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Enterolactone Induces Apoptosis and Inhibits Growth of Colo 201 Human Colon Cancer Cells both *In Vitro* and *In Vivo*

NAOYUKI DANBARA, TAKASHI YURI, MIKI TSUJITA-KYUTOKU, REIKO TSUKAMOTO, NORIHISA UEHARA and AIRO TSUBURA

Department of Pathology II, Kansai Medical University, Moriguchi, Osaka 570-8506, Japan

Abstract. *Background: The mammalian lignan enterolactone (ENL) is produced from plant lignans which are present in large amounts in flaxseed (linseed). The effect of ENL on colon cancer cell growth in vitro and in vivo, and its mechanisms of action, have not been studied in detail. Materials and Methods: The growth of the colo 201 human colon cancer cell line was examined by colorimetric 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) assay, while the expression of apoptosis- and proliferation-related proteins (p53, Bax, Bcl-$x_{L\ and\ S}$, Bcl-2, Caspase-8, Caspase-3 and proliferating cell nuclear antigen (PCNA)) were examined by Western blotting. In vivo tumor growth was examined by transplanting colo 201 cells into ENL-treated and placebo-treated athymic mice. Results: The MTS assay showed that ENL suppressed colo 201 cell growth (IC_{50} for 72 h: 118.4 μM) in vitro. On flow cytometry, induction of apoptosis was confirmed by the appearance of subG1 populations, while cell cycle progression was not affected. The expression of an apoptosis-suppressing protein (Bcl-2) was down-regulated, an apoptosis-enhancing protein (cleaved form of Caspase-3) was up-regulated, proliferation-related PCNA protein was down-regulated and p53, Bax, Bcl-$x_{L\ and\ S}$ and Caspase-8 levels were unchanged. ENL, at a dose of 10 mg/kg given 3 times per week by subcutaneous injection, significantly inhibited the growth of colo 201 cells transplanted into athymic mice without any adverse effects. Conclusion: ENL suppressed colo 201 human colon cancer cell growth both in vitro and in vivo. The tumor-suppressing mechanisms included apoptosis and decreased cell proliferation.*

Correspondence to: Airo Tsubura, M.D., Department of Pathology II, Kansai Medical University, Moriguchi, Osaka 570-8506, Japan. Tel: +81-6-6993-9431, Fax: +81-6-6993-5023, e-mail: tsubura@takii.kmu.ac.jp

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In contrast to Asian countries, colon cancer is one of the most prevalent cancers in the Western world. Nevertheless, though colon cancer incidence and mortality is currently low in Japan, it is increasing. The etiology of human colon cancer is complex and remains poorly understood, although diet is considered to be one of the major causes of this disease (1, 2). Fat consumption is strongly related to colon carcinogenesis, with both the type and amount of dietary fat consumed implicated (3). Epidemiological data indicate that high dietary intake of n-6 polyunsaturated fatty acid (PUFA) increases colon cancer risk and that the consumption of a diet rich in n-3 PUFA correlates with a reduced incidence of colon cancer (3). Experimental studies indicate that linoleic acid, a member of n-6 PUFAs accelerates colon carcinoma cell growth at the primary site and metastases, while eicosapentaenoic acid and docosahexaenoic acid, both of which are n-3 PUFAs, exert significant inhibitory effects (4, 5). Furthermore, conjugated docosahexaenoic acid, a mixture of positional and geometric isomers of docosahexaenoic acid, is more potent than the parent docosahexaenoic acid in suppressing colon cancer cell growth (6). However, fat is not the only dietary component which modifies colon cancer risk.

It has been found that dietary phytochemicals have a preventive action against various cancers (7). Plant lignans are naturally occurring phenolic compounds widely present in vegetables, fruits and whole grains. Flaxseed (linseed) is the richest source of the plant lignan secoisolariciresinol diglycoside (SDG), which can be metabolized by the colonic microflora to two mammalian lignans, enterodiol (END) and enterolactone (ENL) (8). Mammalian lignans show structural similarities to estradiol (9). In experimental studies, rye bran, flaxseed (which has a much higher concentration of lignans than rye) and SDG have been shown to be protective against azoxymethane-induced colon carcinogenesis in rats (10-12). In rats, diets that included 5% and 10% flaxseed protected against colon cancer (11). Flaxseed is also rich in α-linolenic acid, an n-3 PUFA that has anticarcinogenic effects on colon carcinogenesis (13). However, the tumor-protective effect of

flaxseed is mainly due to the presence of lignans and not due to the presence of α-linolenic acid (14). Purified mammalian lignan ENL has been shown to inhibit the growth of 7,12-dimethylbenz(α)anthracene (DMBA)-induced mammary carcinomas in rats (15), and both END and ENL inhibit human breast and prostate cancer cell growth *in vitro* (16, 17). Several epidemiological studies have suggested that lignans may have protective effects against hormone-related cancer, such as those of the breast and prostate, as well as other cancers. However, even though these major mammalian lignans, END and ENL, are produced in the colon, their anticarcinogenic action in the colon has not been thoroughly studied.

There is a limited amount of information that deals with the action of END and ENL on colon cancer cells (18), and there are no available data as to whether they suppress human colon cancer cell growth *in vivo*. ENL is more potent than END in suppressing prostate and colon cancer cell growth (17, 18). Thus, the purpose of this study was to evaluate the effect of ENL on cell growth in colo 201 human colon carcinoma cells both *in vivo* and *in vitro*. Many mechanisms of action for lignans in suppressing cancer cell growth have been suggested, including an antioxidant effect (19), an antiproliferative effect (20), an antiangiogenic effect (21) and an antiaromatase activity (16). To clarify this, the mechanisms of ENL action in reducing the growth of colo 201 cells were also studied.

Materials and Methods

Cell line. Colo 201, a human colon adenocarcinoma cell line, was obtained from the Japan Cell Bank. Colo 201 cells are derived from cells found in the ascitic fluid of a patient with adenocarcinoma of the colon, and are non-adherent floating cells (22). The cells were cultured in RPMI 1640 medium (Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA), 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco). The cells were grown at 37˚C in a humidified atmosphere with 5% CO_2.

Enterolactone. Mammalian lignan ENL (trans-α, β-bis (3-hydroxybenzyl) butyrolactone) was purchased from Fluka Chemie GmbH (Buchs, Switzerland) and stored at 4˚C in the dark. The purity was ≥90%, as analyzed by high pressure liquid chromatography.

MTS assay. Viable cells exposed to ENL were enumerated by a colorimetric 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) assay. Stock solutions of ENL were prepared in ethanol at a concentration of 60 mM. For the experiments, test solutions were prepared from the stock solutions by diluting to 1 of 6 final concentrations that ranged from 1 to 200 μM in a culture medium. Initially, the cells were seeded at a plating density of $1x10^4$/well and cultured for 24, 48 or 72 h. The 6 concentrations of each test compound covered a 5-log range that was chosen to span the 50% inhibitory concentration (IC_{50}) determined in a preliminary assay. The final concentration of ethanol never exceeded 0.2% (v/v). Control cells were exposed to the test medium supplemented with 0.2% ethanol. Following culture with the test compound for 24, 48 or 72 h, MTS (Promega, Madison, WI, USA) was added, and the plate samples were then read at an OD value of 490 nm in 8 wells. Additional controls consisted of the culture medium alone. The percentage of surviving cells relative to untreated controls was calculated.

Flow cytometry. Cells were exposed to ENL at their IC_{50} concentration for 72 h, then in RPMI 1640 supplemented with 10% FBS for 72 h. The cells were washed in phosphate-buffered saline (PBS) (-), then centrifuged and fixed in 70% ethanol. After another centrifugation, the cells were treated with RNase, diluted in (PBS) (-), and stained with propidium iodide (50 μg/ml) in PBS (-). The cells were analyzed with a FACScan (Becton Dickinson, Mountain View, CA, USA) using Lysis II software. CellFIT software with a doublet discrimination module was used to eliminate the possibility of confusing possible multiples of G1 cells with ordinary G2 cells.

Western blotting. Colo 201 cells were examined for the expressions of p53, Bax, Bcl-$x_{L \text{ and } S}$, Bcl-2, Caspase-8, Caspase-3 and proliferating cell nuclear antigen (PCNA) proteins. Cells exposed to ENL at the IC_{50} dose for 72 h were compared with unexposed cells. Immunoblotting of protein was performed as previously described (6). Briefly, cell lysates were prepared and aliquots of each (50 μg of protein) were run on 12% sodium dodecyl sulphate/polyacrylamide gels and blotted onto nitrocellulose membrane (Hybond-p PVDF Membrane, Amersham Biosciences, Piscataway, NJ, USA). The membranes were probed with anti-p53 antibody (clone D0-7, DakoCytomation, Glostrup, Denmark), anti-Bax antiserum (BD Biosciences, San Jose, CA, USA), anti-Bcl-x antibody (clone M-125, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Bcl-2 antiserum (BD Biosciences), anti-Caspase-8 antibody (clone B9-2, Novocastra, Newcastle-upon-Tyne, UK), anti-Caspase-3 antiserum (Novocastra) and anti-PCNA antibody (clone PC10, Novocastra). Next, the membrane was treated with peroxidase-conjugated goat anti-rabbit IgG antiserum (DakoCytomation). Detection of immobilized antigen was performed using ECL plus Western blotting detection reagents and hyperfirm (Amersham Biosciences), according to the manufacturer's instructions. Band intensity was quantified with the NIH Image 1.47 processing and analysis program for use with Macintosh computers.

Colo 201 cell growth in athymic mice. Male BALB/c-nu/nu mice (age, 4 weeks) were purchased from Charles River Japan (Atsugi). The mice were randomized into 3 groups (0, 1 and 10 mg/kg ENL-treated groups), 10 mice per group, 1 group per 2 cages. During the experiment, the animals were housed in plastic cages with sterilized white pine chips as bedding. The animal room was maintained in a specific-pathogen-free condition, and was controlled for temperature (22±2˚C), light (12-h cycle), and humidity (60±10%). After the mice had been acclimatized for 1 week, semi-confluent colo 201 cells growing in RPMI 1640 supplemented with 10% FBS at 37˚C were centrifuged, and $5x10^5$ viable cells/0.25 ml of medium were injected subcutaneously into the backs of the 35-day-old mice using a 26-gauge needle. However, 1 day before tumor cell inoculation (when the mice were 34 days of age), ENL (dissolved in dimethylsulfoxide) at a dose of

0, 1, or 10 mg/kg of body weight was administered subcutaneously to 3 different groups of mice. The same ENL treatment was then given to each group 3 times a week for the entire experimental period. During the experiment, the mice were weighed and the locally growing tumor was measured every 2 or 3 days once it became visible. Tumor volume was measured with calipers and calculated using a standard formula: width^2 x length x 0.5 (23). All mice were sacrificed 23 days after the tumor cell inoculation day. This was followed immediately by autopsy and weighing of the tumors. Locally growing tumors and major organs were processed for hematoxylin and eosin staining. All procedures were approved by the Animal Experimentation Committee of Kansai Medical University, Japan.

PCNA immunohistochemistry and TUNEL staining. The cell kinetics of the primary tumors growing in the athymic mice were evaluated. Cell proliferation was evaluated by PCNA labelling and cell death was evaluated by using the TUNEL index. First, formalin-fixed, paraffin-embedded sections taken from the primary tumors in athymic mice were deparaffinized and washed in distilled water, and antigen was retrieved by microwave heating for 5 min in a pressure cooker. PCNA immunohistochemistry with anti-PCNA antibody (clone PC10, Novocastra) was performed by Dako autostaining (DakoCytomation) using a Dako LSAB II-Kit, according to the manufacturer's instructions. Nuclear antigens were visualized using 3,3'-diaminobenzidine-4HCl (DAB) (Wako, Pure Chemical, Osaka, Japan) and were counterstained with hematoxylin. The average percentage of PCNA-positive cells was determined for the sections, which contained approximately 1000 cells each. To detect apoptotic cells, we used the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-digoxigenin nick end-labelling (TUNEL) reaction from the ApopTag Kit (Intergen, Purchase, NY, USA). Deparaffinized slides were washed with PBS and pretreated with 20 μg/ml proteinase K for 15 min. Equilibration buffer was applied after quenching the endogenous peroxidase with 2% hydrogen peroxide, and the terminal deoxynucleotidyl transferase (TdT) reaction was performed for 60 min at 37˚C in a moist chamber. Sections were treated with anti-digoxigenin-peroxidase for 30 min after the TdT reaction was suspended by the stop/wash buffer. TdT-labelled cells were detected by DAB, and sections were counterstained with hematoxylin. The apoptotic index represents the average percentage of TUNEL-positive cells per section, where each section contained approximately 1000 cells.

Statistics. All results are expressed as the mean±SE. For all the *in vitro* experiments, a two-tailed independent Student's *t*-test for unpaired samples was used, after assuring homogeneity of the variances, to determine whether there were any statistically significant differences. For the *in vivo* experiments, the Mann-Whitney's *U*-test was used to determine if there were any statistically significant differences in body weight, tumor volume and tumor weight. Statistical significance in all analyses was determined by probability values <0.05.

Results

Cytotoxic potential of ENL in vitro. The cytotoxic potential of ENL was examined by MTS assay. The number of viable colo 201 cells decreased after treatment with ENL (Figure 1).

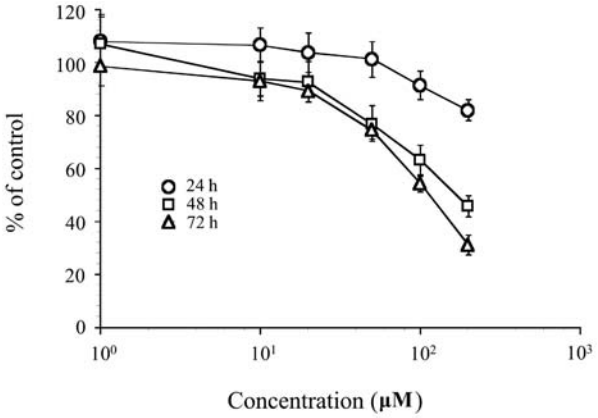

Figure 1. *Growth inhibition curves of colo 201 cells after ENL treatment. Values represent mean±SE of 8 wells. IC_{50} for 72 h: 118.4 μM.*

Significant dose- and time-dependent cytotoxicity (reduction of cell number below the initial plating density) was seen. The IC_{50} for the 72-h treatment was 118.4 μM.

Induction of apoptosis. Flow cytometry was performed to determine whether the inhibition of cell growth by ENL was related to cell cycle progression and/or induction of apoptosis. Colo 201 cells were treated with 118.4 μM ENL (IC_{50} value at 72 h) for 72 h, and the cell cycle fraction was compared with cells kept in the medium alone (untreated control). Representative histograms and data from 3 independent repetitions of the experiments are shown in Figure 2. After 72-h incubation, though the cell cycle progression in cells treated with ENL was comparable to that of untreated control cells, ENL-treated cells showed the subG1 fraction characteristic of apoptosis.

Expression of apoptosis- and proliferation-related proteins. Western blotting was performed to examine changes in apoptosis- and proliferation-related proteins. ENL at 118.4 μM (IC_{50} concentration for 72 h) were applied to cells, followed by incubation for 48 h. Representative Western blotting results are shown in Figure 3, and data from 3 independent repeats of the experiments are summarized in Figure 4. After ENL treatment, there was an increase in the cleaved form of Caspase-3; Bcl-2 and PCNA levels decreased; p53, Bax, Bcl-$x_{L \text{ and } S}$ and Caspase-8 protein levels were unchanged. The pattern of change seen in the apoptosis- and proliferation-related proteins after ENL exposure indicates the acceleration of apoptosis and suppression of cell proliferation.

Growth inhibition of colo 201 cells in athymic mouse. There was no evidence of gross toxicity or histological abnormalities resulting from ENL injection. However, two

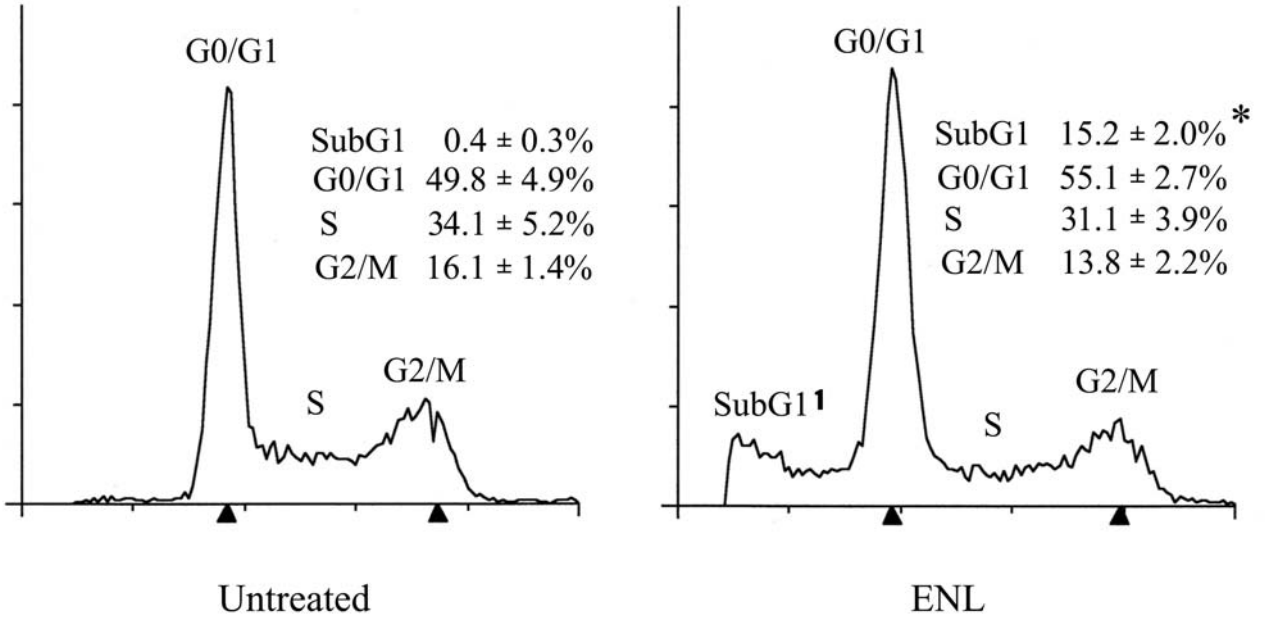

Figure 2. *Flow cytometry of colo 201 cells. Cells were grown in medium alone (untreated control) or treated with 118.4 μM ENL (72 h IC_{50} concentration) for 72 h. The appearance of the subG1 fraction is apparent after 72-h incubation with ENL. *$p<0.05$ vs. untreated control.*

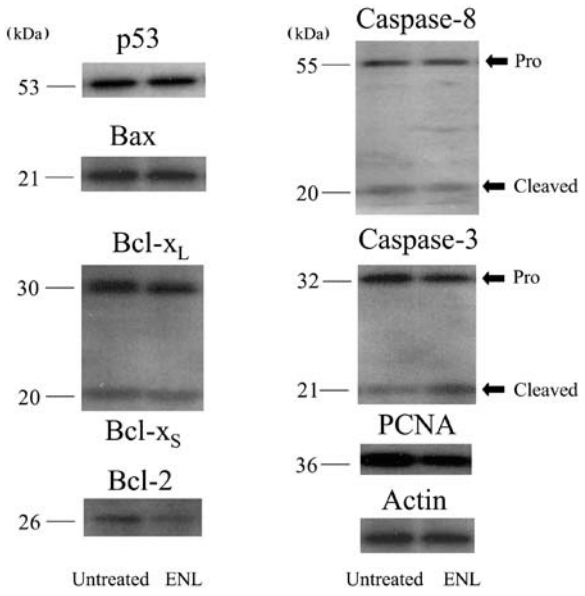

Figure 3. *Expression of apoptosis- and proliferation-related proteins in colo 201 cell grown in untreated medium alone, or treated with ENL. Representative data of apoptosis- and proliferation-related proteins in cells treated with ENL as compared to cells not treated with ENL.*

10 mg/kg ENL-treated animals died and were excluded from the calculation. Growth of the mice was not altered, and there was no significant difference in body weight between 1 mg/kg and 10 mg/kg ENL-treated mice and the ENL-untreated groups (Figure 5). However, the growth of colo 201 cells, as evaluated by tumor volume, was inhibited in the 1 mg/kg and 10 mg/kg ENL-treated mice, compared with the ENL-untreated controls (Figure 6). The final tumor volume and final tumor weight are shown in Figure 7. The mean tumor volume and the tumor weight were both significantly reduced in the 10 mg/kg ENL-treated group, compared with the ENL-untreated group. A similar trend was seen in the 1 mg/kg ENL-treated group, but the differences were not statistically significant. Cell kinetics data are shown in Figure 8. Cell proliferation, as indicated by PCNA labelling, was significantly lower in the ENL-treated groups. Cell death, as reflected by the TUNEL index, was significantly higher in the ENL-treated groups. These results indicate that ENL suppressed colo 201 tumor cell growth *in vivo* by the acceleration of apoptosis and the suppression of cell proliferation.

Discussion

ENL was shown to inhibit colo 201 human colon cancer cell growth both *in vitro* and *in vivo*; the 72-h IC_{50} was 118.4 μM, and the subcutaneous administration of 10 mg/kg ENL, 3 times per week, significantly reduced transplanted tumor growth in athymic mice. In other cancers, such as human breast cancer cell lines, ENL was more effective in suppressing DNA synthesis in estrogen-dependent MCF-7 cells compared with estrogen-independent MBA-MB-231

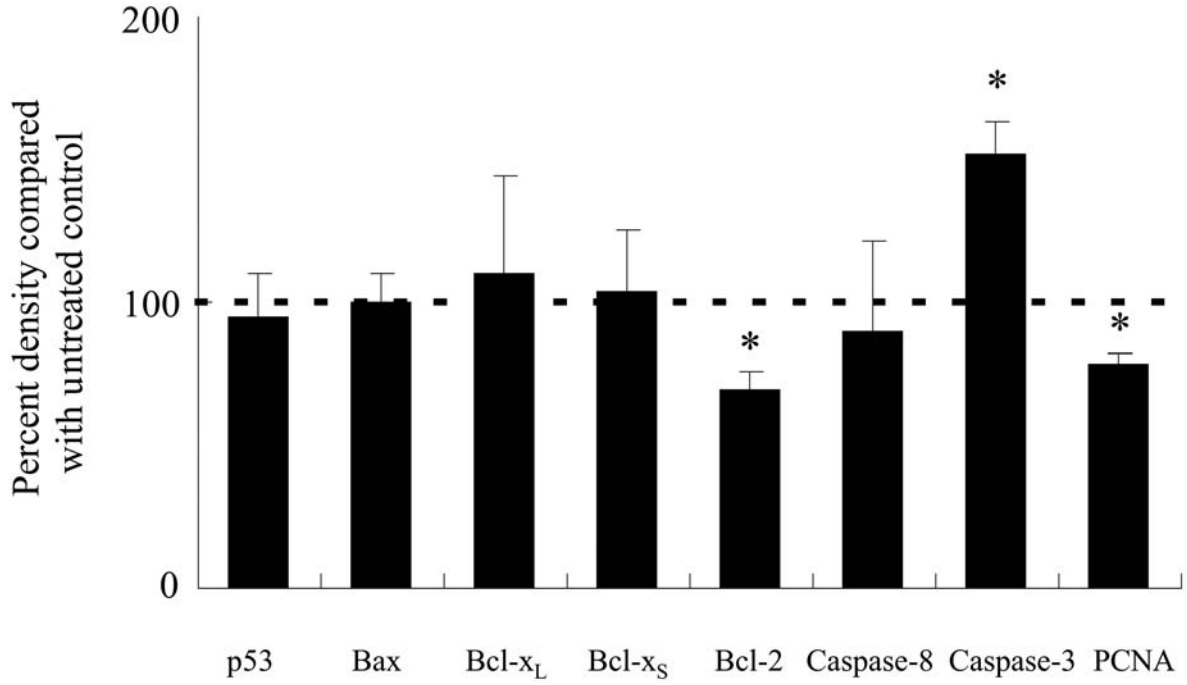

Figure 4. *Quantification of apoptosis- and proliferation-related proteins in cells treated with ENL compared with untreated controls designated as 100%. All results are based on 3 independent samples (*p<0.05 vs. untreated control).*

cells (the 24-h IC_{50} was 82 μM in estrogen-dependent MCF-7 cells *vs.* >100 μM in estrogen-independent MBA-MB-231 cells) (16). Similarly, in estrogen receptor (ER)-positive tumors, competition with estradiol for the receptor binding may be a possible mechanism of tumor growth suppression. In human prostatic carcinoma cells, androgen-sensitive LNCaP cells appear to be more responsive to ENL than androgen-insensitive PC-3 and DU-145 cell lines (17). It has been shown that ENL can suppress circulating free androgen levels by increasing the synthesis of sex-hormone-binding globulin (SHBG) (24). Nevertheless, ENL has also been shown to inhibit the growth of androgen-insensitive prostatic carcinoma cells (17). ENL's ability to inhibit hormone-independent breast and prostate cancer cells suggests that ENL may, in addition to interfering with hormone activity, possess other mechanisms for suppressing cancer cell growth.

ENL, at a 100 μM concentration for 8 to 10 days, has been found to significantly reduce the proliferation of human colon cancer cell lines (LS174T, Caco-2, HCT-15, and T-84) by 55 to 88% (18). In the present study, the 72-h IC_{50} was 118.4 μM for colo 201 human colon cancer cells. Since the growth of the colon cancer cells was not affected by the presence of 17β-estradiol (18), non-hormone-related mechanisms may be involved. Lignans such as nordihydroguaiaretic acid (NDGA) inhibit the growth of colon tumor cells by inducing apoptosis with down-modulation of Bcl-x_L, while Bcl-2 and Bax levels remain unchanged (25). In the present study, apoptosis was seen in the colo 201 cells. Therefore, the apoptosis cascade was affected, with down-regulation of the apoptosis-suppressing protein Bcl-2, and up-regulation of the apoptosis-accelerating protein Caspase-3, while p53, Bax, Bcl-$x_{L\text{ and }S}$ and Caspase-8 levels remained unchanged. ENL also down-regulated PCNA expression. Thus, ENL apparently affects colo 201 cells by modulating the apoptosis cascade and lowering cell proliferative activity.

In adult rats, the continuous consumption of a diet containing 5% flaxseed (or similar amounts of SDG) decreases the incidence, number and size of DMBA-induced mammary tumors (9). Exposure to a diet with 10% flaxseed (or the equivalent amount of SDG) during only the suckling period can also protect against DMBA-induced rat mammary carcinogenesis later in life without adverse effects on the selective reproductive indices in dams of the offspring (14). Supplementation of 10% flaxseed to athymic mice reduces ER-negative MDA-MB-435 human breast cancer cell growth and metastasis (26, 27). Furthermore, daily oral administration of ENL at a dose of 10 mg/kg of body weight for 7 weeks has been found to significantly inhibit DMBA-induced mammary carcinomas in rats (15). In the present study, the subcutaneous injection of 10 mg/kg

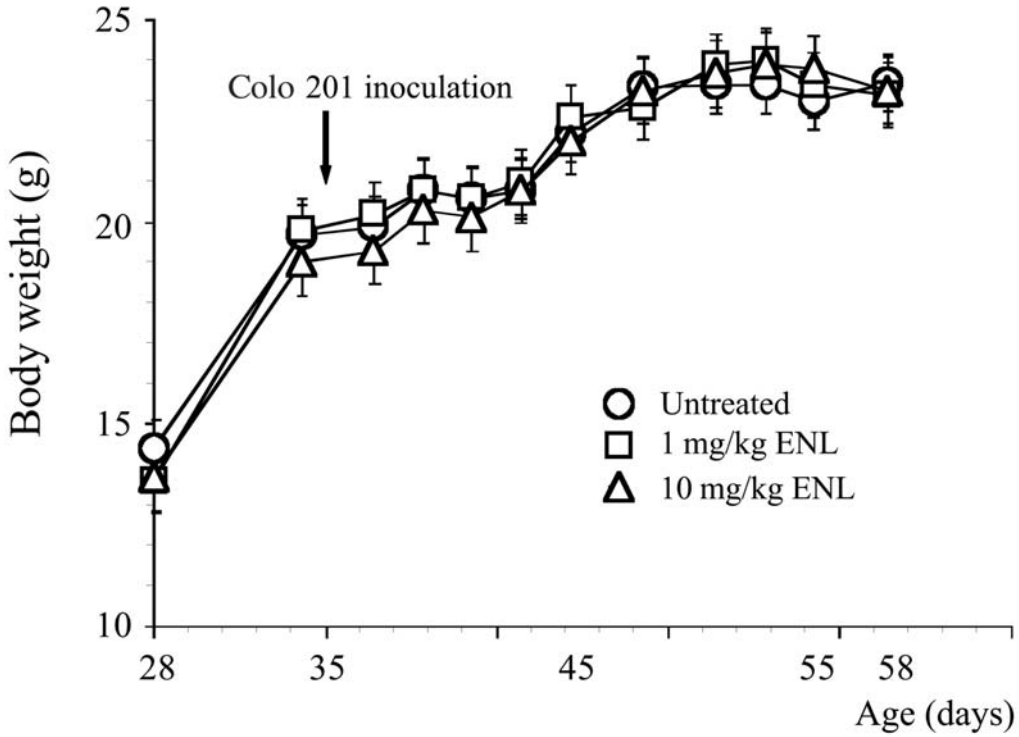

Figure 5. *Effect of ENL on body weight change in male athymic BALB/c-nu/nu mice before and after colo 201 inoculation. Values represent mean±SE.*

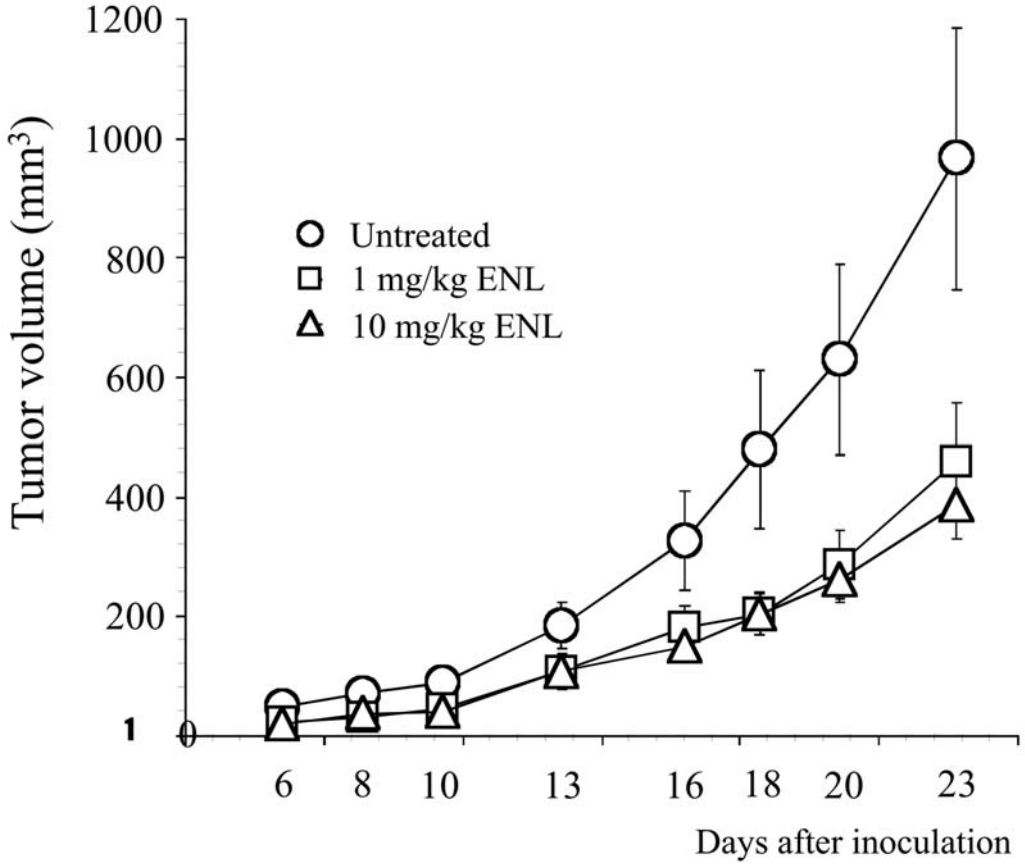

Figure 6. *Growth of colo 201 cells injected subcutaneously into the back of 35-day-old male athymic BALB/c-nu/nu mice. Values represent mean±SE.*

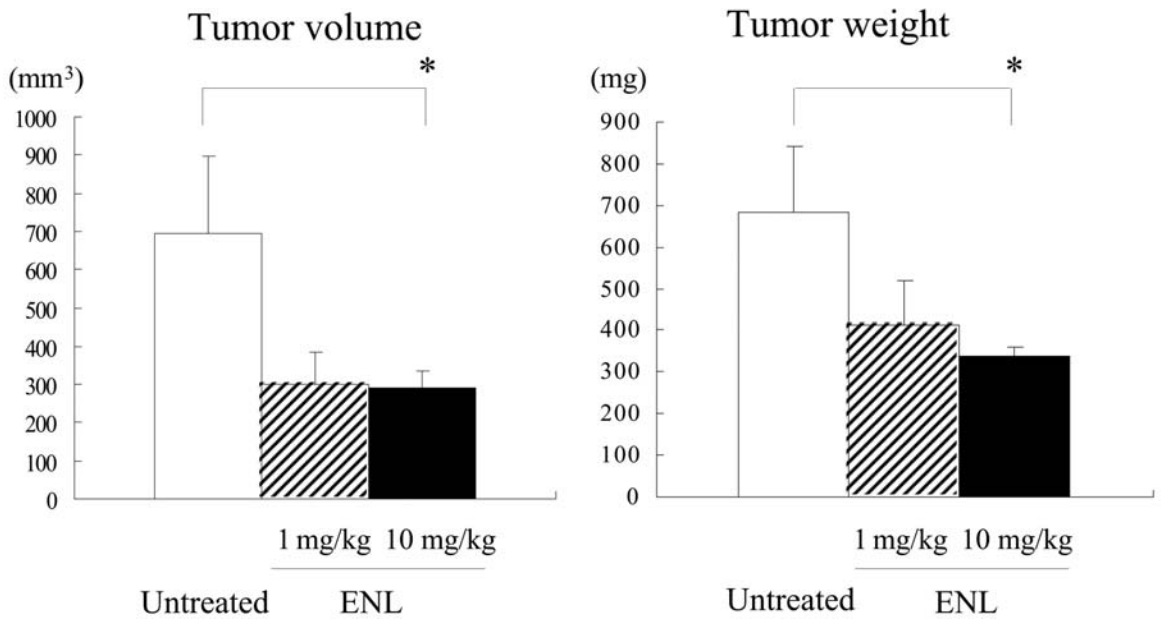

Figure 7. *The effects of ENL on colo 201 tumor volume and tumor weight (*p<0.05 vs. untreated control).*

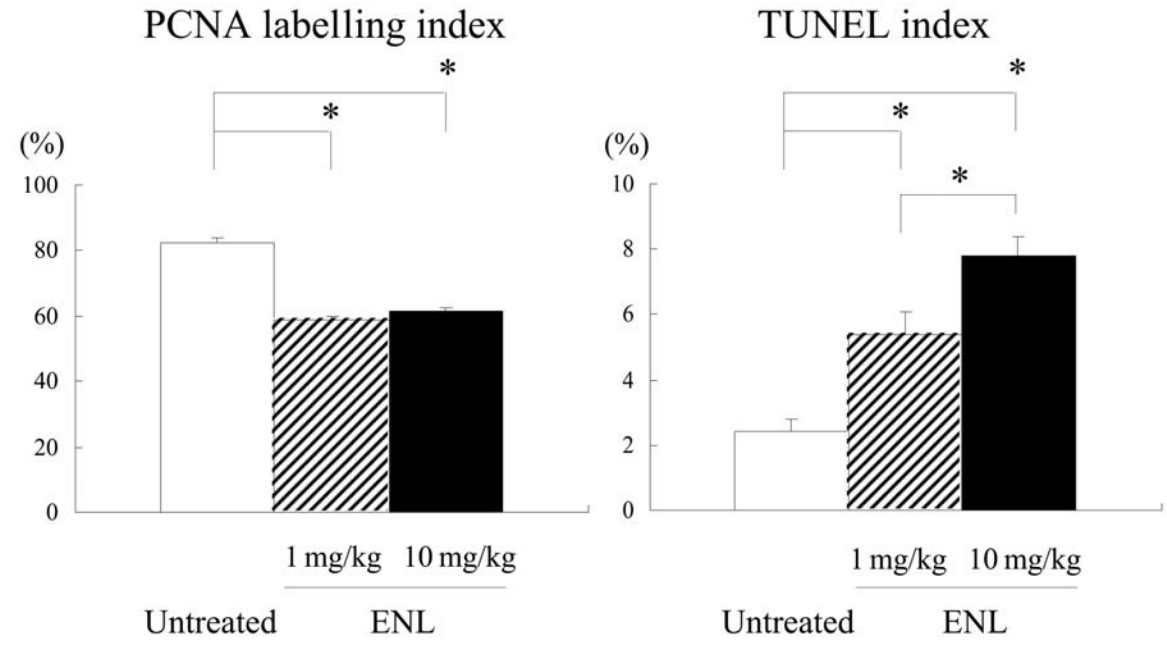

Figure 8. *Changes in cell proliferation (PCNA labelling) and cell death (TUNEL index) of colo 201 cells grown in athymic BALB/c-nu/nu mice given ENL, either 1 mg/kg or 10 mg/kg (*p<0.05).*

ENL, 3 times a week, significantly reduced colo 201 human colon cancer cell growth in athymic mice.

In conclusion , ENL suppressed colo 201 cell growth *in vitro* by modulating the apoptosis cascade and lowering cell proliferative activity, while *in vivo* ENL reduced the growth of colo 201 cells transplanted into athymic mice without causing adverse effects. Therefore, consumption of lignan-containing food may be advantageous in humans, and ENL appears to be a promising molecule for use in human colon cancer control.

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