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Lignan from Safflower Seeds Induces Apoptosis in Human Promyelocytic Leukemia Cells

Jae Hi Kim, Youn Hee Park, Sang Won Choi*, Eun Kyoung Yang and Won Jung Lee†

Department of Physiology, School of Medicine, Kyungpook National University, Daegu 700-422, Korea
**Department of Food Science and Nutrition, Catholic University of Daegu, Gyeongbuk 712-702, Korea*

Abstract

We recently extracted lignans such as matairesinol and 2-hydroxyarctigenin from safflower seeds and found that they exhibit a potent cytotoxic effect on human promyleocytic leukemia HL-60 cells. In this study, we investigated whether mechanisms of the matairesinol-induced cell death are associated with the programmed cell death, apoptosis. Matairesinol dose-dependently reduced viability of HL-60 cells with an IC_{50} value of 60 μM. Staining of cells with Hoechst 33342 revealed distinct morphological features of apoptosis, such as the nuclei broken into chromatin containing fragments of various sizes in the cells exposed to 100 μM matairesinol for 24 hr. Agarose gel electrophoresis of DNA from the cells treated with matairesinol showed internucleosomal DNA degradation into oligonucleosomal sizes. DNA ladder like patterns were easily detected after treatment with matairesinol concentrations ranging from 10 to 100 μM after 24 hr. In cells treated with 100 μM matairesinol for differing time periods, the DNA ladder was detectable from 6 hr onward. A time course histogram of the DNA content analyzed by flow cytometry revealed a rapid increase in subdiploid cells and a concomitant decrease in diploid cells exposed to 100 μM matairesinol. These results indicate that matairesinol-induced HL-60 cell death was due to the DNA damage and apoptosis.

Key words: matairesinol; apoptosis, anti-cancer agents; HL-60 cells

INTRODUCTION

New cancer chemopreventive agents from natural products have long been the subject of intensive investigation. Natural chemotherapeutic agents have been studied, not only for isolation and identification of manifest structure, but also for the elucidation of new target mechanisms that can lead to the inhibition of cancer growth (1-3). Recently, considerable attention has focused on the sequence of events referred to as apoptosis, a programmed cell death, as a target mechanism for the inhibition of cancer growth. Apoptosis is a genetically controlled response of an intrinsic cell suicide system that is required for a critical balance between cell proliferation and cell death in normal development and maintenance of homeostasis of organisms (4). Indeed the process of apoptosis may play an important role in the treatment of various human diseases including cancer. Many cancer chemotherapy agents suppress the growth of transformed or malignant cells by inducing apoptosis (5-7). Therefore, the induction mechanism of apoptosis in tumor cells can be an efficient target for drugs (8,9) and has become a major focus in the study of cancer biology (10).

Increasing evidence suggests that lignans derived from plants have anti-tumorigenic effects in various cancers and have been suggested as potential cancer inhibitory agents (11-14). Lignans isolated from Taiwania exhibited cytotoxicity against A549 human lung cancer, MCF-7 breast cancer, and HT-29 colon cancer cells (15). Antimitogenic activity of lignans has also been demonstrated in human leukemia HL-60 cells (11). However, the anti-leukemic mechanisms of the lignans have not been clarified.

Lignans are phenolic compounds with phytoestrogenic activity due to their structural similarity to estrogen (16,17) and their ability to bind to estrogen receptors with low affinity (18). Flaxseed and other oilseeds are particularly abundant sources of lignans (19,20). We recently isolated lignans such as matairesinol and 2-hydroxyarctigenin from safflower seeds and found strong antioxidative activity (21) and antiproliferative effects on cancer cells. In the present study, we investigated whether mechanisms of the matairesinol-induced cell death of human leukemia HL-60 cells are associated with apoptotic cell death.

MATERIALS AND METHODS

Chemicals

Propidium iodide (PI), 3-(4,5-dimethylthiazol-2-yl)-2,5-

†Corresponding author. E-mail: wjleek@knu.ac.kr
Phone: +82-53-420-6926, Fax: +82-53-424-3349

diphenyl tetrazolium bromide (MTT) and RNAse A were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT, USA). Antibiotics-antimycotes, RPMI 1640 and trypsin-EDTA were purchased from Gibco BRL (Gland Island, NY, USA). Hoechst 33342 was purchased from Calbiochem (San Diego, CA, USA). Matairesinol was isolated from safflower seeds and purified as described previously (21). Stock solution of matairesinol was made in dimethylsulfoxide (DMSO) at a concentration of 100 mmol/L. Working dilutions were directly made in the tissue culture medium.

Cell culture and cell growth assay

Human promyelocytic leukemia HL-60 cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 20 mM HEPES (pH 7.0) and 1% antibiotic-antimycotic. The cytotoxic effect of matairesinol isolated from safflower seeds was analyzed by MTT assay; a method of determining the viability of cells by staining with MTT. For the MTT assay, HL-60 cells (5×10^4 cell/well) were grown for 1~2 days with serial dilutions of matairesinol in a 96-well plate. After incubation, 50 μL of MTT solution (1.1 mg/mL) was added to each well and then incubated for an additional 4 hr. After centrifugation, the supernatant was removed from each well. The colored formazan crystal produced from MTT was dissolved in 150 μL of DMSO and then its optical density was measured at 540 nm by a plate reader (Multiscan@ MCC/340, USA).

Morphological examination

HL-60 cells (1×10^6 cells/mL) grown in 6-well plates were treated with 100 μM of matairesinol at 37°C for 24 hr. Morphological changes occurring in the cells were observed under a phase-contrast microscope and photographed. Ultrastructural examination was performed by conventional techniques using a biological inverted microscope (Nikon T-300, Japan).

Nuclear staining with Hoechst 33342

HL-60 cells (1×10^6 cells/mL) were cultured in 6 well plates in RPMI 1640 medium containing 10% fetal bovine serum in the absence or presence of 100 μM matairesinol. After 24 hr, the cells were stained with 10 μM of the DNA specific fluorochrom Hoechst 33342 for 30 min and observed under fluorescent microscopy (Axioplan2 imaging and Aiophot2 Universal Microscope, USA).

Analysis of DNA fragmentation by agarose gel electrophoresis

To determine apoptotic DNA fragmentation induced by the treatment of matairesinol, HL-60 cells at a density of 1×10^6 cells/mL were treated with various concentrations of matairesinol for 24 hr as well as other time periods. The isolation of apoptotic DNA fragments was performed as described by Hèrrmann et al. (22). Briefly, the cells were harvested by centrifugation at 1200 rpm for 3 min and then treated with a lysis buffer (1% Nonidet P-40, 20 mM EDTA, 50 mM Tris-HCl, pH 7.5). After centrifugation for 5 min the supernatant was collected and brought to 1% SDS and treated for 2 hr at 50°C with RNase A and subsequently with proteinase K for 2 hr at 37°C. The DNA fragments were precipitated with 2.5 volume of ethanol in the presence of 10 M ammonium acetate. The DNA fragmentation was visualized by electrophoresis on a 1.6% agarose gel.

Flow cytometry analysis

HL-60 cells were either untreated or treated with 100 μM matairesinol for various time periods (0~24 hr). After the treatments, approximately 1×10^6 cell pellets were prepared, washed twice with PBS (without Ca^{2+} and Mg^{2+}) plus 2% FBS, suspended in 100 μL of PBS, and then 200 μL of 95% cold ethanol was added while vortexing. The cells were incubated at 4°C for 1 hr, washed twice with PBS-containing 2% FBS and resuspended with 12.5 μg of RNase in 250 μL of 1.12% sodium citrate buffer (pH 8.45). Incubation was continued at 37°C for 30 min before staining the cellular DNA with 250 μL of propidium iodide (50 μg/mL) for 30 min at 4°C. The stained cells were analyzed on a FACScan flow cytometer (Becktin & Dickinson, USA) for the relative DNA content, based on an increased red fluorescence.

RESULTS AND DISCUSSION

Cytotoxic effect of matairesinol on HL-60 cells

The effects of matairesinol on the viability of HL-60 cells determined by MTT assay are shown in Fig. 1. When cells were incubated with several doses of matairesinol, ranging from 10 to 200 μM for 0 to 48 hr, matairesinol decreased cell viability in a dose-dependent manner. The concentrations of matairesinol needed to kill 50% of HL-60 cells incubated for 24 and 48 hr were approximately 100 and 60 μM, respectively. Incubation with 200 μM matairesiol for 24 to 48 hr resulted in greater than 80% cell death. The cytotoxic effects of matairesinol on HL-60 cells were almost equivalent to the effects of various flavonoids, lignans, and anti-cancer drugs (11).

Apoptosis induced by matairesinol

The induction of apoptotic cell death is accompanied by characteristic morphological and ultrastructural changes such as chromatin condensation, internucleosomal fragmentation of chromosomal DNA and the collapse of cells

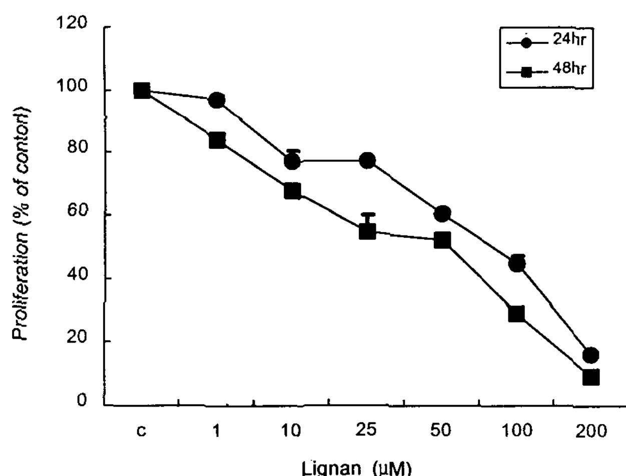


Fig. 1. Inhibitory effects of matairesinol on the growth of the HL-60 cells. Cells were treated with matairesinol at the indicated concentrations. Values are the mean and SE of triplicate samples analyzed in a typical experiment.

into apoptotic bodies (23). To determine whether matairesinol-induced cell death is associated with apoptosis, we examined morphological changes, DNA fragmentation and fluorecent-activated cell sorter (FACS) analysis.

Morphological changes: After incubation of HL-60 cells with 100 μM matairesinol for 24 hr, the cells exhibited typical morphological features characteristic of apoptosis such as cellular shrinkage and apoptotic body formation (Fig. 2A, right). Staining of the matairesinol treated cells with a DNA binding dye, Hoechst 33342, visualized the nuclei broken into chromatin-containing fragments of various sizes, which was then examined by fluorescence microscopy (Fig. 2B, right).

DNA fragmentation: Since the DNA fragmentation is a hallmark of apoptosis in most cells, the induction of DNA strand-breaks was confirmed. Agarose gel electrophoresis of DNA from the HL-60 cells incubated with matairesinol showed internucleosomal DNA degradation into oligonucleosomal sizes. DNA fragmentation was observed in dose and time-dependent manners, ranging from 185 bp and its multiples up to 2500 bp (Fig. 3). The concentrations needed to induce DNA fragmentation corresponded to the doses required for cytotoxic effects. When the cells were treated with 100 μM matairesinol for different time periods, DNA ladder like patterns were detected from 6 hr and sustained until 48 hr, indicating that the cytotoxic effect of matairesinol was attributable to the induction of apoptotic DNA fragmentation.

FACS analysis: Flow cytometric analysis of DNA content was used to corroborate the apoptosis mode of

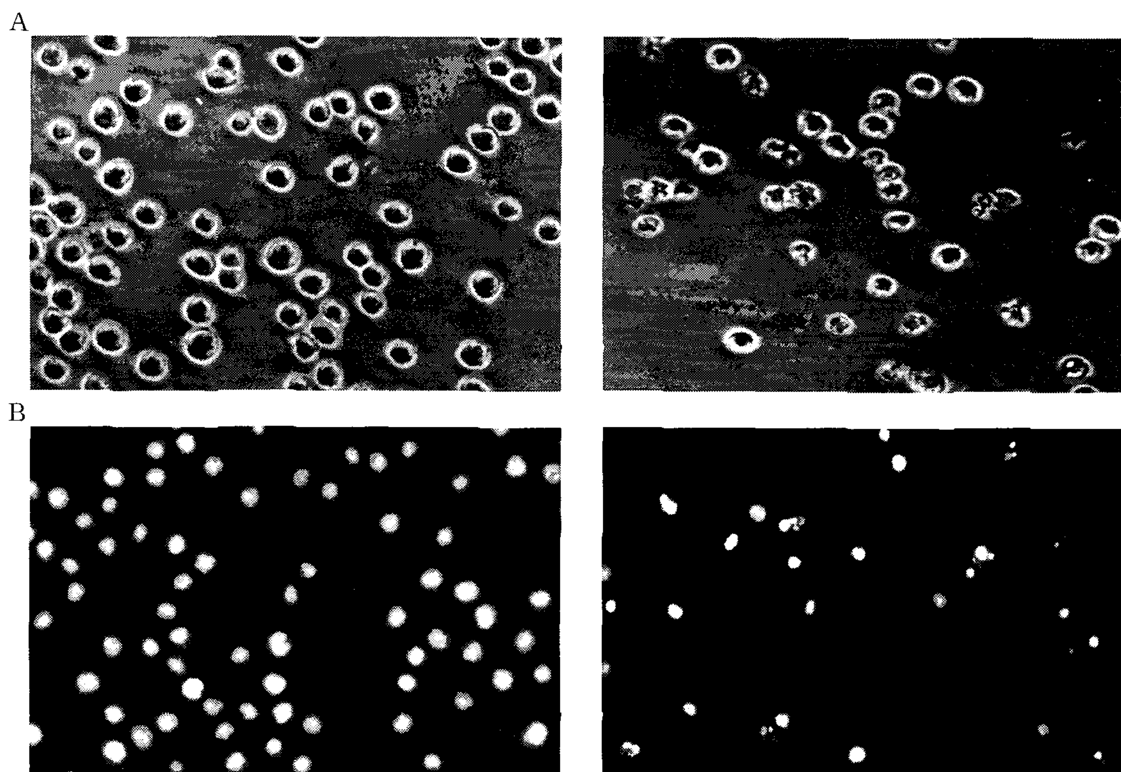


Fig. 2. Effect of matairesinol on apoptosis in HL-60 cells. Cells were exposed to 100 μM matairesinol for 24 h. (A) Morphologic analysis by phase-contrast microscopy (×100) of untreated control cells (left) and matairesinol-treated cells (right). (B) Programmed cell death in cultured cells was assessed by nuclear morphology after staining with bisbenzimide (Hoechst 33324). Nuclei of untreated control cells (left) and matairesinol-treated cells (right) were assessed using an Axioplan2 imaging fluorescence microscope, at a final magnification of 400×.

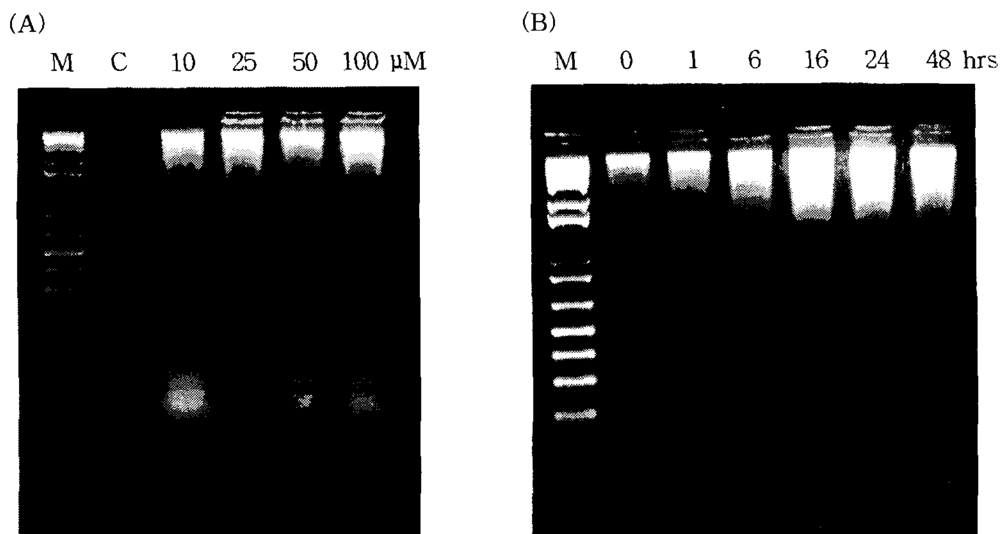
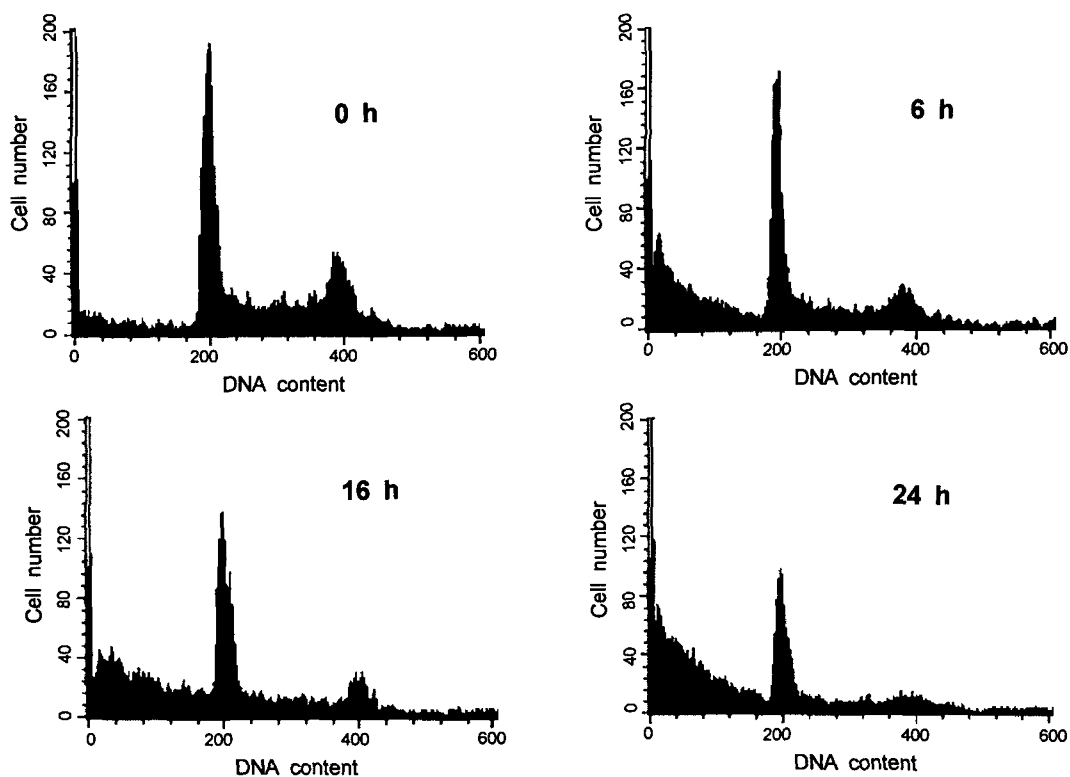


Fig. 3. DNA fragmentation of HL-60 cells treated with matairesinol. HL-60 cells (10^6 cells) incubated for the concentration indicated (A) and for the times indicated (B). The DNA fragmentation was analyzed by NP-40 lysis method using 1.6% agarose gel electrophoresis and staining with ethidium bromide.



Time (hr)	Cell cycle analysis (%)			
	SubG1	G1	S	G2/M
0	3.2	43.9	28.6	21.7
6	35.4	33.1	17.1	12.0
16	42.3	31.5	16.2	8.5
24	57.3	24.5	11.4	6.0

Fig. 4. Flow cytometric analysis of cell cycle distribution in the HL-60 cells treated with 100 μM matairesinol for the times indicated Cell cycle analysis was performed on an equal number of cells (10^6) and staining of DNA was done by propidium iodide.

cell death. Flow cytometry allows a simultaneous estimation of cell cycle parameters and apoptosis. Some anticancer agents are related to cell cycle restriction and a possible role for cell cycle in apoptosis has been suggested (24). The HL-60 cells were treated with 100 μM matairesinol for various periods of time. Then, 1×10^6 cells were immediately fixed in ethanol and stained with propidium iodide for the analysis of DNA content. Time course histograms of DNA content showed a rapid increase in the percentage of the sub G1 peak of cells and a concomitant decrease in diploid cells as shown in Fig. 4. A sub G1 population indicates subdiploid DNA content indicative of apoptotic DNA fragmentation. The percentage of cell populations with subdiploid DNA content was 3% in untreated cells, but was quickly increased to 35, 42 and 57% at 6, 16 and 24 h, respectively. Resveratrol, another polyphenolic compound, also produces G1 phase arrest in HL-60 cells undergoing apoptosis (3).

The present results demonstrate that matairesinol-induced cell death of the HL-60 cells was due to the induction of apoptosis. Chemopreventive agents derived from natural products constitute an important class of therapeutic agents in anticancer drugs (25). Phytoestrogens, including lignans and flavonoids, present in plants and included in the human diet have been suggested as desirable agents for the prevention and intervention of various cancers (11,15,26,27). Although phytoestrogens bind to estrogen receptors with low affinity, limited data are available that suggest a possible involvement of estrogen receptors in phytoestrogen-induced cancer cell apoptosis in cells other than breast cancer cells. Mechanisms of lignan-induced apoptosis in cancer cells have not been explored, but resveratrol has been demonstrated to suppress expression of anti-apoptotic protein Bcl-2 (3). Members of the caspase family of proteases are known to be crucial mediators of the complex biochemical events associated with resveratrol-induced apoptosis in HL-60 cell (3). Bcl-2 overexpression attenuated resveratrol-induced apoptosis in U937 cell by inhibition of caspase-3 activity (28). Further studies are required to elucidate the underlying mechanisms of anticancer activities of lignans both *in vivo* and *in vitro* to develop lignans as useful cancer therapeutic and chemopreventive agents.

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