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Effect of NF-κB inhibitor PDTC on VEGF and endostatin expression of mice with Lewis lung cancer

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ABSTRACT

Objective: To investigate the effects of NF-κB inhibitor pyrrolidine dithiocarbamate hydrochloride (PDTC) on vascular endothelial growth factor (VEGF) and endostatin expression in mice with Lewis lung cance; and its mechanism. **Methods:** Mice survival rate and anti-tumor effects were observed in different concentrations of NF-κB inhibitor PDTC after the Lewis lung cancer mice model was established. VEGF and endostatin expressions were detected by immunohistochemical assay. **Results:** Lewis lung cancer was be inhibited by 0.5 mg/kg, 1.5 mg/kg and 3.0 mg/kg of NF-κB inhibitor PDTC (P<0.05). Microvessel density (MVD) in 0.5 mg/kg, 1.5 mg/kg and 3.0 mg/kg NF-κB inhibitor PDTC groups were significantly lower than the control group (P<0.05). Immunohistochemical assay results showed that VEGF and endostatin expressions in the 0.5 mg/kg, 1.5 mg/kg and 3.0 mg/kg NF-κB inhibitor PDTC groups were significantly lower than the control group (P<0.05). Western blot results also showed that NF-κB inhibitor PDTC could inhibit VEGF and endostatin expressions in tumor tissues. **Conclusions:** NF-κB inhibitor PDTC can inhibit tumor formation and reduce tumor angiogenesis in mice with Lewis lung cancer; and its mechanism maybe associated to VEGF and endostatin down-regulation.

1. Introduction

Pyrroli dinedithiocarbamic acid (PDTC) ammonium salt is a membrane-permeant inhibitor of NF-κB activation that participates in inhibiting the NF-κB pathway in a variety of cells. Relevant reports showed that, as an antioxidant and metal chelator, PDTC is involved in the precipitation of many heavy metal ions in acidic environments; and PDTC can also inhibit apoptosis in smooth muscle cells and leukemia cell HL-60 by apoptosis-related pathway. Furthermore, PDTC has a molecular weight of 164.29 Daltons and its molecular formula is $C_5H_9NS_2 \cdot NH_3$. PDTC is also soluble in water and its solubility in DMSO is up to 100 mM. In recent years, some studies have shown that NF-κB inhibitor PDTC can inhibit tumor cell growth and proliferation; and clinical applications showed that NF-κB inhibitor PDTC has antitumor effects in gastrointestinal tumors[1,2]. Thus, NF-κB inhibitor PDTC is expected to be an effective antitumor drug; however, this requires further studies. The Lewis lung cancer mice model was established in this study and anti-tumor effects of NF-κB inhibitor PDTC were observed. Microvessel density (MVD), tumor vascular endothelial growth factor (VEGF) and endostatin expressions were further observed to explore the possible mechanism of NF-κB inhibitor PDTC.

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2. Material and methods

2.1. Animals and tumor strains

Healthy, random male and female, 6-8 weeks old, weighting 18-22 g, C57/6 mice were provided by the Experimental Animal Center of Southern Medical University; which was raised in a specific-pathogen-free (SPF) laboratory. Lewis lung cancer cells were provided by the Guangdong Academy of Medical Sciences.

2.2. Drugs and Reagents

NF-κB inhibitor PDTC (Japan, Tokyo Chemical Industry Co., Ltd.); Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Guangzhou YingJun Biotechnology Co., Ltd.; FBS, F12, Hanks solution, and PBS were provided by Beijing DingGuo Biotechnology Co., Ltd.; cyclophosphamide powders were purchased from Xi'an Likang Xinte Pharmaceutical Co., Ltd; immunohistochemistry kit and corresponding antibodies were purchased from American Abcam Co., Ltd.

2.3. Establishment of Lewis lung cancer transplantation tumor model

Lewis lung cancer cells were diluted with sterile saline up to 110 mL tumor cell suspension concentration and six C57/6 mice were intraperitoneally inoculated under sterile conditions. When ascites grew vigorous in mice, 3-5 mL of ascites were extracted with medical injectors and transferred into a clean beaker. Then, the ascites were diluted with sterile saline up to the applied concentration, centrifuged for 5 min (1 500 rpm/min.). The supernatant was discarded and lower layer cells were washed 2-3 times with distilled water. Living cells were detected positive by Trypan Blue; Ratio of tumor cells reached 95% was considered as positive. Then, cells were further diluted with sterile saline until a 2 $\times 10^7$/mL concentration was attained. Two hundred C57/6 mice were subcutaneously injected with 0.2 mL of cell suspension to establish the Lewis lung cancer mice model.

2.4. Grouping

In this study, 40 C57/6 mice were randomly divided into five groups (n=8). After 48 hours of subcutaneous inoculation, the control group was injected with equivalent amounts of normal saline; the experimental group or NF-κB inhibitor PDTC group was administered with 0.5 mg/kg, 1.5 mg/kg and 3.0 mg/kg doses, respectively; the cyclophosphamide group or the positive control group was administered with a 20 mg/kg dose by intraperitoneal injection, 0.2 mL, once a day.

2.5. Tumor inhibition rate

After 15 days of continuous injections, mice were sacrificed by breaking their cervical vertebra. Metastasis sites were dissected, and inhibiting rates of the experimental and control groups were calculated as follows: Inhibiting Rate = [(Tumor weight of control group -Tumor weight of treatment group)/Tumor weight of control group] ×100%.

2.6. Determination of MVD in tumor microvessels

After mice were sacrificed, tumors were isolated and sliced into paraffin sections. Detailed procedures and staining method for MVD was according to the kit instructions. MVD measurement methods are as follows: appropriate regions or "hot spots" (rich in tumor microvessels) were first selected under a low magnification optical microscope, the low magnification field was then adjusted into high magnification (400×), and the number of brown dyed microvessels were counted. Five were randomly selected from the field of view; values were averaged and used as MVD values.

2.7. Protein expression determination by immunohistochemistry

After mice were scarified, tumors were isolated and sliced into 5 μm paraffin sections. Immunohistochemical staining method was used to measure MVD according to the kit instructions. Positive cells were brown-yellow particles. Immunohistochemical results were determined by semi-quantitative method or by the percentage of positive cells and dye depth. Positive cells: <10% is 1 point, 10%-50% is 2 points, >50% is 3 points; dye depth: non-positive cells has 0 point, yellow is 1 point, brownish yellow is 2 points, chocolate brown is 3 points. Total immune response score is equal to the percentage of positive cells times dye depth. The strongest staining results were represented by the following: negative (-): 0 point, weak positive (+): 1-3 points, positive (++): 4-6 points, and strong positive (+++): 7-9 points.

2.8. Vascular endothelial growth factor (VEGF) and endostatin expressions detected by western blot

After grinding tumor tissue samples in liquid nitrogen, tissues were placed in RIPA lysis buffer for lysis. A protease inhibitor cocktail was added, tissues were beat by a pipette, and mixed.

After they were placed on ice for 30 min, short shocks of ultrasonic waves at an appropriate frequency were used to break the cells on ice using a probe-type ultrasound. The mixture was extracted at 4 ℃ and centrifuged at 13 000 rpm for 20 min. The supernatant was transferred into a new centrifuge tube and protein concentration was determined by Protein Assay Kit.

After SDS-PAGE electrophoresis, the gel was immersed into a transfer buffer and equilibrated for 10 min. Sandwiched films were assembled and transferred, transfer buffer was added, and electrodes were inserted. After transmembrane, the PVDF membrane was rinsed with TBS for 10-15 min, placed in a TBS/T blocking buffer containing 5% (w/v) skim milk powder, shaked at room temperature for one hour, and an appropriately diluted primary antibody was

added; which was diluted by TBST containing 1% (w/v) skim milk powder. Then, the membranes was incubated at room temperature for two hours and rinsed three times by TBST every 5-10 min. The membranes were incubated with a secondary antibody (1:10 000, HRP labeled), which was diluted by TBST with 0.05% (w/v) skimmed milk powder at room temperature for one hour. Then, the membranes were rinsed three times with TBST every 5-10 min. They were exposed, and photographed. Quantity one v4.62 software molecular bands were used for gray value (Trace Tracking). The optical density curve was drawn according to different subunit bands. Areas under the optical density curves were calculated as a quantity reference for electrophoretic bands and results were statistically analyzed.

2.9. Statistical analysis

All data was analyzed with SPSS 19.0 software. Data were expressed as mean±SD values and compared with single factor analysis of variance. Enumeration data were compared with the *chi*-square test. $P<0.05$/$P<0.01$ has statistical significance.

3. Results

3.1. Effect of NF-κB inhibitor PDTC on survival rate of mice with Lewis lung cancer

Figure 1 illustrates that each group had 40 mice with Lewis lung cancer that all died over time. After 15 days of administration, survival rates of the control group, cyclophosphamide group, 0.5 mg/kg NF-κB inhibitor PDTC group, 1.5 mg/kg NF-κB inhibitor PDTC group and 3 mg/kg NF-κB inhibitor PDTC group were 60.0%, 65.0%, 80.0%, 77.5% and 72.5%, respectively. There were significant differences between the control group, 0.5 mg/kg NF-κB inhibitor PDTC group and NF-κB inhibitor PDTC 1.5 mg/kg group ($P<0.05$). There was also significant differences between the cyclophosphamide group and 0.5 mg/kg NF-κB inhibitor PDTC group ($P<0.05$).

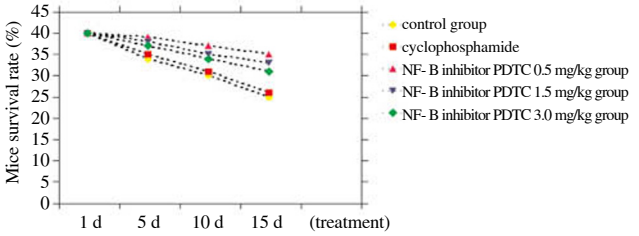

Figure 1. Effect of NF-κB inhibitor PDTC on survival rate of mice with Lewis lung cancer.

3.2. Inhibition effect of NF-κB inhibitor PDTC on mice with Lewis lung cancer

Tumor weight of the NF-κB inhibitor PDTC group was significantly lower than that in the control group; and the 0.5 mg/kg NF-κB inhibitor PDTC group has shown significant tumor suppression effects. There was a significant difference between the 0.5 mg/kg NF-κB inhibitor PDTC group and the control group ($P<0.05$); and its antitumor effect was enhanced as dosage increased. At high concentrations, NF-κB inhibitor PDTC had significant inhibitory effects on mice with Lewis lung cancer (Table 1).

3.3. Effect of NF-κB inhibitor PDTC on MVD of tumor tissue in mice with Lewis lung cancer

Figure 2 illustrates that there was no significant difference on the number of MVD between the 0.5 mg/kg NF-κB inhibitor PDTC group and control group; however, the number of MVD in the 1.5 mg/kg NF-κB inhibitor PDTC group and 3.0 mg/kg NF-κB inhibitor PDTC group were significantly lower than that in the control group ($P<0.05$).

Table 1
Inhibition effect of NF-κB inhibitor PDTC on mice with Lewis lung cancer.

Groups	Dosage	*n*	Tumor weight (g)	Inhibiting rate
Control group	0	12	1.79±0.47	
Cyclophosphamide group	20	12	0.44±0.21**	75.42
NF-κB inhibitor PDTC	0.5	12	1.34±0.34*	25.14
NF-κB inhibitor PDTC	1.5	12	1.15±0.25*	35.75
NF-κB inhibitor PDTC	3.0	12	0.96±0.21**	46.37

Note: * $P<0.05$, ** $P<0.01$ compared with the control group.

Table 2
Effect of NF-κB inhibitor PDTC on VEGF expression in mice with Lewis lung cancer.

Groups	Dosage	*n*	VEGF protein				Positive rate
			-	+	++	+++	
Control group	0	24	4	7	7	6	83.33
Cyclophosphamide group	20	24	13	6	4	1	45.83
NF-κB inhibitor PDTC	0.5	24	9	6	6	3	62.50
NF-κB inhibitor PDTC	1.5	24	11	6	5	2	54.17
NF-κB inhibitor PDTC	3.0	24	13	6	4	1	45.83

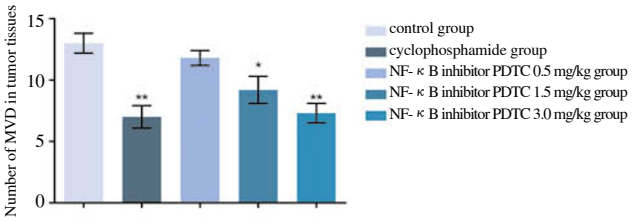

Figure 2. Effect of NF- κ B inhibitor PDTC on MVD of tumor tissue in mice with Lewis lung cancer.
Note: * P<0.05, * * P<0.01 compared with control group.

3.4. Effect of NF- κ B inhibitor PDTC on VEGF and endostatin expressions in mice with Lewis lung cancer

There were significant differences of VEGF protein expression between the NF- κ B inhibitor PDTC group and control group (P<0.05) on a dose-dependent manner. VEGF positive rate decreased as NF- κ B inhibitor PDTC dose increased. Moreover, the 3.0 mg/kg NF- κ B inhibitor PDTC group had the lowest VEGF positive expression (Table 2). VEGF and endostatin expressions in tumor tissues were determined by Western blot. Western blot results revealed that NF- κ B inhibitor PDTC can inhibit VEGF and endostatin expressions in tumor tissues (Figure 3).

Quantity one statistical analysis showed that relative signals in the NF- κ B inhibitor PDTC group and control group were 10.7 ± 1.9 and 17.5 ± 2.3, respectively (P<0.05); and relative signals of endostatin in the NF- κ B inhibitor PDTC group and control group were 3.01 ± 6.8 and 22.3 ± 1.1, respectively.

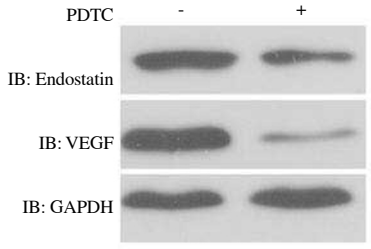

Figure 3. VEGF and endostatin expressions in tumor tissue determined by Western blot.

4. Discussion

Surgery and chemotherapy are currently the main treatments for lung cancer. However, obvious defects exist in both treatments. Firstly, surgical treatment can cause physiological and psychological burden, affecting the patient's quality of life; and secondly, chemotherapy has strong toxicities that brings serious risks to a patient's health[3]. Traditional Chinese medicine can acquire better results; the best choice for patients in clinical treatment. Traditional Chinese medicine has weaker effects and complicated compositions; but combined with Western medicine or chemotherapy, its effectiveness can be improved and side effects reduced. Traditional Chinese Medicine is the inevitable trend for lung cancer prevention and treatment[4]. NF- κ B inhibitor PDTC contains many active ingredients such as flavonoids, phenylpropanoids, and tannins. These ingredients have many good effects such as anti-viral, anti-inflammatory and anti-oxidation of free radicals, and so on[5,6]. It has been confirmed that NF- κ B inhibitor PDTC can inhibit tumor growth, which works through the ethyl acetate extract at the roots of NF- κ B inhibitor PDTC; however, its mechanism is not yet clear.

NF- κ b is an important nuclear transcription factor that has a variety of biological effects, playing a role in immune response, progress of inflammation and tumor genesis. NF- κ b mediates anti-apoptotic signal transduction; thereby, inhibiting cell apoptosis and enabling the cells to lose their normal cell apoptosis process, which subsequently develops into tumor cells. PDTC is a strong antioxidant that can specifically inhibit NF- κ b activity. Specific mechanisms of PDTC include inhibiting the NF- κ b subunit P65 expression and inhibiting I κ b degradation to reduce the nuclear translocation of NF- κ b[5–7]. PDTC is also a metal ion chelating agent (metal chelator), which can precipitate heavy metals such as As, Bi, Cd, Co, Cu, Fe, Mn, Ni, Pb, Sb, Sn, V, Zn in acidic solutions. PDTC can induce vascular smooth muscle cells apoptosis in rats and inhibit HL-60 leukemia cell apoptosis[8–10]. Therefore, PDTC can suppress the excessive activation of NF- κ b and inhibit tumor cell proliferation as a NF- κ b inhibitor[11].

Results of this study revealed that in the NF- κ B inhibitor PDTC treatment groups, NF- κ B inhibitor PDTC can inhibit Lewis lung cancer. Inhibition effects were positive dose dependent and tumor weight in the NF- κ B inhibitor PDTC high dose group was significantly lower than that in the control group (P<0.01). These results suggest that NF- κ B inhibitor PDTC has an important effect in treating mice with Lewis lung cancer. In terms of side effects, its toxicity is minimal compared to the cyclophosphamide group. Studies have not only shown that tumor growth is dependent on blood vessel formation, but it has also shown that angiogenesis plays a crucial role in solid tumor production, development and metastasis[12–15]. Further, the number of capillaries within a tumor tissue is the most objective reference standard in evaluating the tumor angiogenesis index.

The Lewis lung cancer mice model was established in this study. The number of MVD in the NF- κ B inhibitor PDTC treatment groups was lower than that in the control group. The number of MVD in the 1.5 mg/kg NF- κ B inhibitor PDTC group and 3.0 mg/kg NF- κ B inhibitor PDTC group was significantly lower than that in the control group (P<0.05). However, there are no significant differences on the number of MVD between the 0.5 mg/kg NF- κ B inhibitor PDTC group and control group. These results suggest that NF- κ B inhibitor PDTC can inhibit blood vessel formation in mice with Lewis lung cancer and inhibit angiogenesis. In addition, studies have shown that positive regulating factor plays an important role in tumor angiogenesis, such as bFGF, VEGF, PD-ECGF; which can promote the occurrence and development of tumor vessels[16–21]. In recent years, studies have shown that tumor angiogenesis factors such as VEGF can directly induce endothelial cells (ECs) to divide, so as to induce rapid tumor angiogenesis[22–25].

Results of this study has revealed that the percentage of positive

cells and dye depth in the NF-κB inhibitor PDTC treatment group and cyclophosphamide group were lower than that in the control group, which has significant difference (P<0.05). The percentage of positive cells and dye depth in the NF-κB inhibitor PDTC high-dose group were significantly lower than that in the control group, which has significant difference (P<0.05). These results suggest that NF-κB inhibitor PDTC can inhibit VEGF expression in mice with Lewis lung cancer and further inhibit tumor cells to release VEGF; thus, inhibiting tumor angiogenesis.

In conclusion, NF-κB inhibitor PDTC can down-regulate the expression of VEGF, and subsequently inhibit tumor formation and reduce tumor angiogenesis in mice with Lewis lung cancer. Hence, VEGF has a single factor effect or is jointly coordinated with other protein molecules; that is, our future research would focus in studying the mechanisms of VEGF inhibition by PDTC.

Conflict of interest statement

We declare that we have no conflict of interests.

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