (2), ergosterol (3), Inotodiol (4), ergosterol peroxide (5) and trametenolic acid (6) (Ma et al., 2013). Inotodiol, a lanostane triterpenoids, from a chloroform extract of *I. obliquus* sclerotia (Nakata et al., 2007), exhibited significant anti-proliferative effects against mouse leukemia P388 cells (Nomura et al., 2008), Walker 256 carcinosarcoma cells and human mammary adenocarcinoma MCF-7 cells (KahlosK, 1987). In this study, inhibitory rate of HeLa cells was detected by MTT assay after treated with different concentrations of Inotodiol for 24h and 48h. Our

results clearly showed that Inotodiol have inhibitory effect on proliferation of HeLa cells in a dose-dependent manner.

The anti-tumor effect of the drug could be attributed to cytostatic and/or cytotoxic function. Cytostatic substance could prevent cell growth and/or induce cell cycle arrest at various cell cycle checkpoints. On the other hand, cytotoxic substance could induce cell death by apoptosis or necrosis (Pachon et al., 2007). Therefore, cell cycle arrest and the induction of cell apoptosis to prevent cancer cell proliferation becomes the major target of anti-cancer drugs. To further clarify the mechanism of growth inhibition, its ability to arrest cell cycle and induce apoptosis has been studied. Youn et al. advocated for the first time that *I. obliquus* extract inhibited the growth of HepG2 cells in a dose-dependent manner, which was accompanied with G_0/G_1 phase arrest and apoptotic cell death. In addition, G_0/G_1 arrest in the cell cycle was closely associated with down-regulation of cyclinD1, cyclinD2, cyclinE, CDK2, CDK4, and CDK6 expression (Youn et al., 2008). Then, Youn et al. further demonstrated *I. obliquus* extract inhibited the growth of B16-F10 cells by causing cell cycle arrest at G_0/G_1 phase and apoptosis (Youn et al., 2009). Afterwards, Nakajima et al. demonstrated that the extracts from *I. obliquus* induce IMR90 cell cycle arrest in S phase and cause obvious cell apoptosis, associated with κB suppression (Nakajima et al., 2009). In this study, flow cytometric analysis clearly revealed that HeLa cells were induced apoptosis by Inotodiol, and the apoptosis rate increased gradually with higher concentration and longer incubation time. However, cell cycle arrest was not observed neither in G_0/G_1 phase nor S phase. It seems that considerable cell apoptosis in HeLa cells can account for this. Therefore, we infer that Inotodiol inhibit proliferation of HeLa cells mainly through inducing apoptosis. Whether the cell cycle arrest play a role in proliferation inhibition after intervention of Inotodiol deserves further exploration.

Orderly conduct of the cell cycle is the important basis of life activities, mainly depends on the cyclin-dependent kinase (CDK) in the sequential activation and deactivation. Close cooperation of CDKs, CDK inhibitors (CKIs) and cyclins is necessary for ensuring orderly progression through the cell cycle (Lim et al., 2013). CyclinE, which plays an important role in cell proliferation, is one of the main limit factor of G_1/S phase transformation. Over expression of cyclinE can accelerate G_1 phase procession of the cell (Suryadinata et al., 2011). As a cyclin-dependent kinases inhibitor, p27 negatively regulates G_1-G_2 cell cycle progression by binding to and preventing the activation of cyclinD1-CDK4 or cyclinE-CDK2 complexes (Vermeulen et al., 2003). Changes in the level of p27 can alter the progression of the cell cycle and its reduced expression has been shown in some tumors (Slingerland et al., 2000). In present research, cell cycle proteins related with G_1 phase arrest were detected by immunocytochemistry staining. Down-regulation of cyclinE expression, together with up-regulation of p27 expression was observed after Inotodiol treatment. The mechanism may be the high expression of p27 inhibit the activity of CDK, and then arrest the tumor cells in G_1 phase.

Damaged cells could be repaired to maintain the integrity of the cell or be cleared by apoptosis to remove a cancerous cells by G_1 phase arrest. Apoptosis is one aspect of mammalian cell behavior, which is of central importance in growth and development and plays a key role in tumor-oncogenesis. Bcl-2 and bax are two critical regulators of cell apoptosis and play pivotal roles in anti-apoptotic and pro-apoptotic, respectively (Kanthan et al., 2010). Further, it has been proposed that the susceptibility to apoptosis is likely to be determined by the ratio of bcl-2/bax, which are key regulators of apoptosis located mainly at the outer membrane of mitochondria and endoplasmic reticulum, its regulation of apoptosis was achieved mainly through the mitochondria. Overexpression of bcl-2 is known to confer resistance to apoptosis, whereas overexpression of bax results in increasing apoptosis (Krajewski et al., 1994; Kanthan et al., 2010). Our study showed diffuse expression of bcl-2 and bax proteins in contrast to the control group. Expression of bcl-2 was statistically significant decreased, while that of bax was statistically significant increased after treatment with Inotodiol. Apparently, bcl-2/bax ratio of HeLa cells was reduced by Inotodiol, which is consistent with the results of cell apoptosis. The results suggested that Inotodiol induced apoptosis through adjusting the expressions of bcl-2 and bax protein to reduce the ratio of them.

Taken together, the present study demonstrated that the cervical cancer cell lines HeLa is highly sensitive to growth inhibition by Inotodiol, which is associated with apoptosis induction and cell cycle arrest. The mechanism may be affecting the cell cycle by down-regulation the expression of cyclinE and up-regulation of p27, moreover, apoptosis-inducing through increasing the expression of bax and cutting bcl-2. In addition, the present results can provide new prospect for chemotherapy of cervical cancer. Despite this, further studies aimed at whether Inotodiol can reduce tumor growth *in vivo* are clearly warranted. We also need mechanistic studies on Inotodiol.

Acknowledgements

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