Inhibitory Effect of β -Elemene on Human Airway Granulation Tissue in vivo and in vitro

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Key Words

β -Elemene · Human airway primary fibroblasts · Airway stenosis · Interventional bronchoscopic techniques · Granuloma

Abstract

Background: Recurrent airway granulation hyperplasia and scar formation make airway stenosis a clinical challenge. Therefore, a new approach for the treatment of airway stenosis is necessary. **Objective:** To explore the inhibitory effect of β -elemene on the proliferation of fibroblasts and airway granulation. **Methods:** In vivo: (1) study of the effect of local β -elemene injection by bronchoscopy. (2) During bronchoscopy, granulation tissues both before and after treatment were obtained. HE staining was performed and the result compared. In vitro: (1) human airway primary fibroblasts were purified and characterized. (2) Fibroblasts were treated with β -elemene and normal saline (NS) and then examined by optical and electron microscopy. (3) Fibroblasts treated with β -elemene or NS were assessed for viability by tetrazolium salt assay. (4) Apoptotic rates were determined by flow cytometry. **Results:** In vivo: (1) after local injection of β -elemene, airway granulation tissue was reduced. (2) Granulation tissue was found to have less edema, and fibroblasts

turned into mature fiber cells. In vitro: (1) human airway primary fibroblasts were successfully purified and cultured. (2) Compared with the control group, fibroblasts of the experimental group became clumped, the plasma granules were increased, and some fibroblasts lost their nucleus and organelles. (3) Compared with the control group, reduction of cell viability was detected with increased concentrations of β -elemene. (4) With increased concentrations of β -elemene, apoptotic rates of the fibroblasts were raised compared with the control group. **Conclusions:** β -Elemene may induce apoptosis and necrosis of airway primary fibroblasts and inhibit the proliferation of fibroblasts and airway granulation. The results provide a new approach for the treatment of airway stenosis.

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Introduction

Recurrent airway granulation hyperplasia and scar formation represent a major problem of airway stenosis. Airway stenosis patients have dyspnea and a lower quality of life [1–4]. Current treatments include surgery, drugs, and interventional bronchoscopic techniques [1–3, 5–10]. The latter approach represents the main form of

relief [3, 5–7, 10–12]. Unfortunately, this kind of intervention tends to entail recurrent stenosis due to granulation tissue formation. The repeated procedures required [1–3, 9–11, 13, 14] make airway stenosis a clinical challenge. Therefore, exploring new treatments is important for better clinical outcomes. Airway stenosis often occurs due to hyperplasia of airway granulation and scar tissue [1, 4, 9–11, 15]. Fibroblasts are one of the major components of granulation and scar tissue [16–19], and therefore, effective inhibition of the proliferation of airway fibroblasts in granulation tissue and scars could be key to solving the problem of airway stenosis.

β -Elemene, a compound derived from Zingiberaceae *Curcuma wenyujin* rhizome [20–23] used in traditional Chinese medicine, has shown anticancer effects against a broad spectrum of tumors. Its main biological activities were to reduce the ability of tumor cell mitosis, induce apoptosis of tumor cells, and inhibit the growth of tumor cells. Pharmacological experiments have shown that intraperitoneal injection of β -elemene has an obvious inhibitory effect on the synthesis of tumor cell DNA, RNA, and protein. The drug can also have a direct effect on the cell membrane, make tumor cells break, as well as change and enhance tumor cell immunogenicity, and then induce and promote the body's immune response to tumor cells. β -Elemene was less toxic, with less side effects on normal cells. As a promising anticancer drug, it has played an important role in enhancing the effects of many anticancer drugs and has been widely used in the treatment of different kinds of malignancies and in reducing the side effects of chemotherapy. It has also been shown to reverse multidrug resistance [20–27]. In addition, research has shown that β -elemene can inhibit proliferation of human bone fibroblasts and lens epithelial cells and that it can inhibit liver fibrosis [28–34].

The present study aimed to explore the inhibitory effect and mechanism of β -elemene on the proliferation of fibroblasts and human airway granulation as a potential new treatment for airway stenosis. The results show the effect of β -elemene both in vivo by local drug injection and in vitro by treating primary fibroblasts from human airway granulation tissue.

Materials and Methods

Cell Culture Medium

The cell culture medium [high-glucose Minimal Essential Medium, Dulbecco's modified Eagle's medium (DMEM)] was purchased from HyClone USA, 25% trypsin-EDTA and fetal bovine serum were purchased from Gibco Australia, penicillin-strepto-

Fig. 1. Local injection of β -elemene by bronchoscopy. **a** Local drug injection of β -elemene by bronchoscopy at 3 o'clock on the top of the upper stent. **b, c** Overgrowth of granulation tissue was not found 1 month after injection. **d** Repeated local drug injection of β -elemene by bronchoscopy at 6 o'clock and 12 o'clock on the carina. **e** Granulation tissue was absorbed after 4 months.

mycin was purchased from American MP Biomedicals, moxifloxacin was purchased from Bayer Germany, and β -elemene from Dalian Huali Jingang China.

Bronchoscopy with Local Drug Injection

The study protocol was approved by the Institutional Review Board of the Second Affiliated Hospital of Fujian Medical University (approval No. 2015-007) with unanimous acceptance by the Second Affiliated Hospital of Fujian Medical University Ethics Committee. After having obtained informed consent, a 56-year-

Fig. 2. Histology of biopsies before and after treatment with β -elemene. **a** Low magnification ($\times 4$) of a section of granulation tissue from a tracheobronchial stenosis patient before treatment showing marked edema. **b** Low magnification ($\times 4$) of a section from a tracheobronchial stenosis patient after β -elemene treat-

ment showing reduced edema in granulation tissue. **c** At high magnification ($\times 20$), the tissue section from a patient before treatment shows fibroblasts in the area of edema in granulation tissue. **d** At high magnification ($\times 20$), a section from the area of local injection of β -elemene shows reduced edema and more mature fiber cells.

old female patient diagnosed with airway stenosis due to trachea granulation hyperplasia was locally injected with β -elemene into granulation tissue during bronchoscopy (Pentax EB-1970AK, Japan). The patient had two stents in the trachea (Micro-Tech Europe GmbH), with the following injections: 0.5 mg once at 3 o'clock on the top of the upper stent, 1 mg twice at 9 o'clock below the upper stent, 0.5 mg once at 6 o'clock on the carina, and 1 mg twice at 12 o'clock on the carina. The first injection was administered on May 30, 2014, the second injection on June 26, 2014, and the third on October 14, 2014 (needle from Alton Medical Instruments Co., Ltd., Shanghai, China). During bronchoscopy, granulation tissue before and after treatment was obtained by small biopsy forceps. HE staining was performed, and sections were analyzed by optical microscopy.

Cell Culture and HE Staining

Primary fibroblasts were isolated from human airway granulation tissue (this procedure is usually done to alleviate tracheobronchial stenosis; a small biopsy was obtained and the tissue discarded). The 'tissue adherent method' was combined with 'trypsinization' and by using the serum adherent and 'organizing tablet' technology [35]. After passage and purification, cells from passages 3–4 were stained with HE, and the morphology of the cells was determined by optical microscopy. A Clean Bench (Airtect), a Cell Incubator (Sanyo), a centrifuge (Shanghai Medical Device Company), a micropipette (Eppendorf, Germany), an inverted microscope, and an imaging system (Olympus Japan, model U-LH 100HG, Shanghai Instrument Factory) were used.

Fig. 3. In vitro culture of primary airway fibroblasts from granulation tissue. **a** Light microscopy (low magnification) of primary fibroblasts isolated from human airway granulation tissue using the 'organizing tablet' technology. **b** Primary fibroblast culture from tissue derived from the airway (low magnification). **c** Light micro-

scopy (high magnification) shows outgrowth of primary airway fibroblasts from tissue blocks following inoculation and several passages of primary airway fibroblasts. **d** At high magnification, H&E staining of passaged and purified primary airway fibroblast cultures.

Effect of β -Elemene on Cells Assessed by Optical and Electron Microscopy

Human airway primary fibroblasts from passages 3–4 were cultured for 24 h and incubated at indicated concentrations of normal saline (NS) and β -elemene (320 $\mu\text{g/ml}$) in a CO_2 incubator for 72 h. The morphology of the two groups of cells was studied by optical and electron microscopy (Philips EM208).

In vitro Cell Proliferation Assay

Primary fibroblasts isolated from human airway granulation tissue (5×10^3 cells/well) were cultured in 96-well cell culture plates for 24 h prior to treatment and then treated with indicated concentrations of NS and β -elemene (0, 160, 320, 480, and 640 $\mu\text{g/ml}$) in a CO_2 incubator for 48 h. At the end of this period, 20 μl of tetra-

zolium salt (MTS; MTS kit CellTiter 96[®] Aqueous One Solution Cell Proliferation Assay, 200 assays, G3582; Promega Corporation, Madison, Wis., USA) and 100 μl of high-glucose DMEM were added to each well, and the plates were incubated for 0.5 h at 37°C. The absorbance values were determined at 490 nm using an ELISA (Rayto RT-6000).

Annexin-V-FITC/PI Double Staining Assays of the Apoptotic Cells

Double staining with Annexin-V-FITC and PI for flow cytometric analyses was performed using an apoptosis detection kit (DNA Prep Reagent Kit 100 tests, No. 6607055; Beckman Coulter). Human airway primary fibroblasts from passages 3–4 were cultured for 24 h and incubated at indicated concentrations of NS and

Fig. 4. Effect of β -elemene on fibroblast cultures. **a, c** Light microscopy of fibroblast cultures show that fibroblasts in the control group grew well. **b** Image shows weak growth of fibroblasts in the experimental group. The number of fibroblasts was decreased. The

arrow points to the cells with clouds. **d** Cytoplasmic granules in the fibroblasts of the experimental group were increased, and the number of fibroblasts was decreased.

β -elemene (160, 320, 480, and 640 $\mu\text{g/ml}$) in a CO_2 incubator for 48 h. The treated cells were harvested and washed twice with PBS, then resuspended in the binding buffer (calcium buffer, 100 μl). Annexin-V-FITC (5 μl) was added to the cells followed by the addition of 5 μl PI. The samples were then incubated for 20 min in the dark at room temperature. Binding buffer (400 μl) was added, and the cells were subjected to flow cytometric evaluation (Beckman Coulter FC500, USA).

Results

Effect of Local Drug Injection

Bronchoscopy with β -elemene injection was performed three times on a patient with tracheobronchial stenosis (on May 30, 2014, June 26, 2014, and October 14, 2014). Figure

1 shows drug injection (fig. 1a) at a site with granulation tissue. One month later, images show no overgrowth with granulation tissue at the area of injection (fig. 1b, c). Local drug injection was repeated (fig. 1d) and examined by bronchoscopy 4 months later (fig. 1e). No granulation tissue was visible at this time, indicating full resorption.

Histology of Biopsy from the β -Elemene-Injected Site of a Tracheobronchial Stenosis Patient

Comparison of tissue biopsied at bronchoscopy before (fig. 2a, c) and after the injection of β -elemene (fig. 2b, d) shows a marked change after treatment. The lower magnification shows marked edema before treatment. At higher magnification, the pretreatment sample shows a large number of fibroblasts (fig. 2c). These appear absent

Fig. 5. Electron micrographs of β -elemene-treated cultures and controls. **a, b** Electron micrograph showing fibroblasts of the control group, exhibiting structural integrity and few cytoplasmic granules. **c, d** Electron micrograph of fibroblasts in the experimen-

tal group. Cytoplasmic particles were increased; some fibroblasts that appear disrupted are devoid of their nucleus and organelles. Arrows indicate cell nucleus.

Table 1. Inhibitory effects of β -elemene on fibroblast proliferation

OD value group	Density				
	0 $\mu\text{g/ml}$	160 $\mu\text{g/ml}$	320 $\mu\text{g/ml}$	480 $\mu\text{g/ml}$	640 $\mu\text{g/ml}$
β -Elemene group	1.1165 ± 0.2046	1.1985 ± 0.0636^c	1.0523 ± 0.0460^d	$0.8548 \pm 0.0295^{b, c}$	$0.3044 \pm 0.0384^{a, b}$
NS group	1.0344 ± 0.0830	1.0680 ± 0.1044^c	1.0370 ± 0.0798	1.0239 ± 0.0395^c	1.0870 ± 0.0339^a

OD = Optical density. ^a $p < 0.01$, compared with the control group; ^b $p < 0.01$, compared with the low-density group; ^c $p < 0.05$, compared with the control group; ^d $p < 0.011$, compared with the low-density group.

Fig. 6. Dose-dependent effect of β -elemene on fibroblast viability. Fibroblast viability of treated and control cultures was assessed by MTS assay. OD = Optical density.

in the posttreatment biopsy section (fig. 2d), and mature fiber cells are present instead.

Analysis of in vitro Culture of Fibroblasts from Human Granulation Tissue and the Effect of β -Elemene

Figure 3 shows cultures of primary fibroblasts from human granulation tissue at low (fig. 3a, b) and high magnification (fig. 3c, d). Fibroblasts grew well in vitro. A comparison of control cultures with treated cultures is shown in figures 4 and 5. The fibroblasts of the β -elemene-treated cultures grew poorly (fig. 4b). Cytoplasmic granules were increased and the number of cells decreased (fig. 4d). Electron microscopy of treated and control cultures is shown in figure 5. Cells from the control cultures show structural integrity and few granules (fig. 5a, b), whereas cells from treated cultures show an increased number of cytoplasmic particles and some disruption of fibroblast nuclei and organelles (fig. 5c, d).

The viability of cell cultures treated in vitro with β -elemene was assessed by MTS assay. The control groups (NS) showed no significant changes in viability, whereas treatment with β -elemene at the two higher doses, 480 and 640 $\mu\text{g/ml}$, showed a significant reduction in viability (fig. 6; table 1).

Apoptosis of in vitro treated cells and controls was analyzed by flow cytometry (fig. 7). At the concentration of 160 $\mu\text{g/ml}$, there was no difference in apoptotic rates between the control group and the experimental group. At the concentration of 320 $\mu\text{g/ml}$, the late apoptotic rate of the experimental group was 18.1% and for the control

group, it was 5.2%. At a concentration of 480 $\mu\text{g/ml}$, the early apoptotic rate of the experimental group was 13.1%, whereas the late apoptotic rate was 42.6%. The early apoptotic rate of the control group was 2.91%, and the late apoptotic rate was 15.32%. At a concentration of 640 $\mu\text{g/ml}$, the late apoptotic rate of the experimental group was 13.4%, and the necrosis rate was 67.8%. The late apoptotic rate of the control group was 5.91%, and the necrosis rate was 4.7%. The results show pronounced apoptosis induced by β -elemene in a dose-dependent fashion.

Discussion

Airway granulation hyperplasia and scar formation have been found to be the main component of airway restenosis [1–4]. A variety of interventional bronchoscopic techniques have recently been used to treat airway stenosis. This approach produced some beneficial effects, but airway restenosis occurred in some of the patients [1–3, 9–11, 13, 14]. Reported data show a rate of restenosis from 40 to 70% [36]. Therefore, new interventional bronchoscopic techniques to inhibit airway restenosis have important clinical significance. Fibroblasts were shown to be one of the major components of granulation and scar tissue [16–19]. To effectively inhibit the proliferation of airway fibroblasts in granulation and scar tissue may lead to better clinical treatment.

Fibroblast proliferation and collagen metabolism disorders play an important role in the process of wound healing. Increased collagen and reduced collagen breakdown in the wound is an important factor in wound granulation and scar formation and is present in airway stenosis. Currently, the traditional Chinese medicine β -elemene is widely used in the complementary treatment of different types of malignancies [20–27]. β -Elemene has also been found to inhibit the expression of angiotensin II and RhoA/ROCK signaling in hepatic stellate cells and to delay the progression of hepatic fibrosis [28–32]. It may also be effective in antiosteogenic differentiation of ankylosing spondylitis fibroblasts by inhibiting the BMP/SMAD signal pathway and subsequently blocking the expression of ossification marker genes RUNX2 that initiate osteogenic differentiation [33]. In the present study, local drug injection of β -elemene by bronchoscopy in a tracheobronchial stenosis patient prevented an overgrowth of granulation tissue, and some tissue resorption was noted. Granulation tissue before and after treatment was obtained, and HE staining was performed for analysis. Compared with the control, local drug injection of

Fig. 7. Apoptotic rates in β -elemene-treated fibroblast cultures and controls. **a** At 160 $\mu\text{g/ml}$ of β -elemene, there was no difference in the apoptotic rates between the control group and the experimental group. **b** At 320 $\mu\text{g/ml}$, the apoptotic rate of the experimental group was 18.1% and for the control group, it was 5.2%. **c** At 480 $\mu\text{g/ml}$, the early apoptotic rate of the experimental group was 13.1%, and the late apoptotic rate was 42.6%. The early apoptotic rate of the control group was 2.91%, and the late apoptotic rate was 15.32%. **d** At 640 $\mu\text{g/ml}$, the late apoptotic rate of the experimental group was 13.4%, and the necrosis rate was 67.8%. The late apoptotic rate of the control group was 5.91%, and the necrosis rate was 4.7%.

β -elemene by bronchoscopy reduced the edema and may have caused an increased differentiation of fibroblast into mature fiber cells. It appears that β -elemene can affect fibroblasts in vivo and thereby inhibit airway granulation

hyperplasia. This could reverse airway stenosis. To date, few drugs have been found to effectively regulate wound healing. These include dexamethasone and mitomycin C [8]. Therefore, many clinicians have begun to focus on

natural herb extracts for their curative effects and low cytotoxicity [37].

Primary fibroblasts isolated from human airway granulation tissue were successfully cultured in vitro. The drug-treated fibroblasts became clumped, and cytoplasm particles were increased. Some fibroblasts lost their nuclei and organelles. The MTS assay to measure cell viability showed no obvious change in cell viability in the control group, but a dose-dependent reduction in cell viability in the treated group. Apoptotic rates determined by flow cytometry also showed a marked effect of β -elemene. Fibroblast apoptosis and necrosis were affected, suggesting a possible mechanism for the observed in vivo effect.

The inhibition of fibroblast proliferation seen in the present study provides a new model for the treatment of airway stenosis. The exact mechanism of β -elemene action, such as apoptosis and necrosis, or other additional pathways, need to be further explored.

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The authors report no conflicts of interest in this work.

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