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Phytol from Italian ryegrass (*Lolium multiflorum* Lam.) induces apoptosis in human lymphoid leukemia Molt 4B cells

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Abstract

The exposure of human lymphoid leukemia Molt 4B cells to phytol which was isolated from *Lolium multiflorum* Lam. and identified by MS, and ^{1}H- and ^{13}C-NMR, led to both grown inhibition and the induction of programmed cell death (apoptosis) . Morphological change showing apoptotic bodies was observed in the cells treated with phytol. The fragmentation by phytol of DNA to oligonucleosomal-sized fragments that are characteristics of apoptosis was observed to be concentration - and time -dependent. These findings suggest that growth inhibition by phytol of Molt 4B cells result from the induction of apoptosis in the cells.

Introduction

We have purified several compounds extracted from plant and evaluated the antitumor activity of those compounds(1-3). The efficiency of the antitumor compounds seems to be related to the propensity of tumor cells to respond to these compound by apoptosis. Thus, apoptosis may be a primary mechanism of antineoplastic agent (4). Apoptosis is an orchestrated series of events through which the cells precipitated its own death. The stages of apoptosis include cell shrinkage , chromatin condensation , nuclear segmentation, and internucleosomal fragmentation of DNA, resulting in the generation of apoptotic bodies(5). In the previous studies , we demonstrated induction of apoptosis by honokiol(1) , sesamin(2) and pheophorbide a (3) in cultured human lymphopid leukemia Molt 4B cells .

In the present study, we have obtained a fraction which exhibits anti-proliferative activity from Italian ryegrass(*Lolium multiflorum* Lam.) and have determined of the compound contained in the fraction. In this report, we demonstrate the identification of phytol from Italian ryegrass and show the inhibitory effects of phytol on the growth of human lymphoid leukemia Molt 4B cells which results from the induction of apoptosis.

Materials and methods

Cell culture and reagents. Human lymphoid leukemia Molt 4B cells were cultivated as previous described(6) . Molt 4B cells were grown in RPMI 1640 medium (Gobco Labolatories, Gland Island, NY) with 10% fetal calf serum, penicillin G (50 IU/ml) and streptomycin (50 μg/ml) at 37℃ under humidified 95% air-5% CO_2 atmosphere, and passaged every 7 days. Mycoplasma testing was routinely negative.

Apparatus. ^{13}C-NMR spectra were taken with JEOL, JNM-500, Tokyo, Japan. Secondary ion mass spectrometry (SIMS) were done by a Hitachi M-800 instrument, Tokyo, Japan. Fopr preparation of phytol as described in the next paragraph, high pressure liquid chromatography (HPLC) was performed with a JASCO 880-PU, Tokyo, Japan.

Isolation and identification of phytol. The air-dried leaves of *Lolium multiflorum* Lam (300 g) , harvested on the experimental farm of Mie University, were soaked in 700 ml of 80% ethanol for 3 weeks at room temperarure , and resulting supernatant was concentrated to give 82 g of extracts. The extracts (6.5 g) were charged into an Amberlite XAD-2 column , eluted with successive equal volume (100 ml) of H_2O,

20%, 40%, 60%, 80% and 100% methanol. Antiproliferative effects of these fractions were investigated on human lymphoid leukemia Molt 4B cells . One hundred % methanol fraction (1.0 g) having the comparatively higher antiproliferative activity was subjected to silica gel chromatography and eluted with a hexane - ethyl acetate and methanol solvent system. A hexane - ethyl acetate (8 : 2 v/v) fraction , having the comparatively higher antiproliferative activity, showed one main spot on silica gel TLC which was detected by heating after spraying with 10% phosphomolybdic acid in ethanol. This fraction (130 mg) was then purified by preparative HPLC under the following conditions: column, Develosil ODS-5 (ϕ 10 mm x 250 mm); mobile phase, methanol/H_2O = 95 : 5 (v/v); flow rate, 1.5 ml/min; detection wavelength, 210 nm and then compound I (6 mg) having a retention time of 46.6 min in the HPLC was obtained. Next, structural determination of compound I was carried out using IR, MS, ^{1}H-NMR ($CDCl_3$), ^{13}C- NMR ($CDCl_3$) , DEPT, ^{1}H -^{1}H-COSY, ^{13}C - ^{1}H COSY and HMBC. The number of carbon in the compound was revealed to be twenty by ^{13}C - NMR data. The DEPT spectra revealed the presence of five methyl carbons (δ 16.2, 19.7. 19.7, 22.6, 22.7 ppm), ten methylene carbons (δ 24.5, 24.8, 25.1, 34.3, 36.7, 37.3, 37.4, 39.4. 39.9, 59.4 ppm) four methine carbons (δ 27.9, 32.7, 32.8, 123.0 ppm) and one quaternary carbon (δ 140.4 ppm) . These carbons showing signals of 140.4, 123.0 and 59.4 ppm in ^{13}C-NMR spectrum, respectively , were abbreviated as A, B and C , and their protons attached to B and C were done as Hb and Hc, respectively. At first , C was considered to be attached to B by ^{1}H - ^{1}H COSY data (Hc , δ 4.09 ppm coupled with Hb , δ 5.34 ppm). Chemical shifts of C showed proton signal at 4.09 and catbon signal at 59.4 ppm . Moreover, the presence of an OH group was shown by the IR band at 3300 cm^{-1}. These data suggested that C was attached to the OH group. ^{13}C - ^{1}H COSY spectral data

suggested that B and C were correlated to Hb and Hc, respectively. Also, the proton at 1.60 ppm was correlated with the carbon at 16.2 ppm . HMBC spectral data suggested that methyl proton (δ 1.60 ppm) was correlated with A (δ 140.4 ppm), and B (δ 123.0 ppm) and that the methine proton (δ 5.34 ppm) was correlated to A, B and C. These data suggest the partial structure as shown in Fig. 1. As a result, the compound I identified as phytol from the spectral ^{1}H - NMR, ^{13}C - NMR and Ms of authentic sample as shown in Fig. 2.

Assay for growth inhibition. Exponentially growing cells were placed at 3 to 4 x 10^5 cells /ml in the culture flask and cultivated in the presence of vehicle or phytol . After cultivation for 3 days, the cell number was counted by a hemocytometer.

Microscopic observation of morphorological change of Molt 4B cells. Exponentially growing human lymphoid leukemia cell were plated at the initial density of 3 to 4 x 10^5 cells/ml. After cultivation for 2 days in the presence of vehicle or phytol , the cells were fixed with 1% glutaraldehyde and stained with Hoechst 33258. The morphology of the stained cells was examined by an epifluorescence microscope with a cooled CCD camera digital imaging system and Fuji pictography 3000 as described by Okumura et al (7)

Assay for DNA fragmentation . Exponentially growing cells were placed at 3 to 4 x 10^5 cells/ml. After cultivation in the presence of vehicle or phytol for 1, 2 and 3 days , the cells were pelleted by centrifugation, respectively. DNA was isolated from the cell pellets as described previously(8). Equivalent amounts of DNA (2 μg) were loaded into wells of 2% agarose gel and electrophoresed in 40 mM Tris- acetic acid

(pH 7.5) buffer containing 2 mM EDTA.

Results and Discussion

Inhibition of growth of Molt 4B cells by phytol. As shown in Table I, the proliferation of Molt 4B was inhibited as the concentration of phytol was increased. At 100 μM the growth of Molt 4B cells was perfectly inhibited.

Induction of apoptosis by phytol. The significant growth inhibitory activity of phytol led us to investigate whether part of the effect was a result of apoptosis induction. Morphological change showing apoptotic body and fragmentation of genomic DNA into oligonucleosomal - sized fragments are characteristics of the occurrence of apoptosis . The morphology of the treated leukemia cells shows apoptotic bodies after 3 days of treatment with 80 μM (Fig. 3B) and 100 μM phytol (Fig. 3C) . As shown in Fig. 4, the amount of oligonucleosomal - sized fragments in the leukemia cells treated with phytol was increased as the concentration of phytol was increased from 80 to 100 μM. On the other hand, the amount of the DNA fragments in the treated cells also was increased as the cultivation time was increased from 1 to 3 days (Fig. 5) . The search for better cancer chemotherapeutic agents is ongoing all over the world . The present study demonstrates that phytol, a natural product prepared from *Lolium multiflorum* Lam. have significant growth inhibitory effects in human lymphoid leukemia cells and provide the first evidence that phytol can induce apoptosis. In our experiment, oligonucleosomal - sized DNA fragmentation resulted from the continuous exposure of leukemia cells to phytol. Yanagihara et al (9)reported that isoflavone

derivatives inhibited the cells growth of a stomach cancer cells line through the activation of a signal transaction pathway for apoptosis. Diomede et al (10) reported that ether-linked glycelo - phospholipids induced apoptotic death in HL - 60, Molt 4B, and U 937 cells. These findings demonstrate that phytol might exert antitumor activity by triggering apoptosis. A strategy to selectively induce apoptosis of tumor cells without altering healthy cells is a major goal for the development of new therapeutic techniques.

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イタリアンライグラス（*Lolium multiflorum* Lam）からのフィトールによるヒト白血病 Molt 4B 細胞のアポトーシス誘導

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要　　旨

イタリアンライグラス（*Lolium multiflorum* Lam）から単離し，MS，^{1}H- NMRや^{13}C-NMRにより構造決定したフィトールはヒト白血病Molt 4B 細胞の増殖を阻害し，且つ，プログラム細胞死（アポトーシス）を誘導した。フィトールで処理したこの細胞の形態を顕微鏡観察したところ，アポトーシス小体が認められた。この処理細胞から抽出したDNA はオリゴヌクレオソームの単位で断片化され，その変化は濃度と時間に依存的であった。

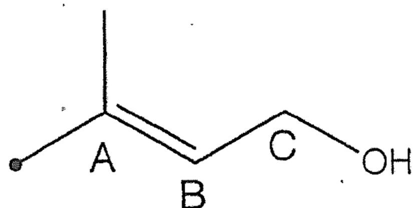

Figure 1. Partial structure of compound I.

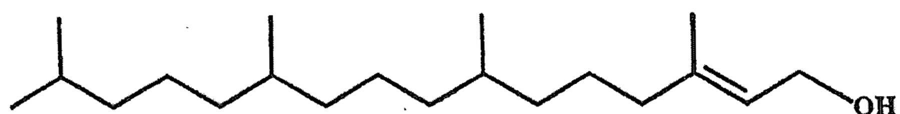

Figure 2. Structure of phytol.

Table I. Effect of phytol on the growth of Molt 4B cells.

Compound	Concentration (μM)	Inhibition (%)
Vehicle	-	-
Phytol	10	9.4
	20	50.8
	40	86.0
	80	98.3
	100	100.0

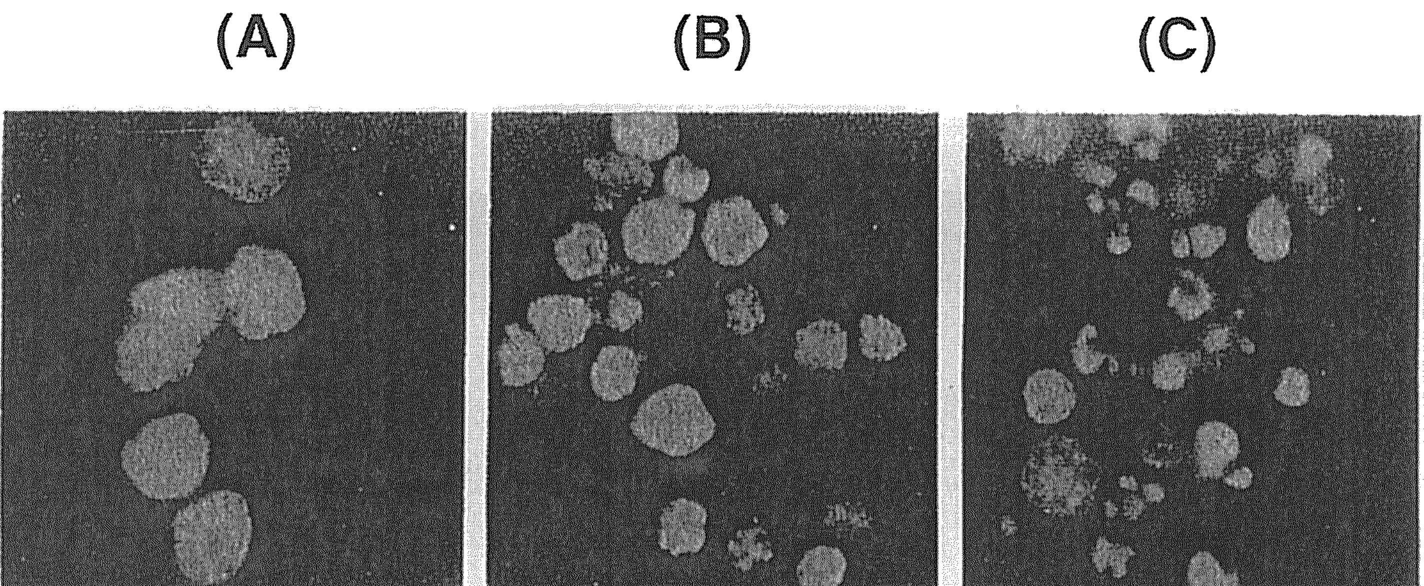

Figure 3. Morphological changes of Molt 4B cells. A, Non-treated Molt 4B cells. B, Molt 4B cells treated with 80 μM phytol. C, Molt 4B cells treated with 100 μM phytol. These cells were cultivated with phytol for 3 days, fixed with 1% glutar-aldehyde, stained with Hoechst 33258, and then observed under an epifluororescence microscope equipped with cooled CCD camera (phometrics, P x L 1400) digital imaging system and Fuji pictography 3000.

(5) (4) (3) (2) (1) M

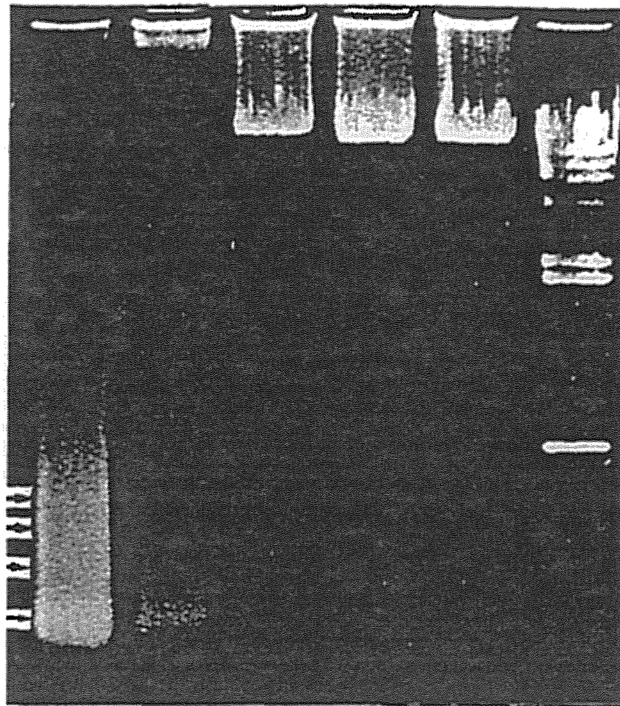

Figure 4. Dose-dependency of DNA fragmentation by phytol in Molt 4B cells. The cells were cultivated in the absence (lane 1) or presence of phytol of 40 μM (lane 2), 60 μM (lane 3), 80 μM (lane 4), and 100 μM (lane 5) for 3 days. After the isolation of DNA from the phytol-treated cells, equivalent amounts of DNA (2 μg) were loaded into wells of 2% agarose gel and electrophoresed in 40 mM Tris-acetic acid, pH 7.5 containing 2 mM EDTA. Arrows indicate DNA ladders. M, λ DNA digested with Hind III.

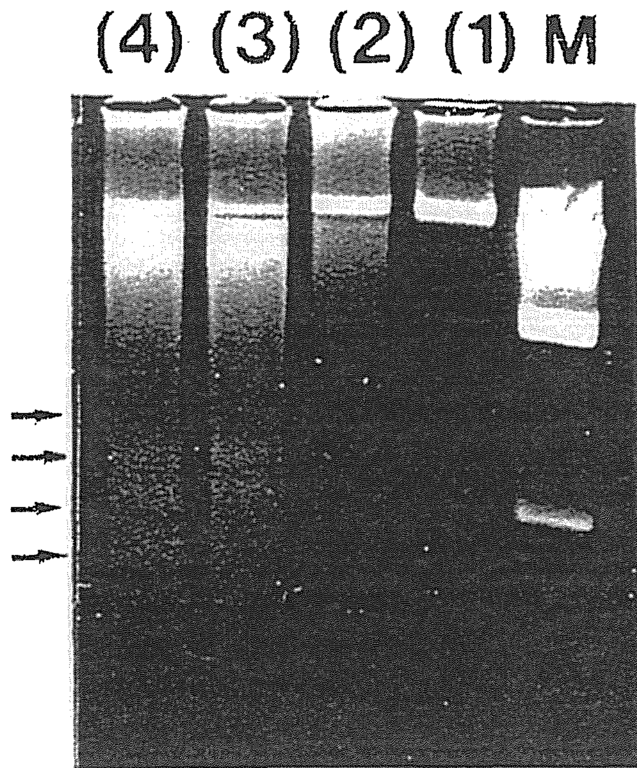

Figure 5. Time-course of DNA fragmentation by phytol in Molt 4B cells. The cells were cultivated in the absence (lane 1), or presence of 100 μM phytol for 1 day (lane 2), 2 days (lane 3), and 3 days (lane 4). After the isolation of DNA from the phytol-treated cells, equivalent amounts of DNA (2 μg) were loaded into wells of 2% agarose gel and electrophoresed in 40 mM Tris-acetic acid, pH 7.5 containing 2 mM EDTA. Arrows indicate DNA ladders. M, λ DNA digested with Hind III.