Sodium 1-monolinolenin isolated 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 sodium 1-monolinolenin (SML) which was isolated from the leaves of Italian ryegrass (*Lolium multiflorum* Lam) and identified by Mass, and ^{1}H- and ^{13}C-NMR, led to both growth inhibition and induction of programmed cell death (apoptosis). Morphological change showing apoptotic bodies was observed in the Molt 4B cells treated with SML. The fragmentation by SML of DNA to oligonucleosomal-sized fragments, that is a characteristic of apoptosis, was observed to be both concentration - and time - dependent. These findings suggest that growth inhibition by SML of Molt 4B cells results 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 compounds by apoptosis. Thus, apoptosis may be a primary mechanism of antineoplastic agents (4). Apoptosis is an orchestrated series of events through which the cell precipitates 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 previous studies we demonstrated induction of apoptosis by phytol (l), sesamin (2) and pheophorbide a (3) in cultured human lymphoid 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 the structure of the compound contained in the fraction. In this report, we demonstrate the identification of sodium 1-monolinolenin(SML) from Italian ryegrass and show the inhibitory effects of SML on the growth of human lymphoid leukemia Molt 4B cells which results from the induction of apoptosis.

Materials and methods

Chemicals. RPMI 1640 medium and phytohemagglutinin-M(PHA-M) were purchased from Gibco Laboratories, Gland Island, NY, USA. Lymphocyte separation medium was purchased from ICN Biomedicals lnc., Aurora, OH, USA. All other chemicals were of the highest grade available from Nacalai Tesque lnc., Kyoto, Japan.

Apparatus. ^{13}C-NMR spectra were taken with JEOL, JNM-500, Tokyo, Japan. Secondary ion mass spectrometry (SIMS) were done using a Hitachi M-80 instrument, Tokyo, Japan. For preparation of SML as described in the next paragraph, high pressure liquid chromatography (HPLC) was performed with a JASCO 880-PU, Tokyo, Japan.

Isolation and identification of SML. The air-dried leaves of *Lolium multiflorum* Lam (400 g), harvested in the experimental farm of Mie University, were soaked in

10 l of 80 % ethanol for 3 weeks at room temperature and the resulting supernatant was evaporated to give 100 g of concentrate. The concentrate was then extracted stepwise with equal volumes (1 1) of distilled water, methanol and acetone. The thus obtained methanol extract (18 g) exhibited a high degree of antiproliferative activity and was suspended in 1 l of 80% methanol and then extracted with l l of n-hexane. The 80%methanol fraction (ca 8 g) exhibited a higher degree of antiproliferative activity than the n-hexane fraction. This 80% methanol fraction was suspended in distilled water and then extracted with ethyl acetate. The ethyl acetate fraction (400 mg) having a higher antiproliferative activity was charged into a HP-20 column, and eluted stepwise with equal volumes (0.5 1) of H_20, 50% methanol, 75% methanol and 100% methanol. The methanol fraction (125 mg) having a comparatively higher antiproliferative activity, was submitted for preparative HPLC under the following conditions: column, Develosil ODS-5 (A) 10 x 250 mm); mobile phase, methanol/H_20=85:15; flow rate 2.3 ml/min; detection wavelength, 210 nm, and then the main peak (compound I, 10.3 mg) having a retention time of 32.6 min was obtained. The structural determination of compound I was carried out, and compound I was assigned to be sodium 1-monolinolenin (SML) by using MS, ^{1}H-NMR, ^{13}C-NMR, DEPT, ^{1}H-^{1}H-COSY and ^{13}C-^{1}H-COSY as follows. ^{1}H-NMR (500 MHz,$CDC1_3$): 8=0.9 (t, 3H, CH_3 - CH_2), 1.2 (m, 2H x 4, CH_2-CH_2-CH_2), 1.6 (m, 2H, OC=OCH_2-CH_2), 2.0 (m, 2H, CH_2-CH =CH), 2.3 (t, 2H, OC=OCH_2), 2.8 (m, 1H x 3, CH=CH-CH_2-CH=CH), 3.6 (dd, 2H, CH_2-OH), 3.9 (m, 1H, CH-OH), 4.2 (dd, 2H, CH_2-OC=O), 5.3 (m, 1Hx 6, vinylic-H). ^{13}C-NMR (90 MHz, $CDC1_3$): δ =14.3, 20.5, 24.9, 25.5, 25.6, 27.2, 29.0, 29.0, 29.1, 29.5, 34.1, 63.2, 65.1, 70.2, 127.1, 127.7, 128.2, 128.3, 130.2, 131.9, 173.0. MS=375.4, ($C_{21}H_{35}NaO_4$).

The chemical structure of SML is shown in Fig. 1.

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

Preparation of human lymphocyte cells. Three ml of lymphocyte separation medium was aseptically transferred toa centrifuge tube and the diluted blood (heparinized blood: physiological saline =1:1) was layered over lymphocyte separation medium in the tube. The tube was centrifuged at 400 x g at room temperature for 20 min. The top layer of the clear plasma was removed, and the lymphocyte layer was transferred to a new centrifuge tube. An equal volume of PBS (−) was added to the lymphocyte layer in the tube and centirfuged for l0 min at room temperature at 260 x g. After the centrifugation, the precipitate lymphocyte was washed again with PBS (-) and suspended in RPMI 1640 medium containing l0% FCS and 2% PHA−M.

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

Microscopic obsevation of morphological change of Molt 4B. Exponentially

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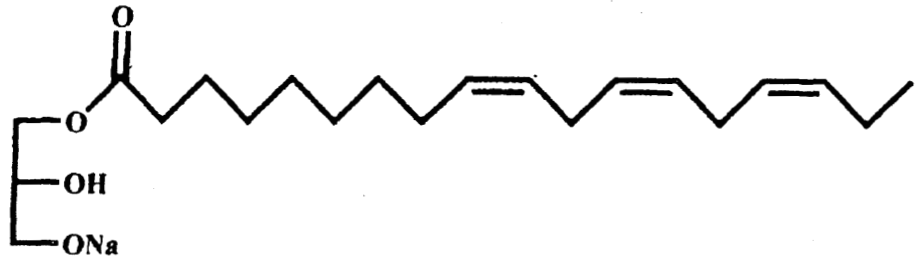

Figure 1. Chemical structure of sodium 1-monolinolenin (SML).

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

Compound	Concentration (μM)	Inhibition (%)
Vehicle	-	-
SML	20	11.6
	40	23.0
	60	39.2
	80	64.0
	100	100.0

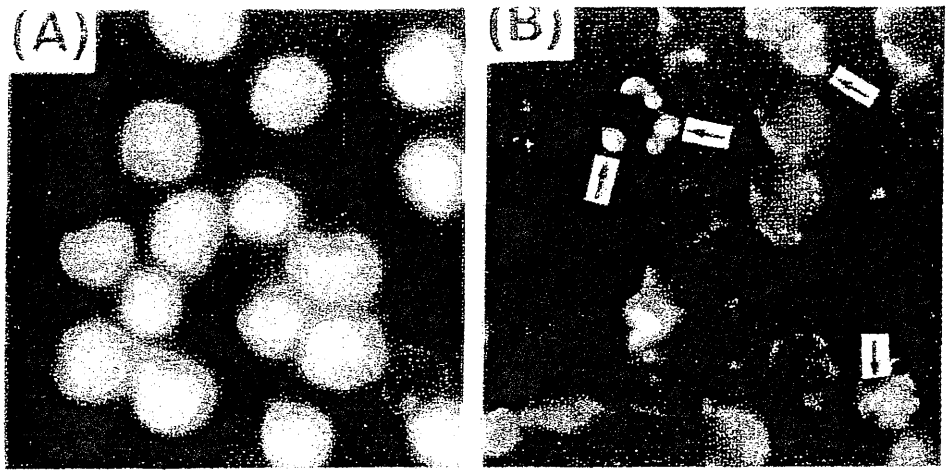

Figure 2. Morphological changes in Molt 4B cells. A, non-treated Molt 4B cells. B, Molt 4B cells treated with 100 μM SML. These cells were cultivated with SML for 3 days, fixed with 1% glutaraldehyde, stained with Hoechst 33258, and then observed under an epifluorescence microscope equipped with a cooled CCD camera (phometrics, P x L 1400) digital imaging system and Fuji pictorography 3000. Arrows indicate apoptotic cells.

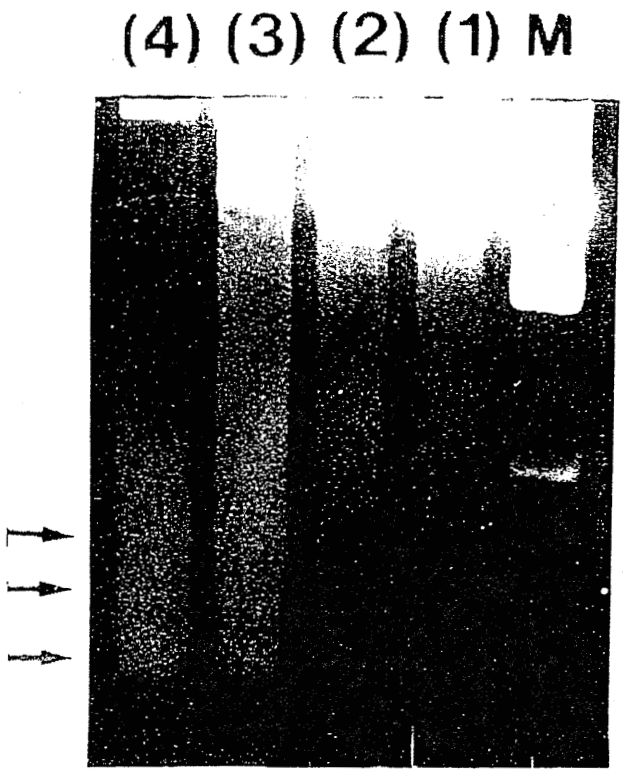

Figure 3. Dose-dependency of DNA fragmentation by SML in Molt 4B cells. The cells were cultivated in the absence (lane 1) or presence of 60 μM (lane 2), 80 μM (lane 3), and 100 μM (lane 4) SML, for 3 days. After the isolation of DNA from the SML-treated cells, equivalent amounts of DNA (2 μg) were loaded 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 *Hin*dIII.

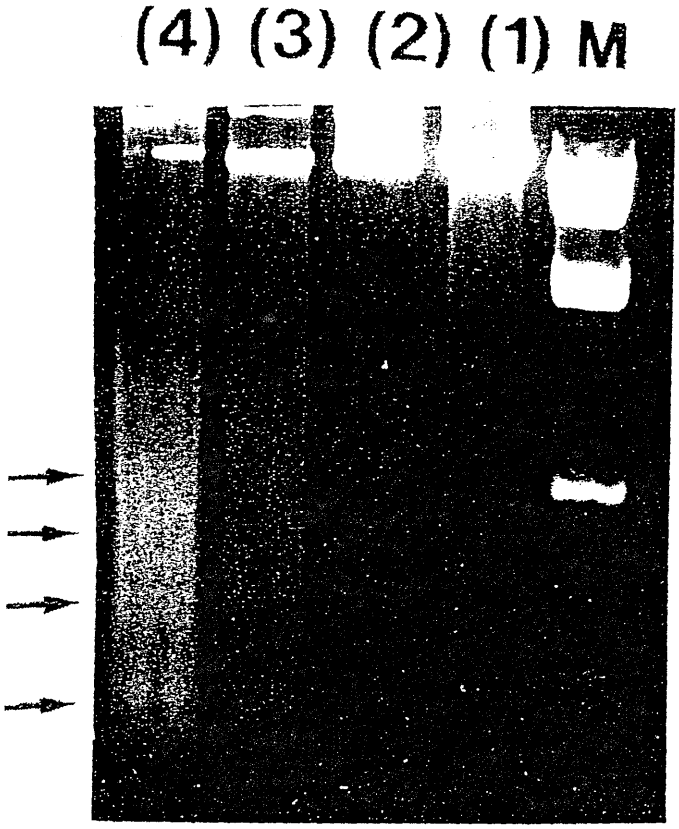

Figure 4. Time-course of DNA fragmentation by SML in Molt 4B cells. The cells were cultivated in the absence (lane 1), or presence of 100 μM SML for 1 day (lane 2), 2 days (lane 3), and 3 days (lane 4). After the isolation of DNA from the SML-treated cells, equivalent amounts of DNA (2 μg) were loaded 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 *Hin*dIII.

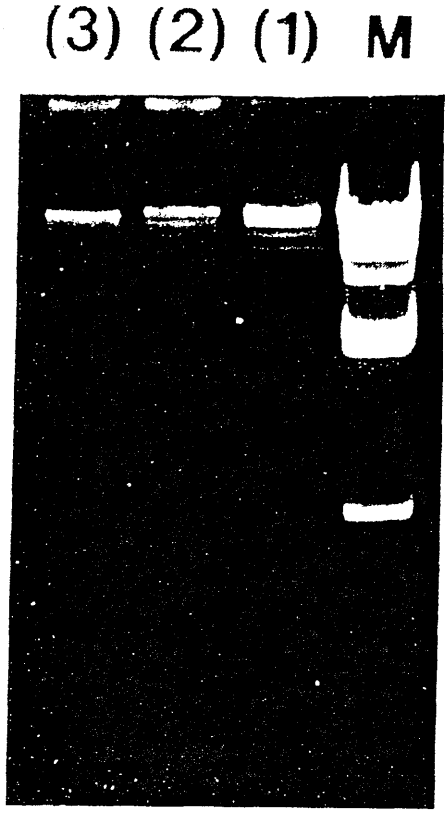

Figure 5. Effect of SML on normal lymphocyte cells. The cells were cultivated in the presence of a vehicle (lane 1), or 100 μM (lane 2) and 150 μM (lane 3) SML for 3 days. After the isolation of DNA from the SML-treated cells, equivalent amounts of DNA (2 μg) were loaded into wells of 2% agarose gel and electrophoresed in 40 mM Tris-acetic acid, pH 7.5 containing 2 mM EDTA. M, λDNA digested with *Hin*dIII.

イタリアンライグラス(*Lolium multiflorum* Lam)から単離したsodium 1- monolinolenin 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sodium1-monolinolenin (SML)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。SML 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。