· Original article ·

Growth inhibition and apoptosis of human colon cancer cells induced by vitamin E succinate

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[**ABSTRACT**] **Objective**: To investigate the growth inhibition and apoptosis induction effect of vitamin E succinate (VES) on human colon cancer cells and to analyze the modulation of apoptosis-mediator Fas expression in this process. **Methods**: Human colon cancer cell line LS174T was treated with VES for 12 h, 24 h and 48 h at the concentrations of 5 mg/L, 10 mg/L and 20 mg/L. 1-(4,5-dimethylthiazo-2-yl)-3,5-diphenylformazan (MTT) assay was employed to detect the inhibitory effect of VES on the growth of colon cancer cells. Flow cytometry was then used to analyze the cell cycle of the colon cancer cells after being treated with VES and the apoptotic rate was calculated at the same time. To find out whether the Fas protein expression was modulated in this process, Western blotting assay and flow cytometry were used to detect the Fas protein level in whole cell lystates and on cell surface. **Results**: VES exhibited a significant inhibitory effect on the growth of human colon cancer cells in a dose- and time-dependent manner. After being treated with VES at 5 mg/L, 10 mg/L and 20 mg/L for 48 h, the apoptotic rate of LS174T cells rose from 0.90% to 15.9%, 46.7% and 64.5%, respectively. Fas neutralizing antibody can significantly block VES-induced apoptosis. After the administration of VES, total Fas protein in whole-cell extracts increased in a dose-dependent manner. The flow cytometry showed that the mean fluorescence intensity rose from 5.43 to 9.88, 13.21 and 18.0 after being treated with VES. **Conclusion**: VES can induce significant growth inhibition and apoptosis in human colon cancer cells. The modulation of Fas expression is one of the mechanisms involved in this process and may be related to the upregulation of Fas molecule on the cancer cell surface.

[**KEY WORDS**] vitamin E succinate; colonic neoplasms; apoptosis

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Cancer is one of the most common causes of death, hence the search for anticancer drugs has been intense. Naturally occurring agents and their derivatives are appealing anticancer agents. Fat-soluble vitamins (A, D, E and K) have been investigated for antitumor properties and found to possess antiproliferative effects.

Vitamin E and its derivatives can negatively regulate tumor growth *in vitro*[1,2] and *in vivo*[3,4]. The advantage for using vitamin E and its derivatives in human cancer therapy is their low toxicity *in vivo* [5]. Vitamin E succinate (VES), a derivative of natural vitamin E but lacking the antioxidant activity, has been shown to trigger apoptosis in 28%-65% of the cells in leukemia cell lines and adherent carcinoma cell lines of lung, breast and colorectal cancer. In contrast, normal cell types including haematopoietic cells, fibroblasts, endothelial cells, cardiomyocytes, hepatocytes and smooth muscle cells showed no susceptibility to apoptosis induced by VES[6]. The selective toxicity of VES for malignant cells suggested it a promising anticancer drug.

Colon cancer is one of the most common malignancies in China and the morbidity and mortality have a tendency of increase in recent years. In this study, the inhibitory effect of VES on the growth of human colon cancer cells was investigated. We also focused on the function of Fas/FasL apoptotic signaling pathway and studied whether Fas was modulated in the VES-mediated cell death.

1 MATERIALS AND METHODS

1.1 Cell culture and treatment Human colon cancer cell line LS174T was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and incubated in a humidified atmosphere containing 5% CO_2 at 37℃. VES(Sigma) was dis-

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solved in anhydrous ethanol and diluted to the corresponding concentrations in the medium. The final concentration of ethanol in the medium was lower than 0.2%. The LS174T cells were incubated for 12 h, 24 h and 48 h in the presence of VES. The VES concentrations selected were 5 mg/L, 10 mg/L and 20 mg/L, respectively. Cells receiving no VES treatment served as blank control.

1.2 MTT assay LS174T cells were seeded in 96-well plate at a density of 5×10^3/well. Twenty-four hours later, cells were treated with VES according to the scheme introduced above. Each treatment had 5 duplicate wells. 50 μl 1-(4,5-dimethylthiazo-2-yl)-3, 5-diphenylformazan (MTT, 5 g/L) was added to each well and incubated for another 4 h at 37℃ until purple formazan crystal developed. Then MTT-containing medium was removed and 200 μl DMSO solution was added to each well and shaken for 5 min at room temperature. The absorbance at 492 nm was read with an ELISA plate reader. The inhibitory rate was calculated as (absorbance value of blank control group − absorbance value of treatment group)/absorbance value of blank control group.

1.3 Flow cytometry assay Colon cancer cells were assayed for DNA content using the propidium iodide (PI) staining method. Briefly, after the treatment completed, cells (2×10^6) were collected, rinsed in PBS twice and then incubated with 50 mg/L propidium iodide at 4℃ for 30 min in the dark. 100 mg/L Rnase was added to remove RNA interference. Cell cycle distribution at different phases was analyzed with FACScan flow cytometry. More than 10 000 events of each sample were acquired for analysis.

1.4 Blocking assay Colon cancer cells were treated with VES at the concentrations of 5 mg/L, 10 mg/L and 20 mg/L for 48 h. Apoptosis-neutralizing Fas monoclonal antibody(0.5 mg/L, Coulter & Immunotech) was added with VES in the medium. Flow cytometry assay was then applied to detect the apoptotic rate in colon cancer cells.

1.5 Western blot assay LS174T cells were treated with VES at the concentrations of 5 mg/L, 10 mg/L and 20 mg/L for 48 h. Cells were harvested and protein extracted from the whole-cell lysates was quantified. 20 μg protein sample was loaded in each lane, separated on 12% SDS-polyacrylamide gel and then transferred onto nitrocellulose membranes with a semi-dry apparatus. After membranes were transferred, they were blocked for 1 h with 50 g/L non-fat milk in PBS. Membranes were incubated with murine monoclonal antibody to human Fas(1∶500 dilution, Jingmei Company) overnight at 4℃, washed 3 times for 5 min with PBS before incubated with goat anti-mouse HRP-conjugated secondary antibody (1∶1 000 dilution, Gene Company) for 1 h at room temperature. The membranes were washed 3 times for 5 min with TBS, and the peroxidase activity on the nitrocellulose sheet was visualized on X-ray film with the ECL Western blotting detection system.

1.6 Cell surface Fas detection by flow cytometry Cancer cells treated and those untreated were collected and washed twice with PBS. PE-conjugated anti-human Fas antibody was added and incubated for 30 min in the dark, then washed 3 times with PBS and the fluorescence intensity was analyzed with flow cytometry.

1.7 Statistical analysis ANOVA were used to analyze the inhibitory function of VES on the proliferation of colon cancer cells. *P*-value less than 0.05 was considered statistically significant.

2 RESULTS

2.1 Inhibition of human colon cancer cell proliferation by VES VES has been previously shown to function as a growth inhibitory agent. The proliferation of LS174T cells was significantly inhibited after the administration of VES($P<0.05$). The inhibitory effect showed a time- and dose-dependent manner. At the same VES dosage, the longer the colon cancer cells incubated with VES, the higher the inhibitory rate was ($P<0.05$). We also found that the inhibitory rate increased with the elevation of drug dosage at a set time point ($P<0.05$,Fig 1).

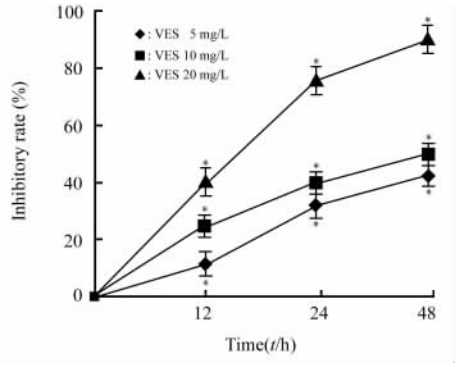

Fig 1　Inhibitory effect of VES on colon cancer cells

* $P<0.05$ *vs* control group

2.2　Apoptosis induced by VES treatment　Cell cycle studies showed that the apoptotic rate of LS174T cells was 0.9%. After administration of VES at 5 mg/L, 10 mg/L and 20 mg/L for 48 h, the apoptotic rate rose to 15.9%, 46.7% and 64.5%, respectively (Fig 2).

2.3　Blocking assay　Blocking assay was performed to find out whether Fas-mediated signals were involved in VES-induced apoptosis. The co-administration of VES with neutralizing antibody significantly blocked VES-induced apoptosis (Fig 3).

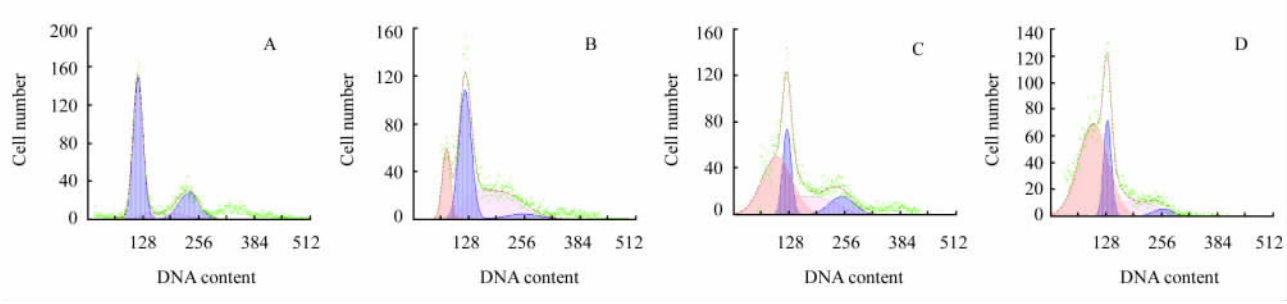

Fig 2　Apoptosis of LS174T colon cancer cells

A: Untreated cells; B: VES 5 mg/L, 48 h; C: VES 10 mg/L, 48 h; D: VES 20 mg/L, 48 h

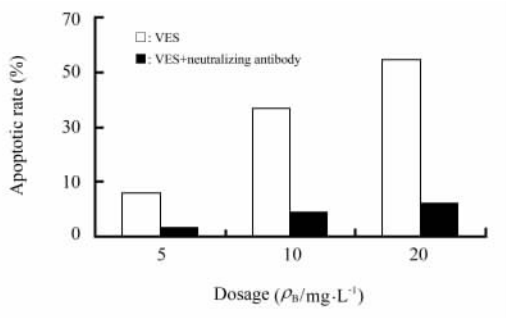

Fig 3　Effect of Fas-neutralizing antibody on VES-induced apoptosis

2.4　Effect of VES on Fas protein level　After the administration of VES at different concentrations for 48 h, Fas protein extracted in the whole-cell lysates showed a dose-dependent increase compared with untreated control group in Western blotting analysis. The levels of Fas protein increased 1.1, 1.8 and 3.9 folds, respectively(Fig 4).

2.5　VES increases cell surface Fas expression　The apoptosis was mainly induced by membrane Fas and the expression of Fas on cell surface was detected with flow cytometry. After the administration of VES at the concentrations of 5 mg/L, 10 mg/L and 20 mg/L for 48 h, the fluorescence intensity increased compared with the untreated control cells, rising from 5.43 to 9.88, 13.21 and 18.0, respectively.

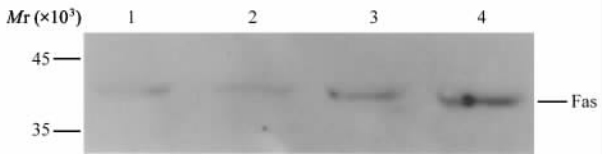

Fig 4　Western blotting result of Fas protein level

1: Untreated cells; 2: VES 5 mg/L, 48 h; 3: VES 10 mg/L, 48 h; 4: VES 20 mg/L, 48 h

3　DISCUSSION

VES has been characterized for its chemopreventive[7] and chemotherapeutic potentials[8-10] and has been shown to be a widespread potent growth inhibitor of human cancer cells *in vitro*[11-14]. The first report of VES inhibiting tumor growth was by Turley[15] and colleagues in which VES was shown to inhibit the growth of human B lymphoma. Fur-

ther research found that not only the tumor from the lymphatic sources, but malignant tumor from the epithelium source such as the breast cancer[16], prostate cancer[13] and gastric cancer[17,18], can also be inhibited by VES, indicating that VES can be used as a chemotherapeutic agent against tumors. In this study, VES was found to inhibit the growth of LS174T human colon cancer cells and the inhibitory effect also presented in a dose- and time-dependent manner. VES also manifested as an active apoptosis inducer as shown in the cell cycle analysis with flow cytometry that the apoptotic rate increased significantly after the administration of VES. VES possessed the potential of inhibiting colon cancer cell proliferation, suggesting its promising prospective use in tumor chemotherapy.

The mechanisms of VES-induced inhibition of tumor cell growth have been a topic of great interest[19-22]. Wu[22] and colleague found that VES can activate biologically TGF-β, which increases the kinase activity of c-jun N-terminal kinase followed by phosphorylation of c-jun. Finally, the activated c-jun triggers apoptosis in human gastric cancer. It implicated that TGF-β played a crucial role in VES-induced apoptosis[23-25]. However, some authors consider the Fas/FasL system another most important pathway by which VES inhibits tumor cell growth[26,27].

Fas is a type Ⅰ transmembrane protein which belongs to tumor necrosis factor (TNF) receptor superfamily. As a member of the 5 death domain-containing receptors, Fas initiates a signal-transduction cascade leading to programmed cell death upon trimerazation with Fas ligand or upon cross-linking with Fas specific agonistic antibody[28-31]. Fas protein has been shown to be constitutively expressed in a variety of epithelial cells including T cells, B cells as well as cells from the liver, lung, breast and colon epithelium[32]. However, colon cancer cells could either down-regulate the expression of Fas[33] or have a signal-transduction obstruction[34,35], thus render them the resistance to apoptosis. Due to the importance of Fas in signaling the pathway to apoptotic cell death of tumor cells, modulating Fas expression and then making tumor cells sensitive to Fas-mediated apoptosis are among the strategies of treatment to cancer[36-38]. Turley[39] et al found that VES increased both Fas protein synthesis and cell surface Fas expression in human breast cancer cells and the VES-induced apoptosis was blocked when Fas neutralized antibody or transfection of Fas antisense oligonucleotides were applied to cancer cells, implicating Fas as the mediator of VES-induced apoptosis. Our data corresponded with this result. The blocking assay suggested the importance of Fas in VES-induced apoptosis. Western blotting assay showed that the Fas protein extracted in the whole-cell lysates including the membrane and cytosolic Fas increased in a dose-dependent manner, indicating the stimulative effect of VES on Fas synthesis. The increase of fluorescence intensity of cell surface Fas, mediator of apoptosis, suggests the upregulation of cell surface Fas expression. Accompanied with increase of cell surface Fas expression, apoptotic rate of colon cancer cells rose accordingly, indicating the important role of Fas in VES-mediated apoptosis.

In summary, this study demonstrates that VES has a marked growth inhibition and apoptosis induction function on human colon cancer cells and upregulation of cell surface Fas plays a crucial role in this process.

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维生素 E 琥珀酸酯对人大肠癌细胞的生长抑制和凋亡诱导作用

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[**摘要**] **目的**:检测维生素 E 琥珀酸酯(VES)对人大肠癌细胞的生长抑制和凋亡诱导作用并分析此种作用的可能机制。**方法**:VES 分别以 5、10 和 20 mg/L 浓度作用于人大肠癌细胞株 LS174T,12、24 以及 48 h 后应用 MTT 法检测 VES 对大肠癌细胞的生长抑制作用。应用流式细胞仪分析不同浓度 VES 处理 48 h 后大肠癌细胞的细胞周期并计算凋亡率;以 Western 蛋白印迹法和流式细胞术检测 VES 处理后肿瘤细胞 Fas 蛋白水平和细胞表面 Fas 表达的变化。**结果**:VES 能够显著抑制人大肠癌细胞的增殖并表现为剂量和时间依赖关系。以 5、10 和 20 mg/L VES 作用 48 h 后,肿瘤细胞的凋亡率由 0.9%分别升高至 15.9%、46.7%和 64.5%。Fas 中和性抗体能够明显阻断 VES 介导的凋亡。VES 处理后,细胞 Fas 蛋白水平升高。流式细胞仪检测细胞表面 Fas 平均荧光强度由 5.43 升高至 9.88、13.21 和 18.0。**结论**:VES 能够诱导人大肠癌细胞的生长抑制和凋亡。调控凋亡诱导分子 Fas 的表达是主要机制之一。其作用主要与肿瘤细胞表面 Fas 分子表达的上调有关。

[**关键词**] 维生素 E 琥珀酸酯;结直肠肿瘤;细胞凋亡

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Construction and characterization of a humanized anti-human CD3 monoclonal antibody 12F6 with effective immunoregulation functions

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[**ABSTRACT**] 12F6 is a murine anti-human CD3 monoclonal antibody, which competes with OKT3 for binding to human T cells and possesses more effective T-cell suppression and activation properties compared to OKT3. It thus exhibits the potential to be developed as an immunoregulation agent for manipulating T-cell functions and preventing acute allograft rejection. In an attempt to minimize the immunogenicity of murine 12F6 (m12F6) for potential clinical application, a humanized version of 12F6, denoted as hu12F6, was successfully constructed by complementary determining region (CDR) grafting and shown to maintain both T-cell activation and suppression activities similar to m12F6. Furthermore, in order to reduce the first dose reaction syndrome caused by T-cell activation following the first administration of anti-CD3 antibodies, two amino acid mutations were introduced into the Fc region of hu12F6, resulting in the Fc-mutated 12F6 humanized antibody (hu12F6mu). This Fc-mutated version displayed a similar antigen-binding affinity and specificity compared with hu12F6 and m12F6 but with much weaker FcR binding activity. hu12F6mu was shown to be much less potent in the induction of T-cell proliferation, cytokine release (tumour necrosis factor-α, interferon-γ and interleukin-10) and early activation marker expression on the cell surface (CD69 and CD25) than parental 12F6 and OKT3 did. In contrast, hu12F6mu was effective in modulating T-cell receptor/CD3 and inhibiting mixed lymphocyte reaction with a similarity as compared to m12F6 and OKT3. In conclusion, the resultant hu12F6mu was much less mitogenic to T cells but retained potent immunosuppression, suggesting it might be an alternative to OKT3 as an immunosuppressive drug with less immunogenicity and toxicity for clinical application.

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