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Cotylenin A, a Plant-Growth Regulator, Induces the Differentiation in Murine and Human Myeloid Leukemia Cells[1]

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Protein factors playing a significant part in differentiation and development have been recently elucidated. However, low molecular factors which also seem to be essential remain still unknown, although only retinoic acid has become such a candidate. Cotylenins had been isolated as the plant-growth regulators, and have been found to affect a number of physiological processes of higher plants. Here we report that at the concentrations above 12.5μg/ml (20μM) cotylenin A induced the functional and morphological differentiation in murine (M1) and human myeloid leukemia (HL-60) cells. Although cotylenin A has some similarity to PMA both in carbotricyclic diterpene structure and in biological activity (*i.e.* differentiation-induction of HL-60 cells into macrophages), the activation of PKC and the elevation of Ca^{2+}-levels by cotylenin A were not observed. Quite recently it has been reported that fusicoccin (closely related to cotylenin A)-targets are 14-3-3 proteins, which are at the crosspoint of a huge array of signalling and regulatory pathways. These results suggest that cotylenin A might become a useful tool for the elucidation of molecular mechanisms of differentiation and development.

Searching for the low-molecular factors which play an important role in differentiation and development beyond species, we found that cotylenins induce the lysozyme activity in murine myeloid leukemia M1 cells, which is one of the typical differentiation-associated properties (1, 2). Cotylenins had been isolated from the metabolites of a simple eucaryote, a *Cladosporium* sp., as the plant-growth regulators (3-5). As shown in Figure 1, all members of cotylenin group contain the diterpenoid tricarbocyclic skeleton, cotylenol, as a common aglycon (5). They have been found to affect a number of physiological processes of higher plants, *e.g.* a) stimulation of cell enlargement of cotyledons, b) the promotion of seed germination, and c) the opening of stomata (6, 7).

Further studies were performed using cotylenin A and murine and human myeloid leukemia cells. In this paper we report that cotylenin A induces the differentiation in M1 and HL-60 cells.

MATERIALS AND METHODS

Materials. Cotylenins A, C, E and cotylenol were isolated from the metabolites of a *Cladosporium* sp., as described previously (3-5). They were dissolved in ethanol, and diluted to appropriate final concentration in medium.

^{32}Pi and [γ-^{32}P]ATP were from New England Nuclear. Histone H1 was purchased from GIBCO BRL. PMA was from Sigma. PKC was obtained from Calbiochem-Novabiochem. FuraPE3/AM and thapsigargin were from Wako Pure Chem. (Osaka, Japan).

Cells and cell culture. The M1 cell line had been established from a spontaneous myeloid leukemia in an SL strain mouse by Dr. Ichikawa (8), who kindly provided us this cell line. The cells were cultured at 37°C in the presence of 7% carbon dioxide in Eagle's minimum essential medium (Nissui Seiyaku Co., Tokyo, Japan) with a double concentration of amino acids and vitamins and 10% (v/v) heat-inactivated (56°C for 30 min) horse serum (GIBCO). Human leukemia HL-60 cells (9) were cultured in RPMI 1640 medium (GIBCO) with 10% heat-inactivated fetal bovine serum (JRH Bioscience, Lenexd, KS, USA) and 80μg/ml gentamicin (Schering-Plough,

[1] Studies on the differentiation-inducing substances of animal cells. XI. For the previous paper of this series, see ref.(29).

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Abbreviations: NBT, nitroblue tetrazolium; PKC, protein kinase C; PMA, phorbol myristate acetate; SH-PTP1, Src homology 2-containing protein tyrosine phosphatase 1; CA, cotylenin A; TG, thapsigargin; DIF, morphogen of *Dictyostelium discoideum* (differentiation-inducing factor).

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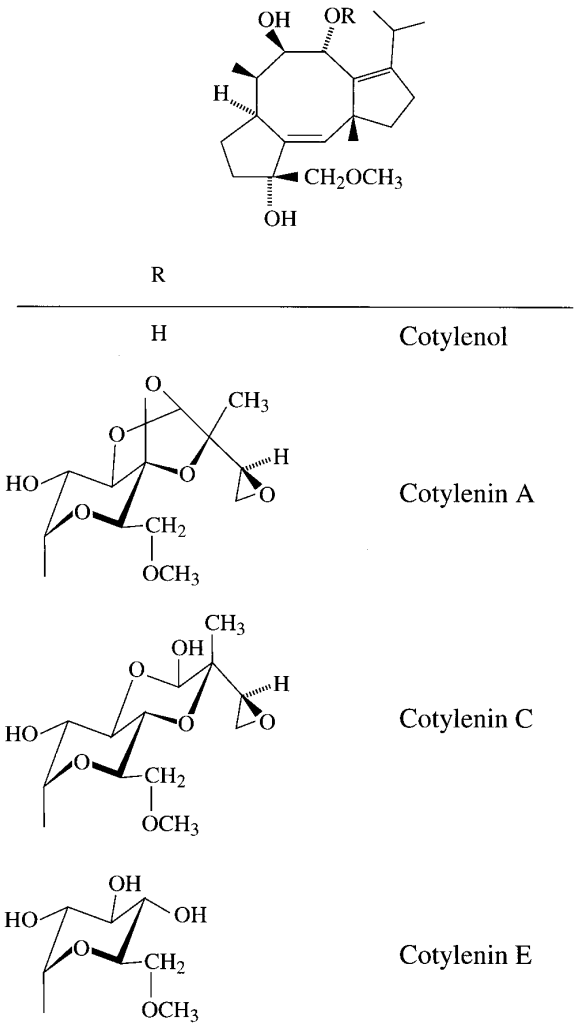

FIG. 1. Molecular structures of cotylenins A, C, E and their common aglycon, cotylenol.

Osaka, Japan) at 37°C in a humidified atmosphere of 5% carbon dioxide in air.

Assays for differentiation. For the assays, the cells (1.0×10^5/ml) were incubated with the medium containing the test samples dissolved in ethanol or ethanol alone as a control for 4 to 6 days. Lysozyme activity was determined by the modified methods of Osserman and Lawlor (10), with lysoplates containing 1% agar (DIFCO), 1/15M sodium phosphate buffer (pH 6.6), 50mM NaCl, and 0.5mg/ml heat-killed *Micrococcus lysodeikticus* (Sigma) (1). Egg white lysozyme (Boehringer-Mannheim) was used as a standard. Phagocytic activity was determined by the methods of Maeda and Ichikawa (11) and Nakayasu *et al.* (12). Nitroblue tetrazolium (NBT) reduction was assayed colorimetrically as described previously (13). Cells were assessed morphologically using a cytospin cytocentrifuge (Shandon Southern, Sewickley, PA, U.S.A.) and cell preparations were stained with May-Grünwald-Giemsa solution (Merck, Darmstadt, Germany). Non-specific esterase activity, using α-naphthyl acetate as a substrate, was determined on cytospin slides in the absence or presence of sodium fluoride as an inhibitor (Sigma assay kit).

Determination of protein kinase C (PKC) activity. Guinea pig neutrophils (5×10^7 cells/ml) were labeled with ^{32}Pi (250 μCi/ml) by incubating at 30°C for 1h in the labeling medium consisting of 10mM HEPES (pH7.4), 136mM NaCl, 4.9mM KCl and 5.5mM glucose. The thus ^{32}P-labeled neutrophils were incubated with cotylenin A or PMA for 10min, and subjected to cell lysis and immunoprecipitation with anti-SH-PTP1 antibody under the ordinary conditions (14). The immunoprecipitates were washed repeatedly, boiled in the sample buffer (1% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.02% bromophenol blue, and 62.5mM Tris-HCl (pH 6.8)), and applied to SDS-PAGE. ^{32}P-Content of the SH-PTP1 spot was determined by a Fuji BAS2000 bioimaging analyzer.

In vitro phosphorylation experiment by PKC was also performed, using PKC from rat brain, [γ-^{32}P]ATP and histone H1 as a substrate, according to the procedure and instructions of Boehringer-Mannheim Biochemica.

Measurement of $[Ca^{2+}]_i$. Cultured cells were collected by centrifugation (1,400rpm, 4min), and incubated (10^6 cells/ml) in a calcium assay buffer (0.1% (w/v) bovine serum albumin, 137.5mM NaCl, 5mM KCl, 2.5mM $CaCl_2$, 0.8mM $MgCl_2$, 5.5mM glucose, 0.6mM $NaHCO_3$, 20mM HEPES (pH 7.2-7.4)) in the presence of FuraPE3/AM (1μg/ml) for 30-40min. Cells were washed with the assay buffer and finally resuspended (10^6 cells/ml) in the same buffer. FuraPE3 fluorometry was carried out at 30°C in a cuvette (2-ml cell suspension per cuvette) as described previously (15).

RESULTS

Four cotylenin-related compounds were tested for their ability to induce the lysozyme activity in murine myeloid leukemia M1 cells (Figure 2). Lysozyme in the growth medium (secreted) was assayed, since about

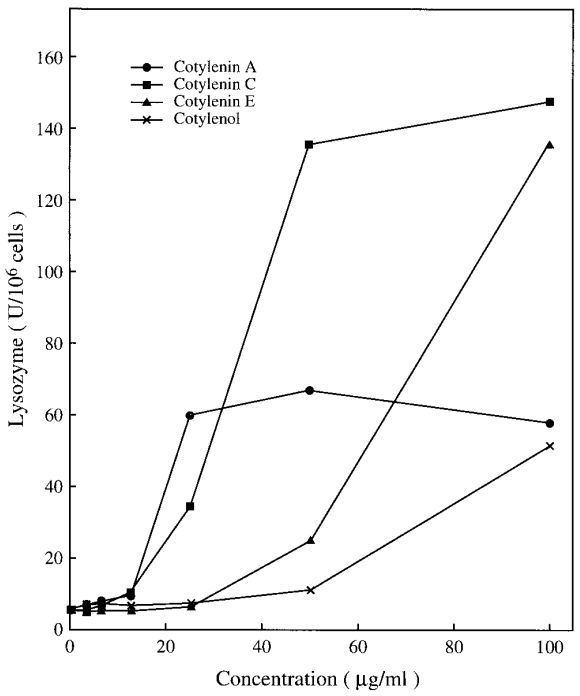

FIG. 2. Effect of cotylenins A, C, E and cotylenol on lysozyme induction. Murine myeloid leukemia M1 cells were cultured with various concentrations of cotylenins for 6 days. Determination of the induced lysozyme activity was carried out as described in Materials and Methods.

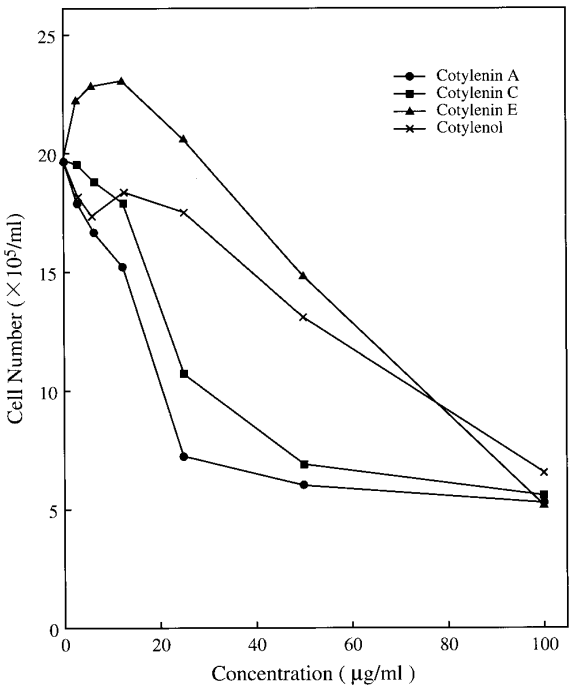

FIG. 3. Effect of cotylenins A, C, E and cotylenol on growth of M1 cells. Cells were cultured with various concentrations of cotylenins for 6 days. For experimental details, see "Materials and Methods".

90% of the induced lysozyme was extracellular (2). Cell growth is shown in Figure 3.

Cotylenins A, C, E and cotylenol induced the lysozyme activity at concentrations above 12.5μg/ml (20μM), 12.5μg/ml (20μM), 50μg/ml (95μM) and 50μg/ml (143μM), respectively. Although even aglycon, cotylenol, itself induced lysozyme, its glycoside-derivatives, cotylenins A, C and E, could induce more strongly. Moreover, the epoxide-ring containing cotylenins (A and C) were effective at lower concentrations than the others. However, there was no correlation between the maximum lysozyme induction by each compound and the existence of the epoxide-ring. Maximum lysozyme activity induced by cotylenin E was higher than that by cotylenin A (Figure 2).

Cotylenin A was used for the further studies, since it induced the differentiation comparatively strongly at the low concentrations and since it could be isolated quantitatively enough to perform the following experiments. After cotylenin A was confirmed to induce the phagocytosis (not shown) as well as the lysozyme activity as the differentiation-markers in murine myeloid leukemia M1 cells, we next examined whether cotylenin A also induces the differentiation in human leukemia HL-60 cells.

The HL-60 cells were cultured with various concentrations of cotylenin A. It suppressed the growth of HL-60 cells in a dose-dependent manner (Figure 4), and induced nitroblue tetrazolium (NBT)-reducing activity, a typical functional marker of the differentiation of HL-60 cells (Figure 5). Furthermore, lysozyme activity, another differentiation marker, and α-naphthyl acetate esterase activity, a typical marker of monocytes and macrophages, were also induced by cotylenin A (Figure 6). Morphological examination indicated that HL-60 cells were induced to differentiate into monocytes-macrophages by cotylenin A (Table 1).

Further, we examined whether cotylenin A activates the protein kinase C (PKC), because cotylenin A has some similarity to phorbol both in molecular structure and in biological activity, *i.e.* differentiation-induction of HL-60 cells into macrophages.

The [^{32}P]orthophosphate-labeled guinea pig neutrophils were incubated with cotylenin A or PMA as a positive control. The intracellular Src homology 2-containing protein tyrosine phosphatase 1 (SH-PTP1) (16) was phosphorylated dose-dependently by PMA, whereas its phosphorylation by cotylenin A was not observed (Figure 7). Another PKC assay was performed using the cell-free system with PKC from rat brain, [γ-^{32}P]ATP and histone H1 as a substrate. Also in this case, similar results were obtained, *i.e.* phosphorylation by PMA was increased dose-dependently, but no

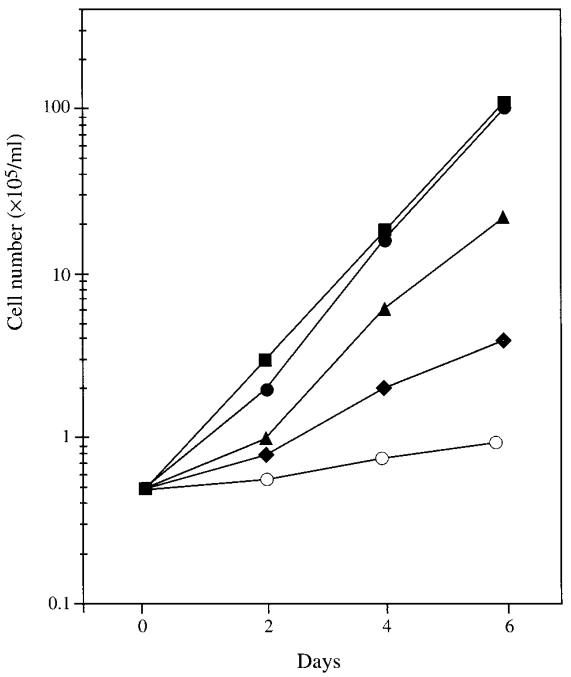

FIG. 4. Effect of cotylenin A on cell growth of human myeloid leukemia HL-60 cells. Cells (5×10^4/ml) were treated with 0 (■), 5 (●), 10 (▲), 20 (♦) or 30 (○) μg/ml of cotylenin A. The density of cells treated with 0 or 5μg/ml of cotylenin A was adjusted at day 4 to 5×10^5 cells/ml, and the cumulative cell numbers were calculated from the cell counts and the dilution used when each culture medium was changed. Each data point represents the mean from four separate experiments.

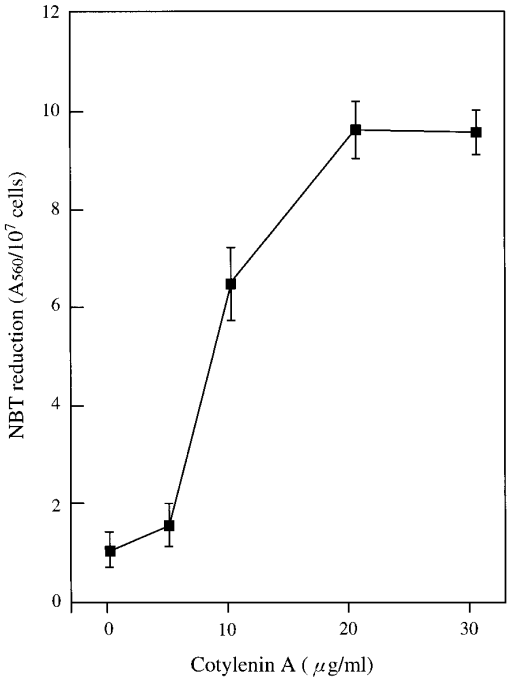

FIG. 5. Effect of cotylenin A on NBT reduction in HL-60 cells. Cells were treated with various concentrations of cotylenin A for 4 days. Data represent the means ± S.D. from three separate experiments.

phosphorylation by cotylenin A occurred (data not shown).

We next examined the intracellular free calcium concentration ($[Ca^{2+}]_i$, Figure 8). Thapsigargin (TG, 1μM), which inhibits the Ca^{2+}-ATPase specifically, raised $[Ca^{2+}]_i$ in HL-60 cells (*upper panel*), and DIF (20μM), the morphogen of *Dictyostelium discoideum* (see "Discussion"), also raised $[Ca^{2+}]_i$ in rat pancreatic AR42J cells, *i.e.* transient surge was observed followed by the stationary level, which was still higher than the basal and continued for more than 15min (*lower panel*), as described previously (15). However, cotylenin A (CA,

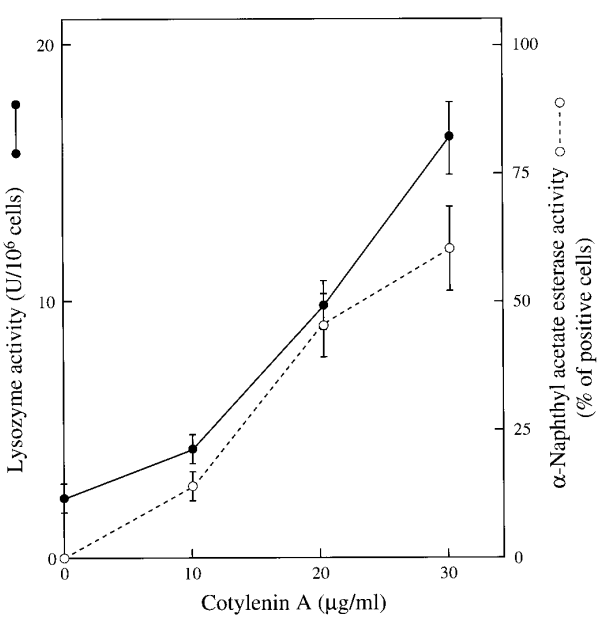

FIG. 6. Induction of lysozyme and α-naphthyl acetate esterase activities by cotylenin A. HL-60 cells were treated with various concentrations of cotylenin A for 6 days. The induced enzyme activities were determined as described in Materials and Methods. Values are means ± S.D. from three separate experiments.

TABLE 1

Effect of Cotylenin A on Morphological Differentiation of HL-60 Cells

Cotylenin A (μg/ml)	Morphological changes (%)				
	Pro	Mye	Neu	Mono	Mac
0	98±2	2±1	0	0	0
10	65±4	14±2	3±1	18±3	0
20	14±2	27±4	3±1	44±6	12±2
30	2±1	16±2	3±1	25±4	54±7

Note. Cells were treated with cotylenin A for 6 days. Values are means ± S.D. from three separate experiments. Pro : promyelocytes, Mye : myelocytes, Neu : metamyelocytes and mature granulocytes, Mono : monocytes, Mac : macrophages.

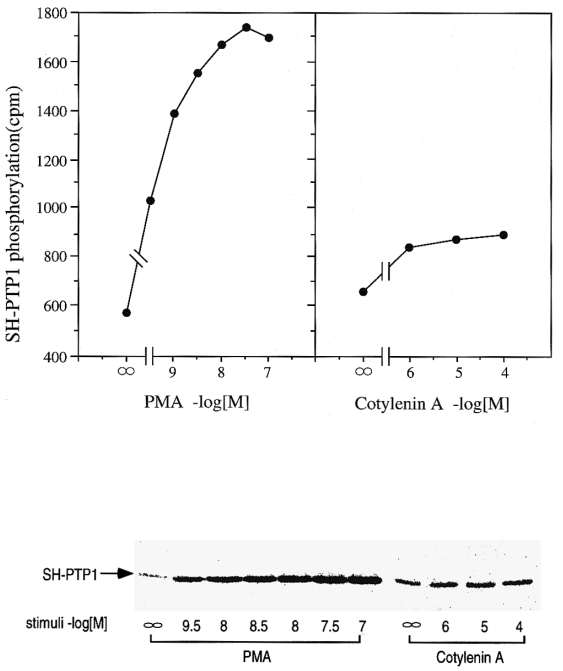

FIG. 7. Effect of cotylenin A on phosphorylation of the intracellular PKC substrate. [^{32}P]Orthophosphate-labeled neutrophils were incubated with various concentrations of cotylenin A or PMA as a positive control, and SH-PTP1 was immunoprecipitated and subjected to SDS-PAGE and autoradiography as described in Materials and Methods. In the lower panel, ^{32}P-content in the SH-PTP1 spots was visualized by a bioimaging analyzer. Similar results were obtained in additional two experiments.

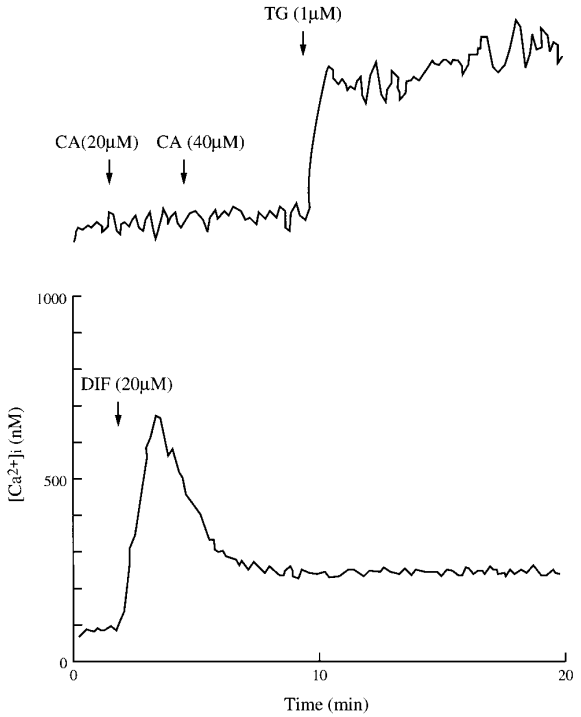

FIG. 8. Effect of cotylenin A on the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Cotylenin A (CA, 20 and 40μM) and thapsigargin (TG, 1μM) were added where indicated. $[Ca^{2+}]_i$ in HL-60 cells was measured as described in Materials and Methods (*upper panel*). When DIF (20μM, see "Discussion") as another positive control was added to rat pancreatic AR42J cells, $[Ca^{2+}]_i$ shown in the *lower panel* was obtained, as described previously (15).

20 and 40μM) did not increase $[Ca^{2+}]_i$ in HL-60 cells at all (*upper panel*).

DISCUSSION

Protein factors playing a significant role in differentiation and development, *e.g.* proteins which cause the induction-chains in early embryogenesis or induce the endoderm and mesoderm have been recently rapidly elucidated, since some pioneer works (17-23) appeared. However, low-molecular factors which might also play an important part in these procedures remain almost unknown, although only retinoic acid is such a candidate.

Previously we isolated and structurally elucidated differanisole A as the differentiation-inducer of murine and human undifferentiated tumor cells (24-26). Three years later, in 1987, Morris *et al.* determined the chemical structure of DIF as the morphogen of cellular slime mold, *Dictyostelium discoideum* (27). Both structures have close similarity. Moreover, we confirmed the cross-activities between differanisole A and DIF (28,29). These results suggest that the molecular structure(s) represented by DIF and differanisole A might play an important role in differentiation and development beyond species. In addition, we found recently that differanisole A enhances synergistically the growth inhibition and differentiation of human myeloid leukemia cells induced by 9-*cis*-retinoic acid (Kanatani, Y. *et al.*, submitted for publication).

In this study we found that cotylenin A which had been isolated as the plant-growth regulator and whose structure is quite different from those of differanisole A and DIF induces the differentiation in murine myeloid leukemia M1 cells and in human leukemia HL-60 cells.

Although cotylenin A has some similarity to PMA both in carbotricyclic diterpene structure and in biological activity (*i.e.* differentiation-induction of HL-60 cells into macrophages), the activation of PKC and the elevation of $[Ca^{2+}]_i$ by cotylenin A were not observed. Hence the studies on other signalling pathways and/or (nuclear) receptors have to be performed.

Quite recently it has been reported that the receptors of fusicoccin (closely related to cotylenin A) are 14-3-3 proteins, which are widely distributed from yeast to human and regulate the signalling pathways triggered and mediated by protein kinases (30).

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