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Oleanolic Acid Induces Apoptosis in Human Leukemia Cells through Caspase Activation and Poly(ADP-ribose) Polymerase Cleavage

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Abstract It has been shown that *Fructus Ligustri Lucidi* (FLL), a promising traditional Chinese medicine, can inhibit the growth of tumors. However, the effective component and molecular mechanism of FLL act to inhibit tumor proliferation are unclear. In this study, we demonstrated that oleanolic acid (OA), a principal chemical component of FLL, inhibited the proliferation of human leukemia HL60 cells in culture. MTT assay showed that treatment of HL60 cells with FLL crude extracts or OA dramatically blocked the growth of target tumor cell in a time- and dose-dependent manner. Morphological changes of the nuclei and DNA fragmentation showed that apoptotic cell death occurred in the HL60 cells after treating with FLL extracts (20 mg/ml) or OA (3.65×10^{-2} mg/ml). Furthermore, flow cytometry assay showed that treatment of HL60 cells with FLL or OA caused an increased accumulation of G_1 and sub-G_1 subpopulations. Western blot analysis showed that caspase-9 and caspase-3 were activated, accompanied by the cleavage of poly (ADP-ribose) polymerase (PARP) in the target cells during FLL- or OA-induced apoptosis. These results suggest that OA acts as the effective component of FLL by exerting its cytotoxicity towards target tumor cells through activation of caspases and cleavage of PARP.

Keywords oleanolic acid; apoptosis; caspase-3; PARP; HL60 cells

The current primary treatment for leukemia is anticancer drug-based chemotherapy that uses one or more drugs to destroy cancer cells. All-trans retinoic acid (ATRA) acting as a differentiation therapy, arsenic trioxide (As_2O_3) acting as a target therapy, and a combination of ATRA and As_2O_3 as well as bone marrow transplant or umbilical cord blood transplantation have shown great progress for the treatment of acute promyelocytic leukemia. However, long-term use of ATRA and AS_2O_3 will lead to drug resistance of the cancer cells causing future treatments on relapsed patients to be ineffective. There are other chemotherapy medicines, such as etoposide, that are very effective in obtaining high clinical complete remission rates in acute promyelocytic leukemia. However, the use of such drugs in treatment might cause chromosome translocation [1,2]. Therefore, the development of antitumor drugs is of great interest both for potential practical uses, such as cancer chemotherapy, and for the understanding of the mechanisms of tumor development.

Suppression of apoptosis might contribute to tumor development by means of accumulation of continuously proliferating cells, and the disruption or elimination of genetically altered cells might decrease tumor potential [3–5]. Thus, the strategy of therapeutic intervention of cancers is to induce apoptosis in the tumor cells [5]. *Fructus Ligustri Lucidi* (FLL) is the dried ripe fruit of *Ligustrum lucidum* Ait. FLL was found to be enriched with oleanolic acid (3β-hydroxy-olea-12-en-28-oic acid; OA), ursolic acid (UA), acetyloleanolic acid, acetylursolic acid, oleic acid, linoleic acid and others, which have the ability to improve

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the stamina, antihypoxia and immunologic function of the body. Recent studies of chemotherapeutic properties have shown that oleanane-type pentacyclic triterpenoids including native OA and its synthetic analogs have antitumor effects [6–16] both *in vivo* and *in vitro*. More and more evidence has proven that there is indeed antitumor activity in triterpenoid derivatives *in vitro* and *in vivo*, but the actual mechanism of how this occurs is still unclear. Because the decoction of FLL is a mixture, OA and UA have structural similarilies, it was necessary to identify the active component. Pure OA ($C_{30}H_{48}O_3$, 456.71) and FLL (containing 14% OA, 6.9% UA) were used as materials in the experiments.

The study was designed to show the mechanism of leukemic cell apoptosis induced by OA and to give an experimental basis for the clinical application of OA.

Materials and Methods

Cell culture and agent treatment

The human myeloid leukemia cell line HL60 was obtained from American Type Culture Collection (Rockville, USA). The cells (37 ºC, humidified, 5% CO_2) were cultured in 90% RPMI-1640 and 10% fetal bovine serum (Gibco BRL, Gaithersburg, USA), supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin. For examining inhibitory proliferation of OA, 1.5×10^6 HL60 cells were incubated with OA (National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China) at the final concentrations of 0, 1.83×10^{-2}, 2.74×10^{-2}, 3.65×10^{-2}, or 4.57×10^{-2} mg/ml for 0, 12, 24, 48, or 72 h.

Decoction of FLL

Fifty grams of dried FLL (Xinxing County, Guangzhou, China) was rinsed and put into a ceramic pot with a snug-fitting lid. The herbs were covered with approximately 300 ml purified water, and soaked for 30 min. The pot was heated to boil and the mixture was simmered for 15 min. The herbs were decocted twice, each time the herbs were boiled down to an infusion of approximately 100 ml. After the second decocting, the herbs were discarded and the resulting liquids were combined and condensed to 100 ml in an 80 ºC water bath, followed by filtration with a Millipore filter (4.5 μm; Millipore Corp., Bedford, USA). Thus, the decoction was equal to 50 mg/ml FLL herbs.

Cell viability analysis

The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay was carried out using a modified method described by Li *et al.* [17]. Briefly, 5×10^4 cells were seeded in each well containing 100 ml RPMI-1640 medium supplemented with 10% fetal bovine serum in a 96-well plate. After incubation for 24 h, OA were added to the culture to final concentrations of 0, 1.83×10^{-2}, 2.74×10^{-2}, 3.65×10^{-2}, or 4.57×10^{-2} mg/ml. After incubation for 48 h, 50 μl MTT (Sigma-Aldrich, St. Louis, USA) (5 mg/ml stock solution) was added and incubated at 37 ºC for another 4 h. Then 0.2 ml of DMSO was added to stop the reactions. The absorbance of each well was determined spectrophotometrically at 490 nm by a microplate reader (Bio-Tek, Rockville, USA).

Morphological analysis

The cells were washed twice with phosphate-buffered saline (PBS; pH 7.4) after being treated with OA for 0, 12, 24, 48, or 72 h, then placed onto sterile cover slides with a Cytospin (Thermo Fisher Scientific Inc., Waltham, USA). The cells were fixed by 4% paraformaldehyde and stained by 6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI) The morphology of the cells was examined using a fluorescent microscope (Leica DMIL, Solms, Germany).

DNA fragmentation analysis

The cells (1.5×10^6) were incubated with OA at the final concentrations of 0, 1.83×10^{-2}, 2.74×10^{-2}, 3.65×10^{-2}, or 4.57×10^{-2} mg/ml for 0, 12, 24, 48, or 72 h. The cells were collected and washed twice with PBS. The cells were lysed with the digestion buffer (pH 7.4) containing 0.2% sodium dodecyl sulfate (SDS), 100 mM Tris-HCl, 0.5 mg/ml proteinase K, and 5 mM EDTA at 37 °C, overnight. After extraction of cell lysates with phenol-chloroform (1:1) and chloroform, DNA was precipitated with 3 M sodium acetate (pH 5.2) and absolute ethanol, and was washed, dried, and resuspended in Tris-EDTA buffer containing 1 μl RNase A (0.5 mg/ml; Sigma-Aldrich) at 37 °C for 1 h. Approximately 15 μg DNA was loaded on a 1.5% agarose gel. After electrophoresis, the gel was visualized with ethidium bromide staining under ultraviolet light.

Analysis of caspase-3, caspase-9 and poly(ADP-ribose) polymerase (PARP) cleavage

The HL60 cells were treated with OA as described in the DNA fragmentation experiments. At indicated times, the cells were washed with PBS and lysed in 50 mM Tris-HCl (pH 7.6) containing 250 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, 1% NP-40 and 50 mM NaF. The cell lysates were centrifuged at 10,000 *g*

for 15 min at 4 ºC, and the protein content of the cytosolic fraction in supernatant was measured by a modified Bradford assay kit (Bio-Rad, San Diego, USA). The cytosolic fraction (50 μg protein) was mixed with 2 volumes of sample buffer [0.1M Tris-HCl (pH 6.8), 200 mM dithiothreitol, 4% SDS, 25 mM EDTA, 20% glycerol, and 0.2% bromophenol blue]. The mixture was boiled at 95 ºC for 5 min, then loaded to a 15% SDS-polyacrylamide gel. Following electrophoresis, the proteins on the gel were electro-transferred onto a nitrocellulose membrane (GE Healthcare, Arlington Heights, USA) with transfer buffer composed of 48 mM Tris-HCl (pH 8.3), 39 mM glycine, and 20% methanol. The membrane was blocked with the blocking solution containing 20 mM Tris-HCl (pH 7.4), 140 mM NaCl, 0.1% Tween-20, 2.7 mM KCl, 1.0 mM Na_2HPO_4, and 1.8 mM KH_2PO_4. The blots were probed with goat anti-human PARP, rabbit anti-human actin (Santa Cruz Biotechnology Inc., Santa Cruz, USA) and goat anti-human caspase-3 and caspase-9 antibodies (Merck, Darmstadt, Germany). The immune complexes were detected by anti-rabbit and anti-goat horseradish peroxidase-conjugated secondary immunoglobulin G antibody, respectively (Santa Cruz Biotechnology Inc.), and were visualized using electrochemiluminescence Western blotting detection reagents (GE Healthcare).

Cell cycle distribution

Cells were harvested and washed twice with cold PBS, then cell pellets were suspended in 200 μl of DAPI solution [containing 10 μg/ml DAPI, 0.1% (*W*/*V*) sodium citrate, and 0.1% RNase]. Cell samples were incubated at 4 ºC in darkness for at least 30 min then analyzed by a flow cytometer (FACSCalibur, Becton Dickinson, Sunnyvale, USA) and CellQuest software (Becton Dickinson).

Statistical analysis

All data were expressed as the mean±SEM. The significance of differences between mean values was analyzed by repeated-measure ANOVA followed by the post-hoc Tukey test. A probability value of $P<0.05$ was considered to be statistically significant. All data were analyzed with SPSS 11.0 for Windows (SPSS, Chicago, USA).

Results

Cell growth inhibition

As revealed by the time and dose effect curves, OA ($\geq 1.83\times10^{-2}$ mg/ml) caused significant growth inhibition of HL60 cells within 12–72 h after the agent treatment. There were significant differences between the OA-treated group and the control group [**Fig. 1(A)**]. Because OA is based on the enriched extracts of the Chinese herb FLL [18], we also assessed the abilities of the crude extract to inhibit the growth of leukemia cells [**Fig. 1(B)**] and showed that the inhibition ability of the crude extract was similar to that of the pure agent.

Changes in cell morphology

To determine whether the inhibition of cell growth by OA was related to apoptotic cell death, morphological analysis of the DAPI-stained HL60 cells was carried out and the results showed that the targeted cells underwent gross morphological changes. After OA (3.65×10^{-2} mg/ml) treatment for 12, 24, 48, and 72 h, the cells showed typical

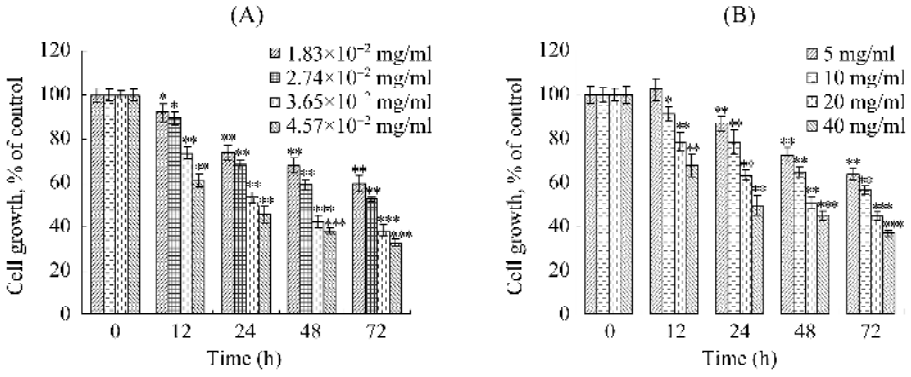

Fig. 1 Effects of oleanolic acid (OA) and crude extracts of *Fructus Ligustri Lucidi* (FLL) on human myeloid leukemia HL60 cell proliferation

The HL60 cells were treated with OA (A) or crude extracts of FLL (B) at the indicated concentrations for 0, 12, 24, 48, or 72 h. The cell proliferation was evaluated with MTT assay. The data represent mean±SEM derived from three independent experiments. *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared with control.

apoptotic changes, including cell shrinkage, chromatin condensation, and loss of normal nuclear architecture. The apoptotic indexes at 12, 24, 48, and 72 h were 26.24%±2.53%, 47.13%±2.45%, 52.71%±2.75%, and 56.71%±2.46%, respectively [**Fig. 2(A)**], showing significant differences (P<0.05) compared with those of the controls. After FLL treatment for 12, 24, 48, and 72 h, the apoptotic indexes were 21.27±4.40%, 36.54%±2.47%, 45.35%±2.69%, and 53.17%±1.53%, respectively [**Fig. 2(B)**].

DNA fragmentation

Because the presence of the genomic DNA ladder has been used extensively as a marker for apoptotic cell death, the next step was to examine DNA fragmentation using agarose gel electrophoresis. As shown in **Fig. 3**, the DNA ladders became apparent after treatment for 48 h and became stronger as more treatment time elapsed. These results were consistent with the observation of morphological changes [**Fig. 2(A)**].

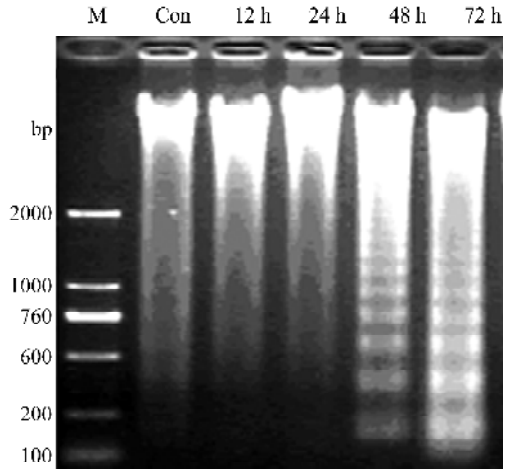

Fig. 3 Analysis of DNA fragmentation in human myeloid leukemia HL60 cells treated with oleanolic acid (OA)

The cells were treated with 3.65×10^{-2} mg/ml OA for 12, 24, 48, or 72 h, and stained with DAPI. The genomic DNA from the treated cells was extracted and loaded onto an agarose gel (1.5%). After electrophoresis, the gel was visualized with ethidium bromide staining under ultraviolet light. Con, 0 h; M, DNA marker.

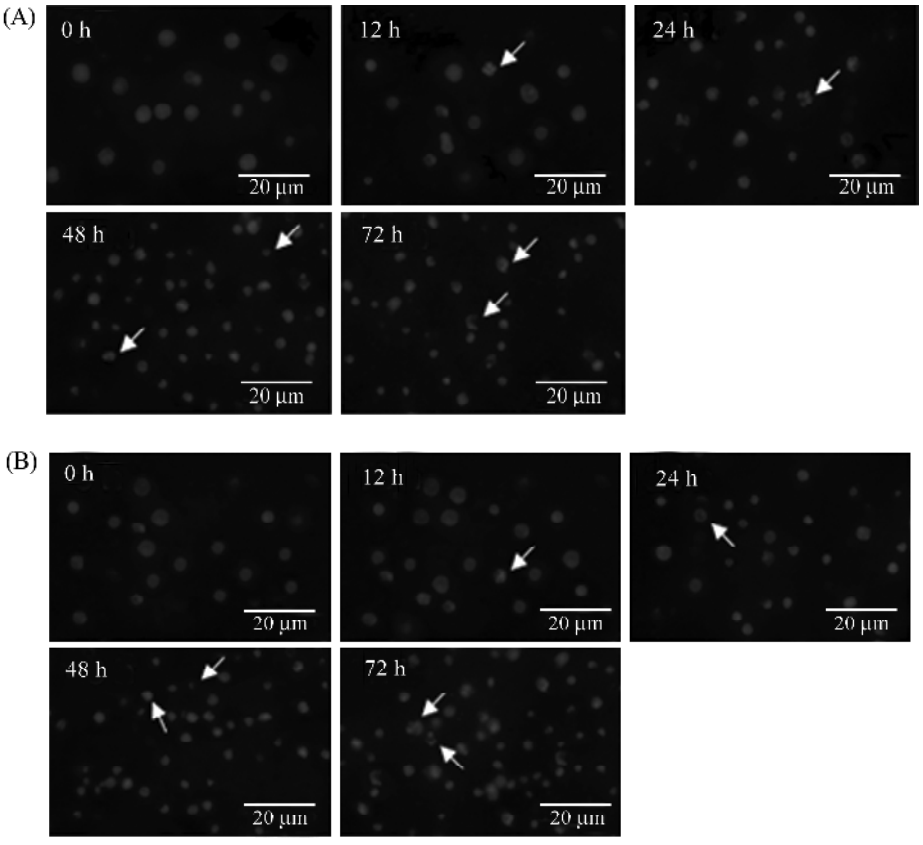

Fig. 2 Changes in nuclear morphology of human myeloid leukemia HL60 cells treated with oleanolic acid (OA) or crude extracts of *Fructus Ligustri Lucidi* (FLL)

The cells were treated with OA (3.65×10^{-2} mg/ml) (A) or crude extracts (20 mg/ml) of FLL (B) for 0, 12, 24, 48, or 72 h. The cells were fixed and stained with DAPI and examined under a fluorescent microscope. The arrows indicate apoptotic fragmented cells.

Increased G_1 and sub-G_1 populations

To show in more detail that the inhibition of cell growth by OA is closely related to cell-cycle control and apoptosis, the cell-cycle distribution of the OA-treated tumor cells was analyzed with a flow cytometer. **Fig. 4(A)** shows that treatment of HL60 cells by OA (3.65×10^{-2} mg/ml) caused an increase from 42.70%±6.30% to 69.91%±6.91% of the G_1 subpopulation within 12–72 h, compared with the untreated cells that showed 36.18%±3.45% in the G_1 subpopulation. There were significant differences between the OA-treated group and the control group after the same duration ($P<0.05$). In the HL60 cells treated with the crude extracts of FLL, the increased accumulation of the G_1 phase was from 50.05%±9.31% to 67.90%±2.73%, compared with 41.20%±12.22% of G_1 in untreated cells. There were also significant differences between the FLL-treated group and the control group after the same duration ($P<0.05$). Both treatments showed a time-dependent increase in the G_1 subpopulation within 24–72 h. The treated HL60 cells increased sub-G_1 DNA content characterizing apoptosis within 48–72 h after treatment [**Fig. 4(B)**].

Activation of caspases and cleavage of its substrate

As caspases are believed to be the central executioners of the apoptotic pathway and caspases selectively cleave target proteins [3,19], we examined the activation of caspase-9 and caspase-3, and the cleavage of caspase-3 substrate, PARP, by Western blot analysis with specific antibodies. In treated HL60 cells, the quantity of procaspase-9 (46 kDa) decreased, and proteolytic cleavage of procaspase-3 (36 kDa) resulted in the formation of the 20 kDa and 17 kDa subunits within 12–72 h. **Fig. 5** showed that complementing the activation of caspase-3, a p85 fragment was yielded by cleavage of PARP (p115) after 12–72 h exposure. These results indicated that OA-

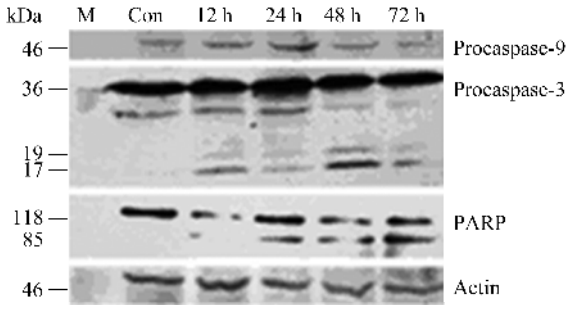

Fig. 5 Western blot analysis of caspase activation and poly (ADP-ribose) polymerase (PARP) cleavage in human myeloid leukemia HL60 cells treated with oleanolic acid (OA)

The HL60 cells were treated with 3.65×10^{-2} mg/ml OA for 12, 24, 48, or 72 h, and stained with DAPI. Cell extracts were prepared and Western blot analyses were carried out for caspase-9, caspase-3, and PARP with specific antibodies (goat anti-mouse PARP, rabbit anti-goat actin and goat anti-rabbit caspase-3 and caspase-9 antibodies). Fifty micrograms of protein was loaded for each lane, and the antigen-antibody complex was visualized by chemiluminescence. Con, 0 h; M, protein marker.

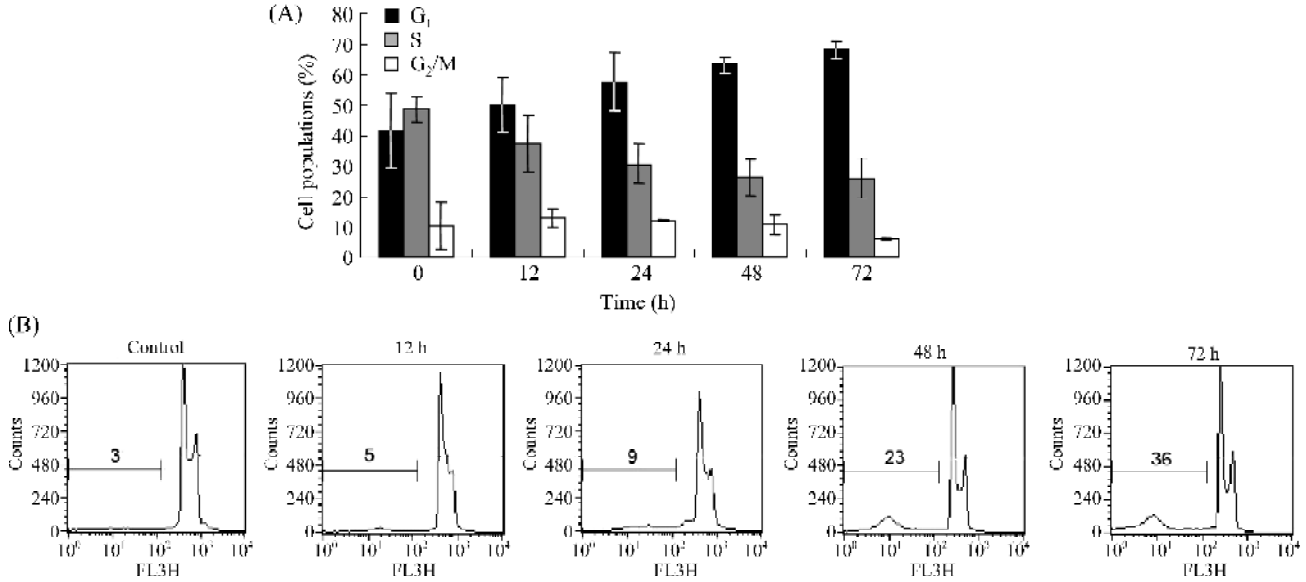

Fig. 4 Flow cytometric detection of apoptosis in human myeloid leukemia HL60 cells treated with oleanolic acid (OA)

The HL60 cells were exposed to 3.65×10^{-2} mg/ml OA for 0, 12, 24, 48, or 72 h, and stained with DAPI. (A) Histograms show the populations in the G_1, S, and G_2/M phases of the cell cycle. (B) Apoptosis is revealed by sub-G_1 (<2N ploidy) cell population. The percentage of apoptotic cells is indicated by the number within each figure, and the duration of OA treatment is indicated above each figure.

induced apoptosis proceeds through caspase activation.

Discussion

This study showed that the inhibitory effect of OA, the principal chemical component of FLL, on tumor proliferation is mainly due to the accumulation of cells in the G_1 phase and apoptotic cell death dependent of caspase activation and PARP cleavage.

FLL, a promising traditional Chinese medicine, can inhibit the growth of tumors [18]. It is well established that the rind of FLL fruit is enriched with OA, acetyl-OA and UA, and that the OA content in FLL reaches 14%. It is, therefore, considered that OA as well as UA contributes to the therapeutic effects of FLL. OA, as an inhibitor of tumor promotion by 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced Epstein-Barr virus activation *in vivo*, was first identified from an anti-inflammatory Chinese herb, *Glechoma hederaceae* L., by Ohigashi and his colleagues [6]. Recently, several lines of evidence have shown that OA and its derivatives have marked antitumor effects and have apparent cytotoxic activity towards many cancer cells *in vivo* and *in vitro* [8,16–22]. Although more effectiveness of the modified synthetic derivatives of OA is reported, we did find that natural OA also shows a significant inhibitory effect on tumor cell proliferation, which is consistent with previous findings [6,7,9,10,13,15].

The chemopreventive effects of OA and UA on tumor promotion by TPA *in vivo* suggest that their inhibitory activities might be caused by some event occurring after the binding of TPA to the receptor in the cells [6]. The examination of the interactions of triterpenoids with kinases in cellular signaling pathways shows that these compounds including OA and UA can selectively inhibit cyclic AMP-dependent protein kinase and phospholipid-dependent protein kinase C [20]. The inhibition of cyclic AMP-dependent protein kinase and phospholipid-dependent protein kinase C might explain the chemoprevention of triterpenoids against tumorigenesis promoted by TPA-induced Epstein-Barr virus activation [6,14], but it does not provide an explanation for apoptosis. Furthermore, OA and its derivatives contribute to antitumor effects by inhibition of DNA topoisomerase I [15] and multidrug resistance-associated protein (MRP1) [16] activities, and angiogenesis [8]. Therefore, the mechanisms of the inhibitory action of triterpenoid against tumors might be complicated, and not easily understood. Both synthetic and natural betulinic acid acting as a triterpene can induce apoptosis of neuroectodermal tumors [8,21,22] and erythroleukemia cell line K562 [13]. Together with previous observations and our data, we suggest that different pentacyclic triperpenes might have different antiproliferative and cytotoxic effects, depending on cell lines. We estimated the proliferation of HL60 treated with FLL or OA by MTT assay and found that both of them can inhibit cancer cell multiplication. The inhibition is time- and dose-dependent, but the effectiveness of OA is better than that of FLL. The lower OA content in decoction of FLL is the most likely explanation for this difference. We observed the effect of OA directly in the successive experiment. In order to clarify the cause of the inhibition of HL60 proliferation, we applied the fluorescent staining method. Karyopyknosis, karyorrhexis, karyolysis, and apoptotic bodies were induced in HL60 cells by OA. The ladder-shaped strand on the agarose gel after electrophoresis confirmed that the method of cell death was apoptosis.

It is well established that apoptosis can occur by either the death-receptor pathway or the mitochondrial pathway. Both pathways are executed by cysteine proteases (caspases) that are activated specifically in apoptotic cells [3,4]. The death-receptor pathway is triggered by members of the death-receptor superfamily, and involves caspase-8 activation [3]. The mitochondrial pathway is mobilized in response to extracellular cues and internal insults such as DNA damage, often through the activation of a pro-apoptotic member of the Bcl-2 family such as Bax or Bid, followed by the alteration of mitochondrial membrane permeability and the release of mitochondrial cytochrome *c* into the cytosol. Cytochrome *c* associates with Apaf-1 then procaspase-9 to form the apoptosome. The death-receptor and mitochondrial pathways converge at the level of caspase-3 activation which is followed by caspase-8 activation or apoptosome formation [3]. With the purpose of identifying the apoptotic pathway that causes the apoptosis of HL60 induced by OA, we determined the activation mechanism of procaspase-8 and procaspase-3, as well as that of the cleavage of PARP by caspase-3, with protein immunoblotting. Procaspase-3 (36 kDa) in OA-treated HL60 cells was cleaved into two fragments of 19 kDa and 17 kDa. The precursor of PARP (118 kDa) was cleaved into two fragments of 85 kDa and 25 kDa (**Fig. 5**). The content of the fragments increased with the prolongation of the OA treatment time. There were no such fragments in the control group. The results indicate that OA can induce apoptosis of HL60 cells by the death-receptor pathway. The activation mechanism of procaspase-9 was also discovered. Although caspase-9 is involved in the mitochondrial pathway, it is a necessary checkpoint to determine whether or not OA can induce

apoptosis by the mitochondrial pathway.

It was shown that the G_1 checkpoint is the key point that can affect the progress of the cell cycle. The transition that occurs at the restriction point (R) in G_1 commits the cell to the S phase, differentiation or apoptosis. It is the ideal interference point to improve the therapeutic efficacy of the tumor. Cell cycle analysis by flow cytometry showed that a marked G_1 arrest occurred when HL60 cells were exposed to OA for 24 h. With the prolongation of the OA treatment time, the G_1 arrest tendency became more obvious. After treatment for 72 h, the percentage of the G_1 phase increased from 41.25% to 67.9%.

Our preliminary study illustrates that OA can induce apoptosis of HL60 cells by the death-receptor pathway. Apoptosis is a complicated process, in which many molecules are involved, and there are great differences between bench work in the laboratory and the application of that work in the clinic. Therefore, further understanding of the mechanism concerning OA-induced apoptosis *in vitro* and the contribution of OA in preclinical animal models of leukemia is required.

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