Original Article

Effects of components present in flaxseed on human colon adenocarcinoma Caco-2 cells: Possible mechanisms of flaxseed on colon cancer development in animals

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ABSTRACT: Previous studies from our laboratory have shown chemopreventive effects of dietary flaxseed on azoxymethane-induced colon tumor development in male Fischer rats and Apc^{Min} mice. Tumorigenesis is associated with uncontrolled cell growth and loss of apoptosis. Accordingly, the objective of this investigation was to study the effects of mammalian lignans (enterodiol and enterolactone) and ω-3 polyunsaturated fatty acid α-linolenic acid, principal active components in flaxseed on cell proliferation and apoptosis in human colon adenocarcinoma Caco-2 cells, thus elucidating possible mechanism of action. BrdU incorporation assay was used for cell proliferation and fluorescence-activated cell sorting (FACS) analysis of annexin V/propidium iodide staining was used for determining apoptotic cells. Results showed that enterodiol, enterolactone and α-linolenic acid at different concentrations caused a significant ($p < 0.05$) increase in apoptotic cells and decrease in cell proliferation. Therefore, dietary flaxseed containing α-linolenic acid and lignans causes a decrease in cell proliferation and an increase in apoptosis resulting in the effective chemoprevention for intestinal and colon tumor development.

Keywords: Flaxseed, enterodiol, enterolactone, α-linoleic acid, colon cancer

1. Introduction

Colorectal cancer is the most common cancer in Western countries and the third leading cause of cancer related deaths. An estimated 102,900 new colon and 39,670 new rectal cases, and 51,370 colorectal deaths are expected in 2010 (*1*). The pathogenesis of colon cancer is a complex interplay of environmental factors such as consumption of high-fat diet, red meat, obesity, alcohol and genetic factors (*1,2*). Among the all risk factors of colon cancer, diet is a major but controllable factor that affects colorectal carcinogenesis; both risk factors and protective factors have been studied extensively (*3*).

Many studies suggest that fatty acid composition of dietary fat plays a vital role in colon tumor development (*4-6*). Corn oil, one of the important vegetable fats in the United States diet, contains high levels of ω-6 polyunsaturated fatty acids (PUFAs) such as linoleic acid and has been shown to enhance colon tumorigenesis in rodents (*7*). In contrast, fish oil, which is rich in ω-3 PUFAs such as α-linolenic acid (ALA), eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) reduces azoxymethane-induced colon tumor development in rats (*2,5*). In addition to ω-3 PUFAs, mammalian lignans such as enterolactone (EL) and enterodiol (ED) have been suggested to prevent breast and colon tumor development in experimental animals (*8-11*).

Flax is perennial plant cultivated from ancient times for its fiber which was used in making linen. Flaxseed was used for nutritional and medicinal purpose of anti-tumoral, relieving pain and anti-inflammatory (*12*). Flaxseed meal contains a high percentage of α-linolenic acid, an ω-3 fatty acid and a high amount of secoisolarciresinol diglucoside, which is metabolized into mammalian lignans ED and EL by the gut microflora (*8*). Studies from our laboratory have reported that dietary flaxseed oil and meal have chemopreventive effects on azoxymethane-induced colon tumor development in male Fischer rats and also inhibits intestinal tumor development in Apc^{Min} mice by increasing ω-3 fatty acid levels, lignans

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and decreasing COX-1 and COX-2 levels (*13,14*). However, the precise mechanism(s) of flaxseed on colon tumor development remain largely unknown. In term of mechanistic studies, it has been well known that tumorigenesis is associated with uncontrolled cell replication and loss of apoptotic death of cells (*15,16*). Accordingly, the purpose of the present investigation was to study the role of these active components, EL, ED and ALA, present in flaxseed on cell proliferation and apoptosis in human colon adenocarcinoma Caco-2 cells.

2. Materials and Methods

2.1. *Materials and reagents*

EL, ED, and ALA were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). The purity for ED, EL is > 95% and for ALA is > 99% by HPLC analysis. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin EDTA and phosphate buffered saline (PBS) were from Mediatech, Inc. (Herndon, VA, USA). Dimethyl sulfoxide (DMSO) was obtained from Fisher Scientific (Fair Lawn, NJ, USA). Cell proliferation ELISA kit was from Roche Diagnostics GmbH (Mannheim, Germany). Vybrant apoptosis kit 2 was purchased from Molecular Probes (Eugene, OR, USA). Other reagents were obtained in their highest purity grade available commercially.

2.2. *Cell culture*

Human colon adenocarcinoma Caco-2 cell line were grown in DMEM supplemented with 10% FBS with 100 unit/mL of penicillin and 100 µg/mL of streptomycin in a humidified atmosphere containing 5% CO_2 and 95% air at 37°C.

2.3. *Preparation of EL, ED and ALA stock solution*

EL, ED, and ALA were dissolved in DMSO respectively as stock solutions. Stock solutions were diluted in DMEM at different concentrations and immediately used. In all the assays, the final concentrations of DMSO in DMEM were less than 0.3%.

2.4. *Cell proliferation assay*

Cell proliferation assay was carried as previously described by Zhang *et al*. (*16*) using Cell proliferation ELISA, BrdU (colorimetric) kit commercially available (Roche Applied Science, Indianapolis, IN, USA). Briefly 1×10^4 cells were plated in 96 well plates and allowed to attach. Following attachment, cells were treated with various concentrations of EL, ED or ALA for desired time points. At time points 24, 48, and 72 h; 10 µL/well of BrdU labeling solution was added to the media and cells were incubated for 3 h in an incubator with 5% CO_2 at 37°C. After incubation, the labeling solution was removed by tapping off the plate; cell plate was then dried using hair-dryer for about 15 min. 200 µL/well of FixDenat solution was added to each well of the plate and incubated at room temperature for 30 min. FixDenat solution was removed by tapping off and anti-BrdU-POD (100 µL/well) was added and incubated for 90 min at room temperature. Antibody conjugate was removed by flicking off the plate, followed by washing with 200 µL of washing buffer three times. After the final wash, the washing buffer was removed and 100 µL of substrate solution was added to each well and incubated for 20 min at room temperature. After incubation, the reaction was stopped by adding 25 µL of 1 M H_2SO_4 per well, the plate was incubated for 1 min on the shaker in the cell plate reader. Absorbance was measured at 450 nm (with a reference wave length of 690 nm). Cell proliferation was expressed as the percentage of the absorbance values of drug treated groups to cells incubated with normal media.

2.5. *Apoptosis assay*

Vybrant Apoptosis Kit 2 (Molecular Probes) was used to quantitate apoptosis. The percent apoptotic cells were determined by using the manufacturer's protocol. Briefly 2×10^5 cells were grown in 6 well plates and allowed to attach. Following attachment, cells were treated with various concentrations of EL, ED or ALA for 72 h. At the end of the treatment, adherent and non-adherent cells were harvested and washed twice with ice-cold PBS, and then resuspended in 1× annexin-binding buffer (approximately 300 µL per treatment). Cells were resuspended 5 µL of Alexa Flour 488 annexin V (component A) and 1 µL of the 100 µg/mL of propidium iodide (PI) working solution was added to each 100 µL of cell suspension. The cells were then incubated at room temperature for 15 min. After incubation, 400 µL of 1 × annexin-binding buffer was added to each sample and the samples were kept on ice until analyzed. Samples were analyzed with BD FACScan™ flow cytometry (BD Biosciences, San Jose, CA, USA). The percentage of apoptotic cells in the cell samples was analyzed using CellQuest Software (BD Biosciences, San Jose, CA, USA).

2.6. *Statistical analysis*

Data were analyzed with INSTAT software (Graph Pad, San Diego, CA, USA). ANOVA followed by Tukey post test was applied to compare the statistical difference of different treatment groups with DMSO groups as controls. Significance in all the experiment was considered at $p < 0.05$. Values were expressed as mean ± the standard deviation (S.D.) of the mean.

3. Results

3.1. *Effects of lignans on cell proliferation*

Caco-2 cells were plated and allowed to grow until 70-90% confluence was observed. At that point the growth media was removed and the cells were treated with increasing concentrations of EL, ED and combination of EL and ED. Cell proliferation data for human colon cancer Caco-2 cells using various concentrations of EL, ED both alone and in combination are shown in Figure 1. IC_{50} for EL, ED was found to be 60 µM and 150 µM, respectively. EL and ED significantly ($p < 0.05$) inhibited cell proliferation of Caco-2 cells starting at 50 µM and 150 µM, respectively, as shown in Figure 1. Thus, EL significantly ($p < 0.05$) decreased cell proliferation at relatively lower concentrations when compared to ED. However, both EL and ED decreased BrdU uptake into the DNA in a concentration-dependent manner.

Various concentrations of a combination of EL and ED were used to study cell proliferation at different time periods as shown in Figure 1C. A combination of 37.5 µM of EL and 37.5 µM of ED showed significant ($p < 0.05$) decrease in cell proliferation.

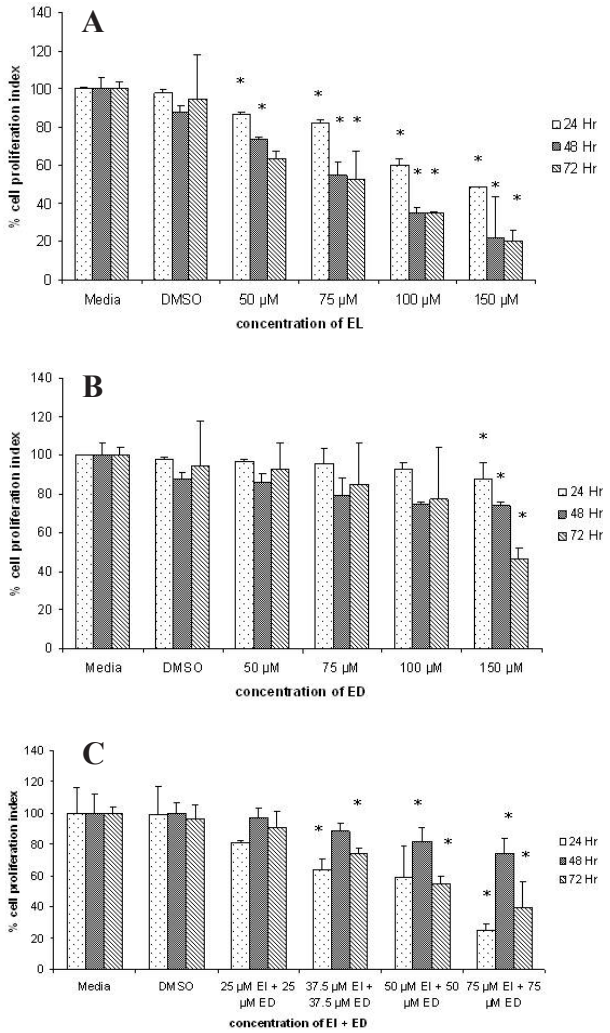

Figure 1. Effects of lignans on cell proliferation in Caco-2 cells. Cells in Media groups were treated with media only, whereas, cells in DMSO groups were treated with media containing same concentration of DMSO of treated groups. Thus, DMSO groups were preformed as control group. (**A**) Cells were treated with different concentrations of EL (0-150 µM) for 24, 48, and 72 h, respectively. (**B**) Cells were treated with different concentrations of ED (0-150 µM) for 24, 48, and 72 h, respectively. (**C**) Cells were treated with combinations of EL and ED for 24, 48, and 72 h, respectively. At the end of respective treatment, BrdU incorporation assays were performed as detailed in the "Materials and Methods". Values of BrdU incorporation assay are mean ± S.D. of three samples in each treatment. *, $p < 0.05$ indicates statistical significance in treated groups as compared to DMSO control groups.

3.2. *Effects of ALA on cell proliferation*

Caco-2 cells were plated and allowed to grow until 70-90% confluence was observed. At that point the growth media was removed and the cells were treated with increasing concentrations of ALA and combination of ALA + EL and ALA + ED as shown in Figure 2. The IC_{50} for ALA was found to be around 750 µM. ALA in concentration of 700 µM significantly ($p < 0.05$)

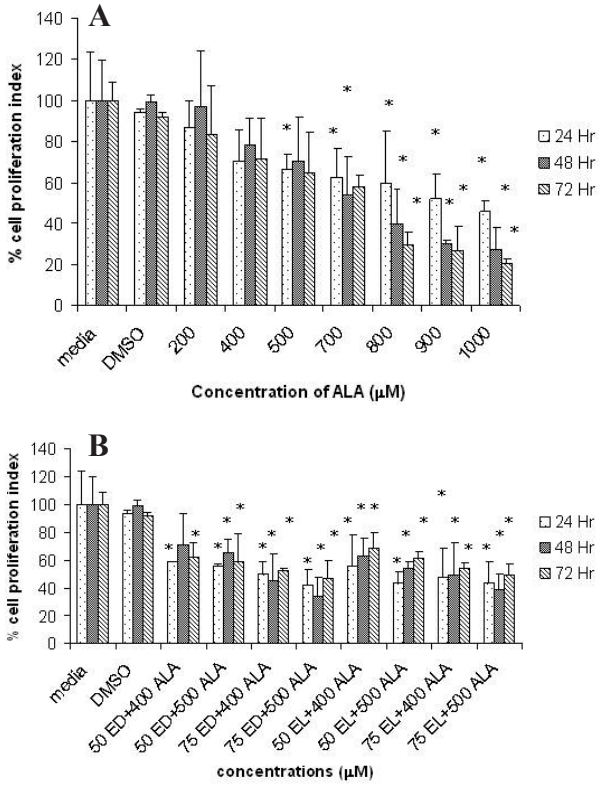

Figure 2. Effects of ALA and combinations of ALA with either EL or ED on cell proliferation in Caco-2 cells. (**A**) Cells were treated with different concentrations of ALA (0-1,000 µM) for 24, 48, and 72 h, respectively. (**B**) Cells were treated with combinations of ALA with either EL or ED for 24, 48, and 72 h, respectively. At the end of respective treatment, BrdU incorporation assays were performed. Values of BrdU incorporation assay are mean ± S.D. of three samples in each treatment. *, $p < 0.05$ indicates statistical significance in treated groups as compared to DMSO control groups.

reduced cell proliferation.

As shown in Figure 2B, a combination of 50 µM of ED and 400 µM of ALA showed significant ($p < 0.05$) decrease in cell proliferation. Similarly, a combination of 50 µM of EL and 400 µM of ALA also significantly ($p < 0.05$) inhibited cell proliferation. As compared to ALA alone, combination of ALA with either ED or EL, ALA exhibited significant inhibition of cell proliferation in Caco-2 cells at a relatively lower concentration.

3.3. *Effects of lignans on apoptosis*

Flow cytometric analysis of Caco-2 cells with various concentrations of EL and ED both alone and in combination is shown in Figure 3. Both EL and ED at the concentration of 100 µM significantly ($p < 0.05$) induced apoptosis in Caco-2 cells as compared to DMSO treated control group. However, EL treated cells showed higher increase of apoptotic cells when compared to ED. The percentage of apoptotic cells was higher at 150 µM of EL when compared to other concentrations of ED alone and combination with 75 µM of ED and 75 µM of EL.

3.4. *Effects of ALA on apoptosis*

Flow cytometric analysis of Caco-2 cells using various concentrations of ALA alone and in combination with EL and ED is given in Figure 4. ALA alone significantly ($p < 0.05$) induced apoptosis in relatively higher concentrations (800 µM and 1,000 µM). Even ALA combined with either ED or EL significantly ($p < 0.05$) induced apoptosis at 500 µM of ALA with either 75 µM of ED or 75 µM of EL, respectively, which are relatively higher concentrations as compared to the combination of EL and ED.

4. Discussion

Colorectal cancer continues to pose a serious health problem in the United States leading to the third most prevalent cancer in the United States and accounting for 10% of cancer deaths (*1*). Colorectal cancer evolves from a multistep process and is a disease strongly influenced by diet. Prevention of colorectal cancer at early stages has been improving due to several advances in diagnosis and cellular biology.

Among dietary factors, there is growing epidemiological, clinical and experimental evidence which suggests a protective role of ω-3 PUFAs found in fish oil, flaxseed oil, perilla oil on colon cancer. In contrast, dietary lipids rich in ω-6 PUFAs found in vegetable oils, corn oil enhance the development of colon tumors. This is significant because the typical Western diet contains 10-20 times more ω-6 than ω-3 PUFAs (*2,7*).

Dietary flaxseed meal which has a high percentage of ω-3 PUFAs and lignans grown in the Dakotas, was

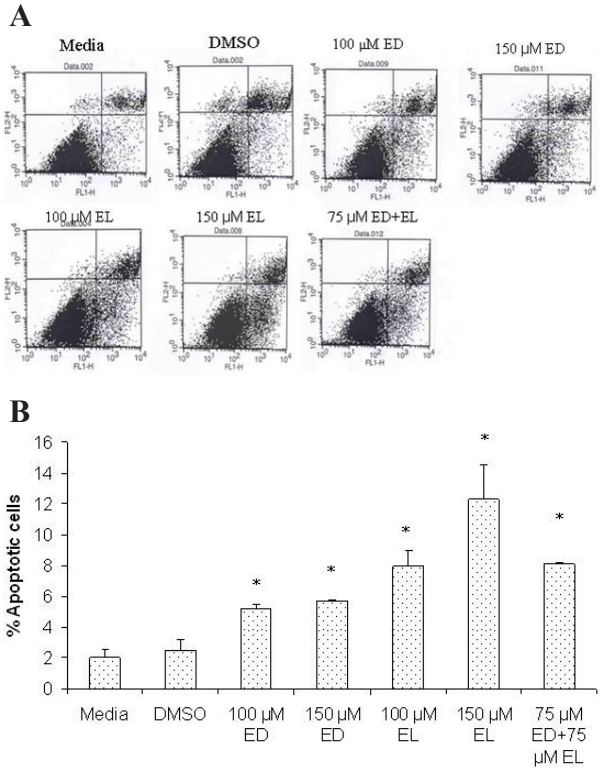

Figure 3. Effects of lignans on apoptosis in Caco-2 cells as measured by annexin V/PI staining. Cells were treated with EL alone, ED alone and combination of EL and ED for 72 h, and then cells were collected by brief trypsinization. (**A**) Dot plot of annexin V (FL1-H)/PI (FL2-H) staining of Caco-2 cells by flow cytometry. The lower left quadrant includes viable cells, which exclude PI and are negative for annexin V staining. The lower right quadrant is apoptotic cells, which exclude PI but bind to green fluorescence labeled annexin V. The upper quadrants represent necrotic cells or dead cells that do not exclude PI. (**B**) shows percentages of apoptotic cells after analysis of the FACS data using CellQuest software. In each case data represent mean ± S.D. of three observations. *, $p < 0.05$ indicates statistical significance in treated groups as compared to DMSO control.

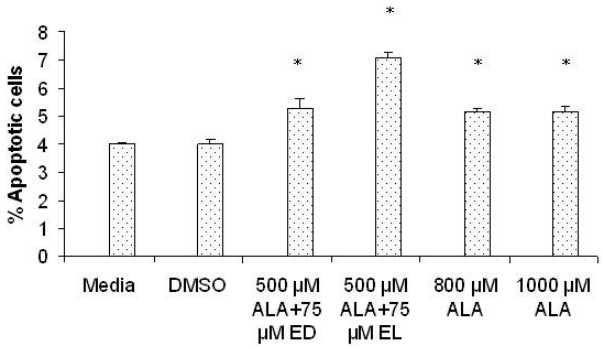

Figure 4. Effects of ALA and combinations of ALA with either EL or ED in Caco-2 cells as judged by annexin V/PI staining. Cells were treated with ALA alone or combination of ALA with either EL or ED for 72 h, and then cells were collected by brief trypsinization. Graph shows percentages of apoptotic cells after analysis of the FACS data using CellQuest software. In each case data represent mean ± S.D. of three observations. *, $p < 0.05$ indicates statistical significance in treated groups as compared to DMSO control.

tested as a possible chemopreventive agent in colon and intestinal tumor development. Studies from our laboratory have been reported that dietary flaxseed meal showed substantial chemopreventive effects on colon tumor development and intestinal tumor development in male Fischer rats and Apc^{Min} mice model, respectively, by increasing levels of ω-3 fatty acid and lignans (*13,14*).

The present study was undertaken to elucidate the possible mechanism of action of flaxseed meal on colon tumor development *in vivo* by investigating the effects of components of flaxseed meal on cell proliferation and apoptosis in human colon adenocarcinoma Caco-2 cells. Results showed that major components of dietary flaxseed meal, EL, ED, and ALA can inhibit colon tumor cell proliferation, with EL being more effective than ED and ALA. Mammalian lignans such as EL and ED have been shown to reduce proliferation of estrogen sensitive breast tumor cells such as ZR-75-1 and MCF-7 in culture (*11*). This effect has been related to a number of mechanisms including the ability of lignans to act as antiestrogen *i.e*., competing with estrogens for binding with estrogen receptors. In case of colon tumor cells, evidence of estrogen receptors is conflicting. Therefore, the growth inhibitory activity of colon tumor cells may be mediated through other mechanisms (*11*).

The protective effects of lignans against colon tumor cell proliferation might be through apoptosis mediated cell death and also through their antioxidant property. A recent study showed that EL suppressed Colo 201 human colon cancer cell growth both *in vitro* and *in vivo* and the suppressive mechanisms were attributed to apoptosis and decreased cell proliferation (*10*).

The current study showed that mammalian lignans along with ALA were effective in inducing apoptosis and inhibiting cell proliferation in human colon adenocarcinoma Caco-2 cells. However, the concentration of ALA which could result in a significant effect was relatively higher than EL and ED. A possible reason may be that ALA is metabolized to EPA and DHA and these metabolites alone or in combination with ED and EL may provide higher effects on inhibiting cell proliferation and inducing apoptosis in Caco-2 cells.

Several *in vitro* studies have already showed that ω-3 fatty acid, EPA has antitumoral effects through inhibition of cell proliferation or induction of apoptosis (*17*). In another study, it was shown that growth inhibitory and cytotoxic effects of PUFAs with methylene-interrupted double bonds such as arachodonic acid and EPA are due to peroxidation products that are generated during lipid peroxidation and COX activity (*18*).

In conclusion, this study demonstrated that components of flaxseed meal are effective in decreasing cell proliferation of colon cancer cells and inducing apoptosis of cancerous cells. Consumption of dietary flaxseed leading to the circulating level of ALA, EPA, DHA, EL, and ED could be effective for the management of colon cancer. Further studies are needed on the effects of ALA, EPA, DHA, EL, and ED on other cell lines on other biomarkers of colon cancer development to completely understand the mechanism of action.

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