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Effects of Fat-soluble Extracts From Vegetable Powder and β-carotene on Proliferation and Apoptosis of Lung Cancer Cell YTMLC-90

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Objective The aim of this investigation was to study the effects of fat-soluble extracts from vegetable powder (FEFVP) and β-carotene on the proliferation and apoptosis of cultured YTMLC-90 lung cancer cells. **Methods** The lung cancer cells were continuously exposed to a broad range of concentration of FEFVP and β-carotene. The proliferation was evaluated in MTT test. The induction of apoptosis was evaluated by morphological change, DNA fragmentation analysis, and DNA content analysis combined with flow cytometric analysis. **Results** Both FEFVP and β-carotene were found to inhibit cell proliferation and to induce morphologic changes consistent with apoptosis in YTMLC-90 cancer cells, including cellular shrinkage, chromatin condensation and nuclear fragmentation. DNA agarose gel electrophoresis showed DNA fragmentation 'ladder'. Flow cytometric analysis revealed decreased DNA content and the presence of a sub-G_1 apoptotic peak. **Conclusion** These findings are consistent with the induction of apoptosis. Moreover, the effects of FEFVP are stronger than those of β-carotene. FEFVP inhibits the growth of YTMLC-90 probably via the induction of apoptosis cancer cells.

Key words: Cell culture; FEFVP; Apoptosis

INTRODUCTION

The relationship between vegetable consumption and human cancer has been studied in our laboratory for ten years. One of our recent studies showed that the intake of vegetables especially celery, alliums, radish, yams, and carotenoids had an inverse association with the risk of lung cancer in high risk population with lung cancer in Yunnan Tin Corporation (YTC)[1]. Other research results *in vitro* and *in vivo* in our laboratory showed that the vegetable powder prepared from mallow had antitumor activities[2]. The objectives of the present study were to investigate how fat-soluble extracts from vegetable powder (FEFVP) induces apoptosis of cancer cell line and to study the antitumor mechanism of vegetables.

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MATERIALS AND METHODS

Materials

Vegetable powder (VP) was made of leaf of celery, spinach, mallow, by dry baking under 100℃. Three kinds of VP were mixed according to 1:1:2.VP was stored at -20℃. The fat-soluble extracts from vegetable powder (FEFVP) were extracted with 3:7 acetone-petroleum ether and the content of β-carotene was determined according to the method described before[3]. The FEFVP containing 30 mg/100 g of β-carotene was used in cell culture. The extract dose was expressed in β-carotene levels. The extract was then diluted in tetrahydrofuran (THF) to the necessary concentration. Chemicals used in this study were analytically pure.

β-carotene: Sigma Co.

TE buffer: 10 mmol/L Tris • Cl (pH7.6), 1 mmol/L EDTA (pH8.0).

The YTMLC-90 lung cancer cell line was established by using the cancer tissue of the miner with lung cancer in 1990, which was generously provided by YTC.

Cell Culture

The YTMLC-90 lung cancer cell line was cultured in RPMI 1 640 with 10% heat-inactivated fetal calf serum, 100U/mL penicillin and 100 μg/mL streptomycin in a humidified atmosphere of 5% CO_2/ 95% air at 37℃. The chemicals in the medium were specific for cell culture.

There were nine groups, including the blank control group (untreated), solvent control group (medium with 0.25%THF), positive control group (medium with 10 μg/mL adriamycin) and 6 experimental groups: medium supplemented with FEFVP at concentration of 2.5×10^{-8}, 2.5×10^{-7}, 2.5×10^{-6} mol/L of β-carotene, medium supplemented with β-carotene at concentration of 1×10^{-6}, 1×10^{-5}, 1×10^{-4} mol/L respectively.

Microculture Tetrazolium (MTT) Assay

Cells (10^3) were seeded into 96-well culture plates in a volume of 100 μL per well. The MTT assay was performed both in untreated and treated cells at 24 h, 48 h, 72 h and 96 h. In brief, 20 μL of 5 mg/mL MTT stock solution was added and incubated to each well and then incubated at 37℃ for 3 h, 150 μL/well DMSO solution was added and incubated for 10 min. The absorbance of each sample was measured at 550 nm with a microculture plate reader.

Morphological Assessment of Apoptosis

Cells (3×10^5 cells/flask) were cultured in 25 mL flasks. Treated cells were continuously exposed to FEFVP at the concentration of 2.5×10^{-8}, 2.5×10^{-7}, 2.5×10^{-6} mol/L of β-carotene and β-carotene at concentration of 1×10^{-6}, 1×10^{-5}, 1×10^{-4} mol/L for 24 h and 48 h. Cells were collected by using the mixture of 0.02% EDTA and 0.25% trypsin (1:1), centrifuged at 1 500 rpm for 5 min, fixed with methanol, stained with HE, and then examined using an Olimpus BH-2 microscope at a magnification of ×280. Apoptotic cells displayed typical morphological features, including shrinkage and chromatin condensation.

Electron microscopic assessment of apoptosis: Cells treated with the highest concentration of FEFVP and β-carotene for 48 h were harvested, trypsinized and then centrifuged at 5 000 rpm for 5 min. The supernatant was removed, the pellet was re-suspended in PBS and

specific fixing solution (9:1) for electroscopic assessment. Cell suspension was centrifuged at 10 000 rpm for 10 min. The supernatant was removed, and the pellet was slowly added with fixing solution. The following was performed in electron microscopic institute.

DNA Fragmentation Analysis

DNA analysis by gel electrophoresis was performed as described by Hermann *et al.*[4]. Briefly, YTMLC-90 cells were cultured in RPMI 1640 medium in a humidified atmosphere of 5% CO_2/95% air at 37℃. Grouping method and the medium used in the test were the same as the above. Treated cells were then collected in 36 h or 48 h as described above. Approximately 10^6 cells/sample were washed twice with ice-cold phosphate-buffed saline (PBS). Cells were centrifuged at 1 500 rpm for 5 min, the supernatant was removed, and the pellet was lysed in 150 μL lysis buffer at 4℃ for 30 min, digested for 1 h at 37℃ with 1mg/mL DNase–free RNase A (Sigma, St.Louis, MO), lysed in proteinase K for 2 h at 37℃. Cells were then centrifuged at 2 000 rpm for 5 min and cellular debris was removed, and supernatants were added with NaAC and ethanol. After staying overnight at 4℃, cell lysates were centrifuged at 14 000 rpm for 10 min. After 2 h at room temperature, the pellet was diluted in TE buffer. DNA from cell lysates was analyzed on a 1.8% TAE-agarose gel containing ethidium bromide. DNA ladder was visualized and photographed under UV light.

Flow Cytometric Assessment of Cell Cycle Phase and Apoptosis

Cells (1×10^5cells/well) were cultured in the medium, medium with THF (0.25%), medium with adriamycin, medium supplemented with FEFVP at concentration of 2.5×10^{-8}, 2.5×10^{-7}, 2.5×10^{-6} mol/L of β-carotene and β-carotene at concentration of 1×10^{-6}, 1×10^{-5}, 1×10^{-4} mol/L for 24, 48 h respectively. Treated cells were then collected in 36 h or 48 h by using the mixture of 0.02% EDTA and 0.25% tryptosin (1:1). Approximately 10^5 cells/sample were washed twice with ice-cold phosphate-buffed saline (PBS). After staying overnight at 4℃ cells were washed twice with ice-cold PBS again and stained with 50 μg/mL propidium iodide (Sigma Chemical Co.) with (0.5% Triton, 200U RNase/mL) and analyzed on Flow cytometery (Coulter Co. Epics Elite ESP). Data were analyzed with ELITE program. DNA cycle phase was analyzed by using multicycle program.

Statistical Analysis

Unpaired, two-sided Student's *t* test was used to compare the effects of FEFVP on *in vitro* YTMLC-90 growth. Statistical significance was defined as $P<0.05$.

RESULTS

Inhibition of in vitro Growth of YTMLC-90 Lung Cancer Cell by VEFVP and β-carotene

Fig.1 demonstrates that both VEFVP (a) and β-carotene (b) could inhibit the proliferation of YTMLC-90 lung cancer cells. There was a dose-response effect on the cells when exposed to different concentrations of FEFVP and β-carotene.

The efficacy of FEFVP was compared with that of β-carotene by exposing the YTMLC-90 cells to different concentrations of either FEFVP or β-carotene for 48 h. Table 1 illustrates that FEFVP was a more potent inhibitor of growth than β-carotene.

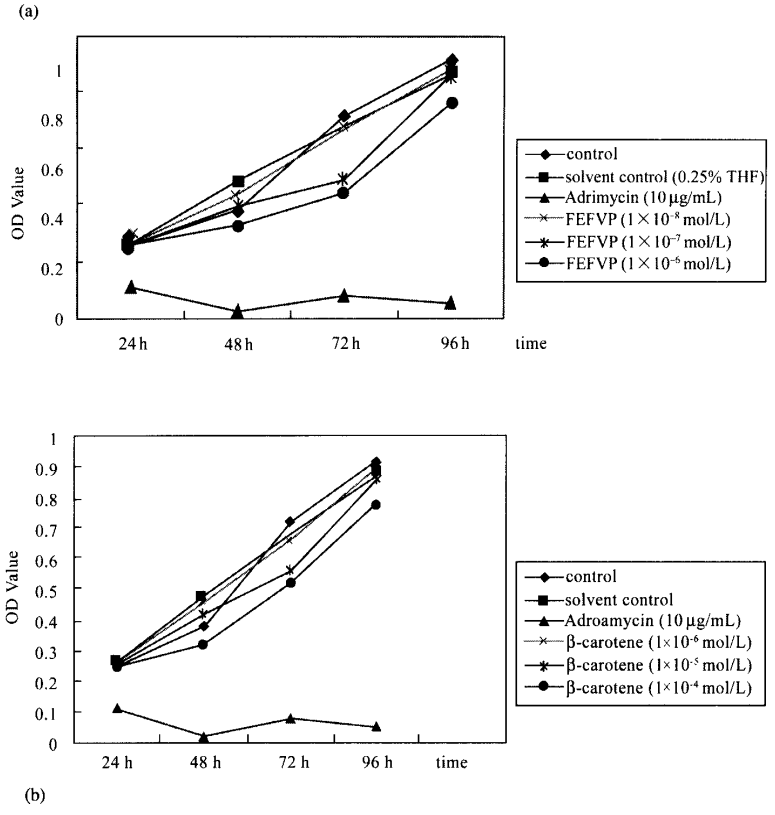


FIG.1. The effects of FEFVP and ß-carotene on the proliferation of YTMLC-90 cells (OD value), (a) treated with FEFVP; (b) treated with ß-carotene.

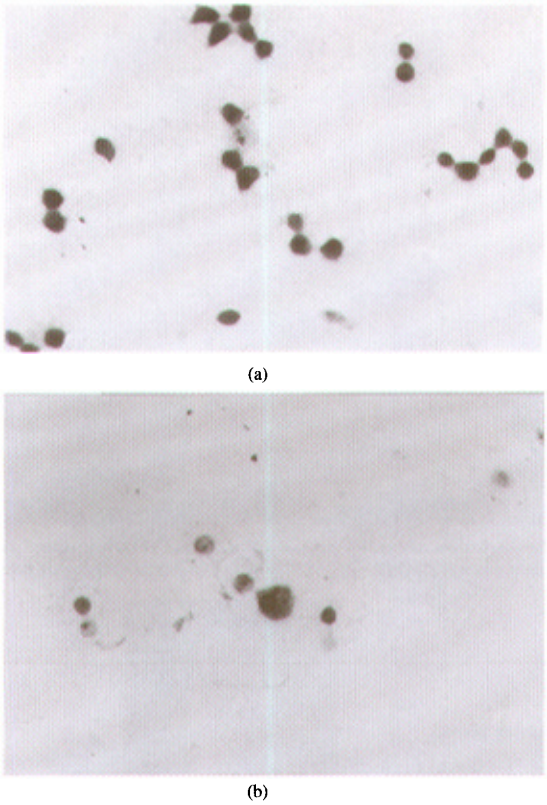
TABLE 1

The Effects of FEFVP and β-carotene on the Proliferation of YTMLC-90 cells (*OD* Value, $\bar{x}\pm s$)

Groups	OD	IR (%)
Control	0.38±0.03	
Solvent Control	0.48±0.12	
Adriamycin (10 μg/mL)	0.02±0.01	94.7[a]
FEFVP (2.5×10^{-8} mol/L)	0.43±0.02	10.4[b]
FEFVP (2.5×10^{-7} mol/L)	0.39±0.08	29.5[c]
FEFVP (2.5×10^{-6} mol/L)	0.32±0.04	33.3[d]
β-carotene(1×10^{-6} mol/L)	0.46±0.03	4.2
β-carotene(1×10^{-5} mol/L)	0.42±0.04	12.5[b]
β-carotene(1×10^{-4} mol/L)	0.36±0.04	25.0[c]

Note. OD:optical density; IR:inhibitory rate(%), $IR=(OD_{control}-OD_{test})/OD_{control}\times100$; Different superscript letters(a-d)represent significant differenece from each other compared with control group at $P<0.05$ level.

Routine microscopic examination revealed that cultured YTMLC-90 lung cancer cells treated with moderate and higher concentrations of FEFVP lost their characteristics of intercellular adhesiveness and underwent shrinkage with nuclear pyknosis (Fig. 2).



(a)

(b)

FIG. 2. Induction of apoptosis by FEFVP (A) in YTMLC-90. Apoptosis-associated DNA strand breaks, cellular shrinkage, nuclear pyknosis. Cells exposed to solvent control (B) had no morphologic changes (×280).

Electron microscopic assessment of apoptosis showed that cells treated with the highest concentration of FEFVP for 48 h displayed typical morphological features, including shrinkage and chromatin condensation (Fig. 3).

To further determine whether apoptosis occurred during growth inhibition, DNA fragmentation analysis, an indicator of apoptotic body DNA, was performed. Agarose gel electrophoresis demonstrated DNA laddering, a typical apoptosis stripe, in YTMLC-90 lung cancer cells treated with FEFVP at higher and moderate levels for 48 h (Fig. 4). However DNA laddering was not present in the cells treated with FEFVP at a lower level (data not shown).

FIG. 3. Transmission electron microscopy appearance of YTMLC-90. Pb-U-stained nuclei after 48 h incubation in medium plus FEFVP (2.5×10^{-6} mol/L). Apoptotic changes were noted (×10400).

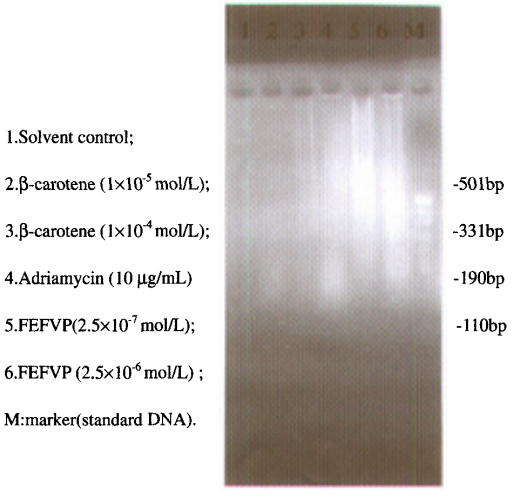


FIG. 4. Induction of apoptosis by FEFVP in YTMLC-90 cancer cells. DNA fragmentation analysis by agarose gel electrophoresis. The cells were cultured for 48 h in the presence or absence of FEFVP at the concentration of 2.5×10^{-7}, 2.5×10^{-6} mol/L of β-carotene. After cultured, DNA was analyzed using a 1.8% agarose gel and photographed under UV light. The results showed the "ladder".

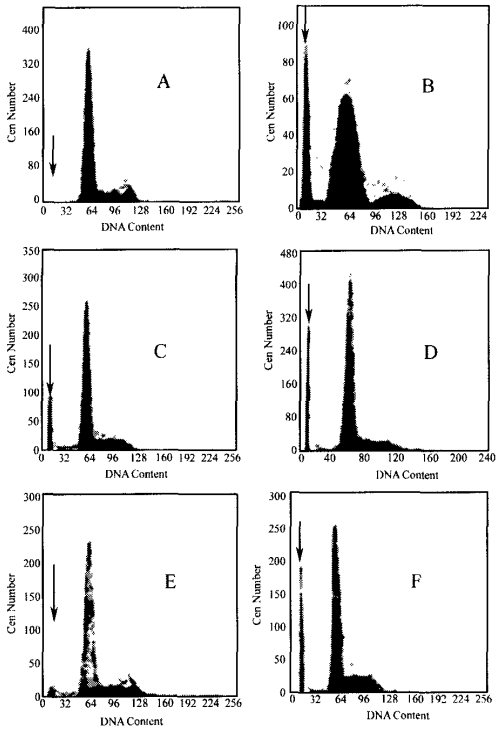


FIG. 5. Quantification of apoptosis induced by FEFVP, β-carotene in YTMLC-90 cells by using flow cytometry, cell cycle histograms of 48 h treated with FEFVP, β-carotene. The results showed sub-G_1 peak (arrow): A. Solvent control; B. Adroamycin (10 μg/mL); C. FEFVP (2.5×10^{-7} mol/L); D. FEFVP (2.5×10^{-6} mol/L); E. β-carotene (1×10^{-5} mol/L); F. β-carotene (1×10^{-4} mol/L).

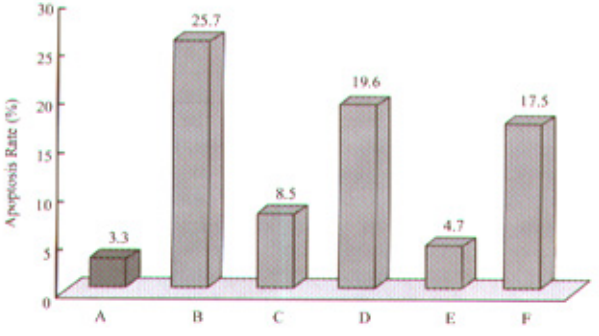


FIG. 6. Quantification of apoptosis induced by FEFVP, β-carotene in YTMLC-90 cells by using flow cytometry. The percentage of cells in the apoptotic peak from a representative experiment. A. Solvent control; B. Adriamycin (10 μg/mL); C.FEFVP (2.5×10^{-7} mol/L); D.FEFVP (2.5×10^{-6} mol/L); E. β-carotene (1×10^{-5} mol/L); F. β-carotene (1×10^{-4} mol/L).

FEFVP-induced apoptosis was also studied in cultured YTMLC-90 cells by means of flow cytometery. Cell cycle analysis in the cultured lung cancer cells treated with FEFVP for 36 h and 48 h at higher and moderate levels revealed the presence of a sub-G_1 apoptotic peak (Fig. 5). Apoptosis varied from 7% to 17% following 48 h FEFVP treatment at different concentrations. In contrast, spontaneous apoptosis in untreated control cells and in blank control cells was about 3%-4% (Fig. 6). They were significantly different from each other at $P<0.05$ (Table 2).

TABLE 2

The Ratio of Apoptosis in YTMLC-90 Cells Treated With FEFVP, β-carotene Respectively (n=3)

Groups	Apoptosis Rate (%)
Solvent Control	3.3±1.0[a]
Adriamycin (10 μg/mL)	25.7±4.3[b]
FEFVP (2.5×10^{-7} mol/L)	8.5±2.1[c]
FEFVP (2.5×10^{-6} mol/L)	19.6±1.8[d]
β-carotene (1×10^{-5} mol/L)	4.7±1.2[a]
β-carotene (1×10^{-4} mol/L)	17.5±1.6[d]

Note. Figures in same horizontal row with different superscript letters (a-d) were significantly different from each other at $P<0.05$ level.

DISCUSSION

There is convincing that diets high in green vegetables protect against lung cancer. The most effective dietary means of preventing lung cancer is consumption of diets high in vegetables and fruits[5].

FEFVP has been shown to function as a growth inhibitor. FEFVP showed an inhibiting effect on the proliferation of human hepatoma cell line 7721, human gastric cancer cell line SGC7901 and human lung cancer cell line YTMLC-90 in a dose-dependent manner[2]. However, the mechanism by which FEFVP inhibited human cancer cell growth is not known yet.

Apoptosis, or programmed cell death, is a highly regulated progress that involves activation of a cascade of molecular events, leading to cell death characterized by nuclear membrane blebbing, shrinkage, chromatin condensation, chromosomal DNA fragmentation, and formation of membrane-bound apoptotic bodies that are eventually phagocytosed by neighboring cells. Apoptosis plays important roles in the development and maintenance of homeostasis and elimination of damaged cells or unnecessary cells for the organism. On the other hand, inappropriate regulation of apoptosis may cause many serious disorders, such as neural degeneration, AIDS, autoimmune diseases, and cancers[6].

A variety of stimulators can induce physiological cell death. This study confirmed that FEFVP can induce apoptosis of cultured human lung cancer cells by means of 3 different techniques, i.e. morphological assessment, DNA fragmentation analysis, and flow cytometry. The results also suggest that the effect of FEFVP is more potent than that of single β-carotene (Fig. 3), which is in line with the conclusion of a previous study[7]. But the mechanism by which FEFVP induces apoptosis remains unclear. Researches are ongoing to further study the mechanisms involved in the induction of apoptosis in YTMLC-90 cell line.

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