3-Oxoolean-12-en-27-oic Acid Isolated from *Aceriphyllum rossii* Induces Caspase-8-Dependent Apoptosis in Human Promyelocytic Leukemia HL-60 Cells

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In the present study, we investigated the effects of 3-oxoolean-12-en-27-oic acid (3-OA) isolated from the underground parts of *Aceriphyllum rossii* (Saxifragaceae) on the viability and apoptosis of HL-60 human promyelocytic leukemia cells, and the mechanisms underlying its action. 3-OA-treated HL-60 cells and HeLa human cervix adenocarcinoma cells displayed several apoptotic features, such as, DNA fragmentation, DNA laddering by agarose gel electrophoresis, and hypodiploid DNA contents by flow cytometry, and 3-OA also caused the activations of caspase-8, -9 and -3. Pretreatment with z-VAD-fmk (a broad-caspase inhibitor) almost completely suppressed 3-OA-induced DNA ladder formation and hypodiploid DNA contents, thereby implicating the caspase cascade in the apoptotic process. In addition, z-IETD-fmk (a caspase-8 inhibitor) and z-DEVD-fmk (a caspase-3 inhibitor) also completely neutralized the apoptotic effect of 3-OA in HL-60 cells. Furthermore, 3-OA increased Fas-related protein contents and the mRNA expressions of Fas ligand (FasL), Fas, and Fas-associated death domain (FADD). Preincubation with anti-Fas or anti-FasL blocking antibodies completely prevented 3-OA-induced apoptosis. Taken together, these results suggest that 3-oxoolean-12-en-27-oic acid induces apoptosis by activating caspase-8 *via* FasL-stimulated death receptor signaling.

Key words 3-oxoolean-12-en-27-oic acid; apoptosis; caspase-8; caspase-3

Information on the molecular mechanisms of drug action is essential for the development of any potential chemotherapeutic. In this context, several cancer drugs have been shown to induce apoptosis in tumor cells.[1] Apoptosis, known as programmed cell death, is a highly organized cell death process that is characterized by distinct morphological features, such as, cell shrinkage, chromatin condensation, plasma membrane blebbing, DNA fragmentation, and the breakdown of cells into smaller units (apoptotic bodies).[2] Many investigators have demonstrated that apoptosis is regulated by two major pathways.[3—5] The first pathway occurs *via* death receptors on the cell surface, such as, TNFR1 (tumor necrosis factor receptor 1) and Fas, the latter of which can directly activate procaspase-8 by DISC (death-inducing signaling complex), which includes the cytoplasmic tails of Fas receptors and procaspase-8.[4] Caspase-8, in turn, activates the proteolytic caspase cascade that transmits and amplifies death signals by activating apoptotic executioner caspases like caspase-3 and -7. These executioner caspases then cleave several substrate proteins including poly (ADP-ribose) polymerase-1 (PARP-1), which results in the self-destruction of cells.[6] Furthermore, interaction between Fas and FasL induces apoptotic cell death and altered levels of Fas/FasL expression have been implicated in the pathogenesis of diseases associated with immune regulation.[7,8]

The second pathway regulates the apoptotic cascade *via* a convergence of signaling at mitochondria, such as, those signals mediated by Bcl-2 family proteins. This pathway involves altered mitochondrial membrane potentials, the release of cytochrome *c* into the cytosol, and the activation of procaspase-9, which is followed by the activations of effector caspases like caspase-3 and -7.[5] The mitochondrial pathway, driven by Bcl-2 family proteins, may involves anti-apoptotic (Bcl-2 and Bcl-xL) or pro-apoptotic (Bax, Bak, and Bid), and regulates cell death by controlling mitochondrial membrane permeability during apoptosis.[9]

Triterpenoids derived from plants are used for medicinal purposes in many Asian countries and some have been reported to have anti-tumor activity.[10] Moreover, it was recently found that the novel synthetic triterpenoid methyl-2-cyano-3,12-dioxooleane-1,9-dien-28-oate potently induces caspase-mediated apoptosis in human lung cancer cells.[11] Furthermore, it is also known that the phytoterpenoid 3-oxoolean-12-en-27-oic acid (3-OA, a pentacyclic compound) is present in *Aceriphyllum rossii* Engler (Saxifragaceae), which grows in scant amounts in damp rocky areas in valleys in the central northern region of Korea.[12] During recent years, pharmacological characterizations of 3-OA have concluded that it has several biological activities, which include; the protein tyrosine phosphatase 1B inhibitory activity,[13] acyl-CoA:cholesterol acyltransferase (ACAT) inhibitory activity,[12] and anti-complementary activity.[14] Furthermore, it has been demonstrated that 3-OA is cytotoxic *in vitro* to several cancer cell lines.[15] Thus, as a part of our screening program to evaluate the chemopreventive effects of natural compounds, we investigated the effects of 3-OA on cancer cells and the molecular mechanism that underlies its induction of apoptosis in HL-60 cells.

MATERIALS AND METHODS

Materials 3-Oxoolean-12-en-27-oic acid used for this study was isolated from *Aceriphyllum rossii* as previously described.[15] The 3-oxoolean-12-en-27-oic acid isolated was checked by LC-MS/MS and was >96% pure. RPMI 1640 medium, fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Life Technologies Inc. (Grand Island, NY, U.S.A.). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-

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tetrazolium bromide (MTT), RNase, 4′,6-diamidino-2-phenylindole-dihydrochloride (DAPI), propidium iodide (PI), phenylmethylsulfonylfluoride (PMSF), dithiothreitol, and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Antibodies for caspase-8, -9, and cytochrome *c* were purchased from BD Biosciences (San Jose, CA, U.S.A.). Antibody for β-actin, caspase-3, Bcl-2, Bcl-XL, PARP-1, Fas, FasL, FADD, cFLIP and the peroxidase conjugated secondary antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). Caspase inhibitors (z-VAD-fmk, z-DEVD-fmk, z-IETD-fmk, z-LEHD-fmk) were purchased from Enzyme System Products, Inc. (Livermore, CA, U.S.A). Antagonistic anti-Fas monoclonal antibody, ZB4, was purchased from Millipore (Temecula, CA, U.S.A). The monoclonal antibody that reacts with Fas ligand NOK-1 was from Pharmingen (San Diego, CA, U.S.A.).

Cell Culture HL-60 human promyelocytic leukemia, HeLa human cervix adenocarcinoma, U-937 human histocytic lymphoma, A172 human glioblastoma, A549 human lung adenoma cell lines were obtained from the Korean cell line bank (KCLB, Seoul, Korea) and cultured in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin sulfate (100 μg/ml). Cells were maintained at 37 °C in an atmosphere of 5% CO_2 in air (standard conditions of incubation).

Quantification of Cytotoxicity MTT assay was used to determine 3-OA cytotoxicity. The MTT assay was performed using a modified method described by Plumb *et al.*[16] Briefly, the cells were seeded in each well containing 100 μl of the RPMI medium supplemented with 10% FBS in a 96-well plate. After 12 h, various concentrations of 3-OA were added. After 48 h, 50 μl of MTT (5 mg/ml stock solution, in PBS) was added and the plates were incubated for an additional 4 h. The medium was discarded and the formazan blue, which formed in the cells, was dissolved with 100 μl of dimethyl sulfoxide (DMSO). The optical density was measured at 540 nm.

Detection and Quantification of DNA Fragmentation DNA fragmentation was quantitated as previously reported.[17] In brief, cells were lysed in a solution containing 5 mM Tris–HCl (pH 7.4), 1 mM EDTA, and 0.5% (w/v) Triton X-100 for 20 min on ice. The lysate and supernatant after centrifugation at 27000×***g*** for 20 min were sonicated for 40 s, and the level of DNA in each fraction was measured by a fluorometric method using DAPI. The amount of the fragmented DNA was calculated as the ratio of the amount of DNA in the supernatant to that in the lysate. The genomic DNA was prepared for gel electrophoresis as previously described.[17] Electrophoresis was performed in a 1.5% (w/v) agarose gel in 40 mM Tris–acetate buffer (pH 7.4) at 50 V for 1 h. The fragmented DNA was visualized by staining with ethidium bromide after electrophoresis.

Apoptosis Assessment by PI Staining After treatment of cells with 3-OA for a certain period of time, cells were harvested and washed twice with ice-cold PBS. Then the cells were fixed and permeabilized with 70% ice-cold ethanol at 4 °C for 1 h. The cells were then washed once more with PBS and resuspended in PI staining solution containing 50 μl/ml of propidium iodide and 250 μg/ml of RNase A. The cell suspensions were incubated for 30 min at room temperature, followed by the fluorescence-activated cell sorting (FACS) cater-plus flow cytometry (Becton Dickinson Co., Heidelberg, Germany). Finally, the extent of apoptosis was determined by measuring DNA content of the cells below the G_0/G_1 peak ($subG_1$).

Preparation of Cytosolic and Mitochondrial Fractionations and Western Blot Analysis HL-60 cells (2.5×10^7) were collected by centrifugation at 200×***g*** for 10 min at 4 °C. The cells were washed twice with ice-cold PBS, pH 7.2, followed by centrifugation at 200×***g*** for 5 min. The cell pellet was then resuspened in ice-cold cell extraction buffer (20 mM HEPES–KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 100 μM PMSF, protease inhibitor cocktail) for 30 min on ice. Cells were then homogenized with a glass dounce and a B-type pestle (80 strokes) and subjected to centrifugation at 1000×***g*** to remove unbroken cells, pellet nuclei, and heavy membranes. The postnuclear supernatant was centrifuged at 14000×***g*** for 30 min to yield a pellet mitochondria-enriched heavy membrane fraction, and the resulting supernatant was further centrifuged at 100000×***g*** to obtain the cytosolic fraction. Supernatants were then frozen in aliquots at −70 °C until required. The mitochondria-rich fraction was washed once with extraction buffer, followed by a final resuspension in lysis buffer [150 mM NaCl, 50 mM Tris–HCl (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, and 1 mM EGTA] containing protease inhibitors. Approximately 40 μg of cytosolic or mitochondrial protein extracts were separated using 15% SDS-polyacrylamide gels and transferred to nitrocellulose. The blots were incubated with monoclonal anti-cytochrome *c*, anti-Bcl-2, and polyclonal anti-caspase-3, -8, and -9 antibodies followed by enhanced chemiluminescence (ECL)-based detection (Amersham Pharmacia Biotech).

RNA Preparation and RT-PCR Total cellular RNA was isolated using Easy Blue® kits (Intron Biotechnology, Seoul, Korea), according to the manufacturer's instructions. RNA (2 μg) was reverse-transcribed (RT) from each sample using MuLV reverse transcriptase, 1 mM dNTP, and oligo (dT_{12-18}) 0.5 μg/μl. Polymerase chain reaction (PCR) analyses were performed on aliquots of the cDNA preparations to detect FasL, Fas, FADD, and cFLIP (using β-actin as an internal standard) gene expression using a thermal cycler (Perkin Elmer Cetus, Foster City, CA, U.S.A.). Reactions were carried out in a volume of 25 μl containing (final concentrations) 1 unit of *Taq* DNA polymerase, 0.2 mM dNTP, ×10 reaction buffer, and 100 pmol of 5′ and 3′ primers. After an initial denaturation for 2 min at 94 °C, 30 amplification cycles were performed for FasL (1 min of 94 °C denaturation, 1 min of 65 °C annealing, and 1 min of 72 °C extension), Fas (1 min of 94 °C denaturation, 1 min of 60 °C annealing, and 1.5 min of 72 °C extension), FADD (1 min of 94 °C denaturation, 1 min of 65 °C annealing, and 1.5 min of 72 °C extension), cFLIP (1 min of 94 °C denaturation, 1 min of 62 °C annealing, and 1 min of 72 °C extension) and β-actin (1 min of 94 °C denaturation, 1 min of 72 °C annealing, and 1 min of 72 °C extension). PCR primers used in this study are listed below and were purchased from Bioneer (Seoul, Korea): sense strand FasL, 5′-CAAGTCCAACTCAAGGTCCATGCC-3′, anti-sense strand FasL, 5′-CAGAGAGAGCTCAGATACGTTTGAC-3′; sense strand Fas, 5′-CACTTCGGAGGATTGCTCAACA-3′ anti-sense strand

Fas, 5′-TATGTTGGCTCTTCAGCGCTA-3′; sense strand FADD, 5′-CTGGGCAAGCGGCGAGAC-3′, anti-sense strand FADD, 5′-AGAGAACCAAAGTCCAGGCTGTGT-AGA-3′; sense strand cFLIP, 5′-GCCCGAGCACCGAGAC-TACG-3′; anti-sense strand cFLIP, 5′-GGTTAAGACATAA-AAATTTTTTGCTTG-3′; sense strand β-actin, 5′-TCTG-GCACC-AGACCTTCTACAATGAGCTGCG-3′, anti-sense strand β-actin, 5′-CGTCATACTCC-TGCTTGCTGATCCA-CATCTGC-3′. After amplification, portions of the PCR reaction products were electrophoresed on 2% agarose gel and visualized by ethidium bromide staining and UV irradiation.

Data Analysis Data presented are the means±S.D. of three independent determinations. All experiments were done at least three times, with three or more independent observations each time. Statistical analysis was performed using Student's *t*-test.

RESULTS

The Cytotoxicity of 3-OA on Various Cancer Cells In a previous study,[15] we reported that 3-OA is cytotoxic to HL-60 and K562 cancer cells. We initiated the present study by examining the cytotoxicity of 3-OA (structure shown in Fig. 1) on five different cancer cells using MTT assay. 3-OA showed different degrees of cytotoxicity on these cells based on IC_{50} values, which ranged from 7.9 to 49.1 μM. In particular, 3-OA inhibited the growths of HL-60 and HeLa cells (Table 1).

Induction of Apoptosis by 3-OA The cytotoxic effects of 3-OA on HL-60 and HeLa cells were attributable to the induction of apoptosis, as demonstrated by the following biochemical features. The induction of DNA fragmentation was demonstrated by incubating HL-60 cells with 3-OA at different concentrations (1.8, 3.7, 7.5, 15, 30 μM) and times (0.5, 1, 2, 4, 8 h). Amounts of fragmented DNA were quantified using a fluorometric method by DAPI staining and were found to gradually increase in a concentration- and time-dependent manner (Fig. 2A). In addition, a ladder pattern of DNA internucleosomal fragmentation was observed after treating cells with 3-OA at 15 μM for 8 h (Fig. 2B). To further characterize 3-OA-induced apoptosis, we performed cytofluorimetric analysis by PI staining. As indicated by Fig. 2C, the percentage of hypodiploid DNA contents increased up to 4 h after adding 15 μM 3-OA to HL-60 cells, at which time the percentage of subG1 cells reached about 35.3%. Similar results were obtained when HeLa human cervix adenocarcinoma cells were treated with 20 μM 3-OA for 24 h (Figs. 3A, B, C). These results show, for the first time, that 3-OA induces apoptosis in HL-60 human leukemia and HeLa human cervix adenocarcinoma cells.

Requirement of Caspase Activities during 3-OA-Induced Apoptosis Because 3-OA was found to induce the apoptosis of human leukemia HL-60 cells (Fig. 2), we examined the mechanism involved. Since the caspase family of cysteine protease plays a central role in apoptosis *via* the controlled demolition of the cellular architecture in response to diverse stimuli,[18] we investigated the involvement of caspase activation in 3-OA-induced apoptosis in HL-60 cells by Western blotting. Treatment of cells with 15 μM 3-OA was found to cause the time-dependent proteolytic cleavages of procaspase-8, procaspase-9, and procaspase-3 (Fig. 4A). In addition, the activation of caspase-3 was accompanied by the degradation of PARP-1 (a target during apoptotic response).[19] Cleavage of PARP-1 was evident at after 3 h of treatment in HL-60 cells and coincided with the appearance of activated caspase-3. To determine whether the activations of caspases are required for the induction of apoptosis by 3-OA, we pretreated HL-60 cells with z-VAD-fmk (a broad caspase inhibitor). As shown in Figs. 4B and 4C, z-VAD-fmk almost completely abrogated 3-OA-induced apoptosis. In addition, to determine whether the activations of caspase-8, -9 and -3 are required for the induction of cell death by 3-OA, we pretreated HL-60 cells with various caspase inhibitors. As shown in Fig. 4D, z-DEVD-fmk (a caspase-3 inhibitor) or z-IETD-fmk (a caspase-8 inhibitor) markedly attenuated 3-OA-stimulated DNA fragmentation, whereas z-LEHD-fmk (a caspase-9 inhibitor) had little effect at a similar concentration. These observations revealed that 3-OA-induced apoptosis in HL-60 cells occurs *via* a caspase-dependent pathway that particularly involves the activations of caspase-3 and caspase-8. Caspase-3 is a well-known downstream effector caspase that can be proteolytically activated by caspase-8 or -9 *via* different signaling pathways. To determine the relationship between caspase-8 and caspase-3 in this apoptotic system, we examined whether z-IETD-fmk influenced caspase-3 activity, and as shown in Fig. 4E, z-IETD-fmk was found to completely inhibit caspase-3 cleavage.

Induction of Apoptosis by 3-OA Is Due to Interaction between Fas and FasL In HL-60 cells, we found that activated caspase-8 is a main stream player in 3-OA-induced apoptosis. To determine whether 3-OA-induced apoptosis involves death receptor signaling, HL-60 cells were treated with 15 μM of 3-OA for various times. Total proteins and mRNAs were then extracted, and expressions were determined by Western blotting and RT-PCR. As shown in Fig.

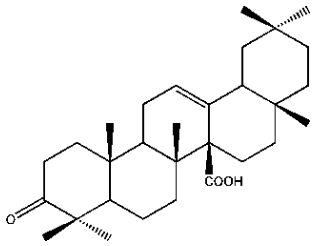

Fig. 1. Chemical Structure of 3-Oxoolean-12-en-27-oic Acid Isolated from *Aceriphyllum rossii*

Table 1. Cytotoxic Activity of 3-OA on Cancer Cell Growth *in Vitro*

Cell line	IC_{50} (μM)[a]	
	3-OA	Cisplatin
HL-60	7.9	2.4
HeLa	11.5	39.5
A172	20.4	137.8
U937	25.9	9.1
A549	49.1	47.1

a) IC_{50} is defined as the concentration that results in a 50% decrease in the number of cells compared to that of the control cultures in the absence of an inhibitor. The values represent the means of results from three independent experiments with similar patterns.

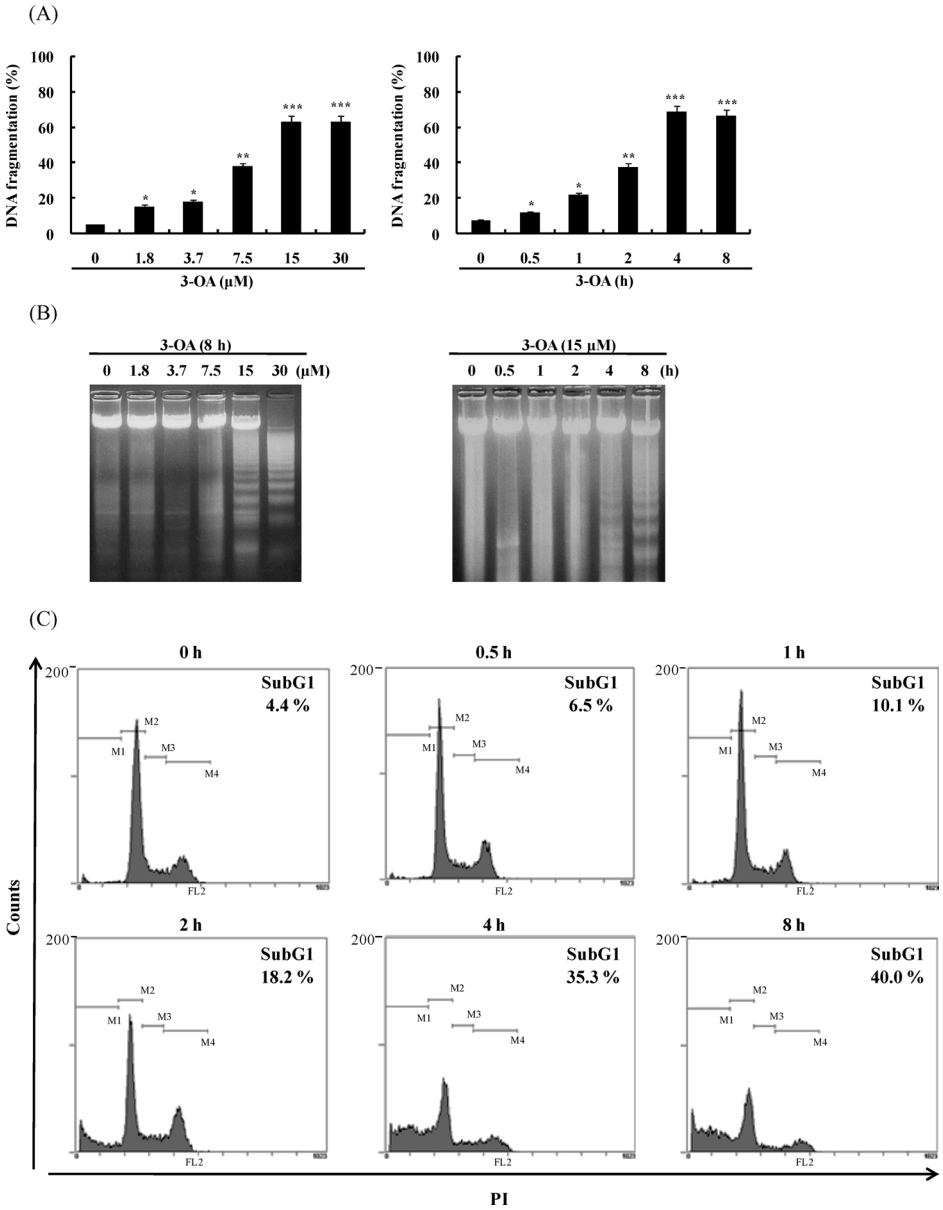

Fig. 2. Effects of 3-OA on the Induction of Apoptosis in HL-60 Cells

(A) HL-60 cells were treated with 3-OA for 8 h with the indicated concentrations and with 15 μM 3-OA for the indicated times. The extents (%) of fragmentation were determined using DAPI, as described in Materials and Methods. Data presented are means±S.D. of three independent experiments. $*p<0.05$, $**p<0.01$, $***p<0.001$, significantly different from the control group by the Student's t test. (B) 3-OA was treated as described above, and DNA fragmentation was analyzed by agarose gel electrophoresis. (C) 3-OA was treated as described above and SubG1 cell populations stained with propidium iodide (PI) were determined by flow cytometry (FACS). Fluorescence intensities are shown on a log scale. Results show a typical experiment which has been repeated three times.

5A, significant increases in the levels of Fas-related proteins, including FasL, Fas and FADD, were observed, whereas cFLIP expression reduced in a time-dependent manner. In addition, RT-PCR analysis showed that the expressions of FasL, Fas, FADD and cFLIP mRNA were correlated with their protein levels (Fig. 5B). On the other hand, 3-OA had no effect on the expression of β-actin (the housekeeping gene). To determine whether the FasL–Fas interaction is involved in 3-OA-induced apoptosis in HL-60 cells, we used an inhibitory antibody against Fas (ZB4) and FasL (NOK-1) that interferes with the FasL–Fas interaction. Preincubation with ZB4 or NOK-1 significantly suppressed the 3-OA-increased subG$_1$ population in HL-60 cells (Fig. 6).

DISCUSSION

Triterpenoids, biosynthesized in plants by the cyclization of squalene, have been used for medicinal purposes in many Asian countries. Furthermore, some, like ursolic and oleanolic acids, are well known anti-inflammatory and anti-carcinogenic agents.[20—22] These triterpenoids and their derivatives are also effective at inhibiting angiogenesis, tumor cell invasion, and metastasis, and have emerged as a new chemotherapeutic class.[10] In particular, oleanolic acid derivatives have been reported to induce the apoptosis of tumor cells in acute myeloid leukemia.[10] Although it has been recently reported that 3-oxoolean-12-en-27-oic acid has substantial cytotoxicity,[14] the molecular mechanisms involved in this process and in its apoptosis-inducing effect have not been elucidated. The

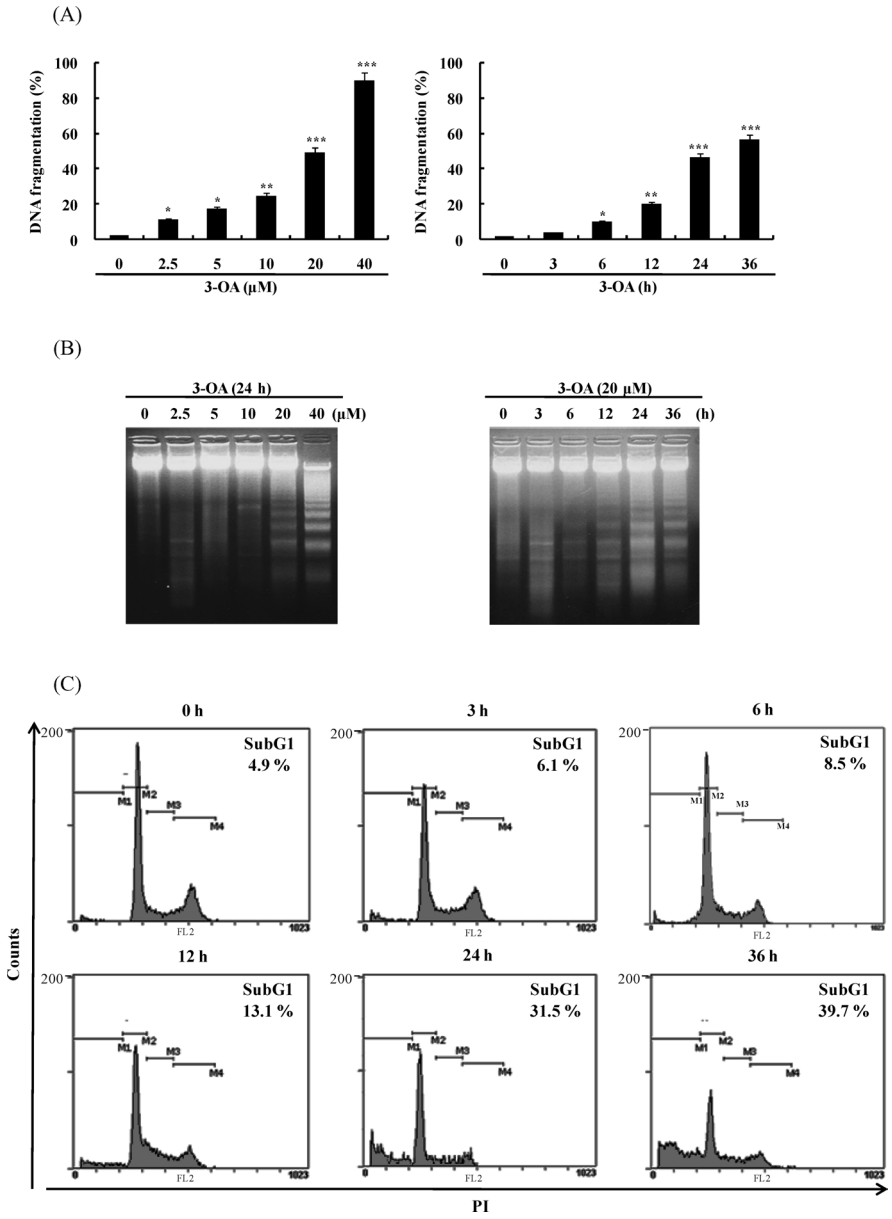

Fig. 3. Effect of 3-OA on the Induction of Apoptosis in HeLa Cells

(A) 3-OA was treated as described in Fig. 2A, and the extents (%) of fragmentation were determined using DAPI, as described in Materials and Methods. Data presented are means±S.D. of three independent experiments. $*p<0.05$, $**p<0.01$, $***p<0.001$, significantly different from the control group by the Student's *t* test. (B) 3-OA was treated as described in Fig. 2B, and DNA fragmentation was analyzed by agarose gel electrophoresis. (C) 3-OA was treated as described in Fig. 3C, and SubG1 cell populations stained with propidium iodide (PI) were determined by flow cytometry (FACS). Fluorescence intensities are shown on a log scale. Results show a typical experiment which has been repeated three times.

present study demonstrates for the first time, based on DNA fragmentation, DNA laddering, and DNA hypodiploid content, that 3-OA can induce the apoptosis of HL-60 human leukemia and HeLa human cervix adenocarcinoma cells (Figs. 2, 3).

The mechanisms of apoptotic activation have been studied intensively under different physiological and pathological conditions.[4] Many genes are known to participate in the regulation of apoptosis, and the activation of the caspase cascade is a central effector mechanism of the apoptosis that occurs in response to death-inducing signals initiated by cell surface receptors, mitochondria, or endoplasmic reticulum stress.[23] The data presented here indicate that following exposure of HL-60 cells to 3-OA, caspase-8, -9, -3 activities and subsequent PARP-1 cleavage are markedly increased in a time-dependent manner (Fig. 4A). PARP-1 is also a substrate for caspase-3, and therefore the activation of caspase-3 could explain the PARP-1 cleavage observed in HL-60 cells. Moreover, PARP-1 cleavage can be used as a sensitive indicator of apoptosis.[5] Furthermore, in the present study, the complete abrogation of 3-OA-induced apoptosis by 50 μM z-VAD-fmk demonstrated the involvement of caspase activation in the apoptotic process (Figs. 4B, C). Caspase-3 is a well-known downstream effector caspase that can be proteolytically activated by caspase-8 and -9 *via* different signaling pathways. In addition, we found that pretreatment with z-IETD-fmk or z-DEVD-fmk markedly prevented 3-OA-induced DNA fragmentation (Fig. 4D), which suggests that the apoptosis induced by 3-OA involves a caspase-8 and caspase-3-mediated mechanism. Furthermore, the activation of caspase-3 (a

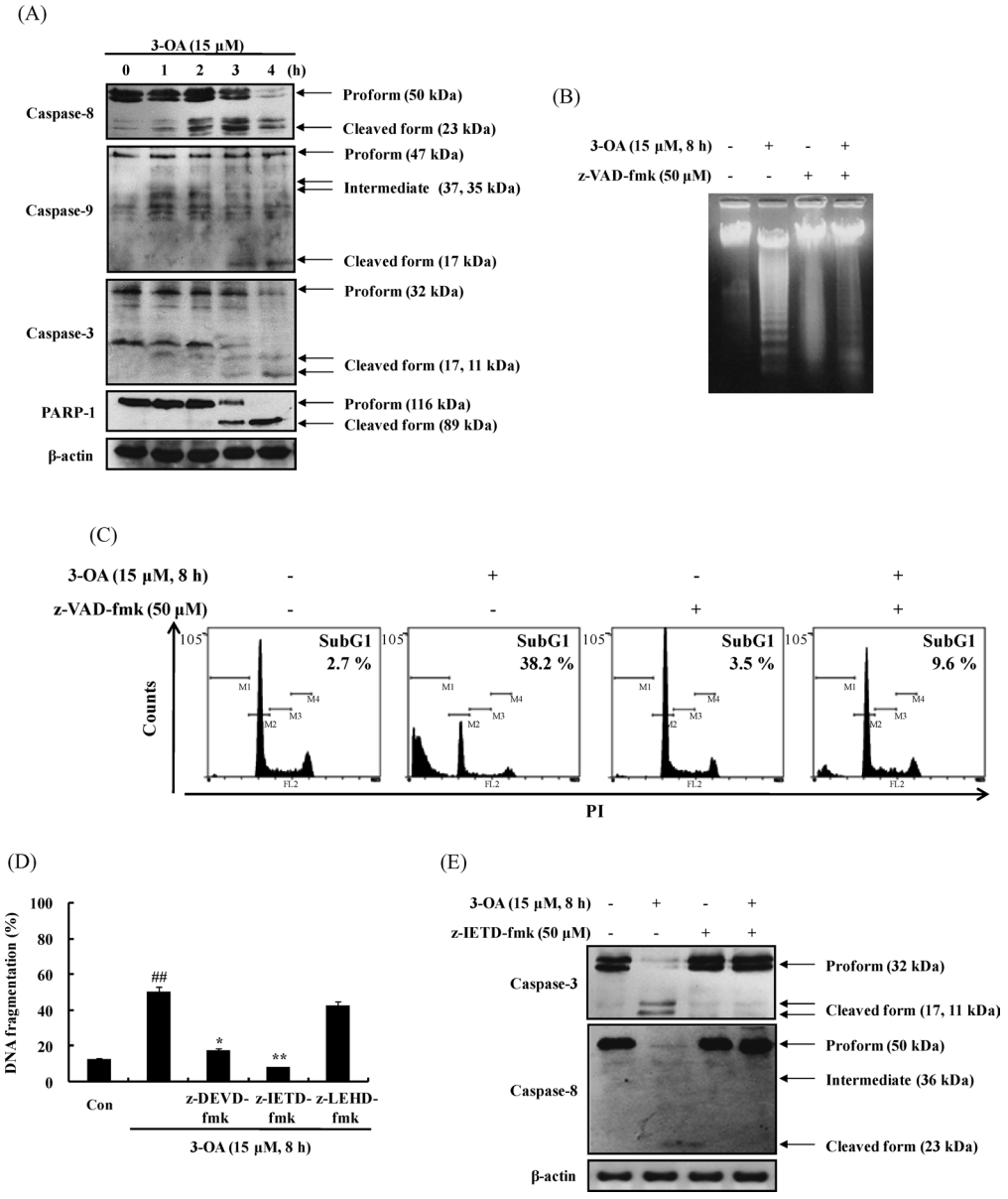

Fig. 4. Effect of 3-OA on the Caspase-8, -9, -3 Activities and PARP-1 Cleavage in HL-60 Cells

(A) 3-OA induced the cleavage of procaspase-8, -9, -3 and PARP-1 in the cytosol. HL-60 cells were treated with 15 μM 3-OA for indicated times, and then cytosolic fractions were separated by SDS-PAGE, transferred onto cellulose membranes, and blotted with caspase-8, caspase-9, caspase-3 or PARP-1 specific antibodies. β-Actin was used as an internal control. (B) Effect of z-VAD-fmk (a broad caspase inhibitor) on 3-OA-induced DNA fragmentation in HL-60 cells. HL-60 cells were pretreated without and with z-VAD-fmk (50 μM for 1 h), and then challenged with 3-OA (15 μM for 8 h). Total genomic DNA was extracted and resolved on 1.5% agarose gel. DNA fragmentation was visualized by ethidium bromide staining. (C) Effect of z-VAD-fmk on 3-OA-induced hypodiploid DNA contents in HL-60 cells. 3-OA was treated as described in Fig.4B and PI-positive cells were determined by flow cytometry. (D) Effect of several caspase inhibitors on apoptosis in response to 3-OA-induced DNA fragmentation. HL-60 cells were pretreated without and with 50 μM of z-DEVD-fmk, z-IETD-fmk, z-LEHD-fmk for 1 h, and then treated with 15 μM 3-OA for 8 h. Data presented are the means±S.D. of results from three independent experiments, ## $p<0.01$ *vs.* control group, * $p<0.05$, ** $p<0.01$ *vs.* 3-OA-treated group; significance of difference between treated groups by Student's *t*-test. (E) Effect of z-IETD-fmk on 3-OA-induced procaspase-3 and -8 cleavage. HL-60 cells were pretreated without and with 50 μM z-IETD-fmk for 1 h, and then treated with 15 μM 3-OA for 8 h. Cytosolic fractions were separated by SDS-PAGE, transferred onto cellulose membranes, and blotted with caspase-8 or caspase-3 specific antibodies. β-Actin was used as an internal control.

downstream protein of caspase-8) by 3-OA was found to be blocked by caspase-8 inhibitor in HL-60 cells (Fig. 4E), indicating that the apoptosis induced by 3-OA proceeds *via* a caspase-8-mediated mechanism.

Caspase-8 acts as the initiator caspase in the apoptosis that results from the ligand-driven engagement of death receptors. On the other hand, Fas (Apo-1/CD95) is a well-characterized member of the TNF (tumor necrosis factor) superfamily of death receptors.[24] In response to the cognate ligand, FasL, it undergoes oligomerization and recruits the adaptor protein FADD, which in turn recruits caspase-8 and/or c-FLIP to form DISC.[25] Upon recruitment by FADD, procaspase-8 oligomerization drives its activation through self-cleavage.[19] cFLIP contains a death effector domain that is similar to corresponding segments in FADD and caspase-8. Moreover, caspase-8 activation can be blocked by the recruitment of the degenerate caspase homologue cFLIP.[26] In the present study, we observed elevated expressions of Fas-related proteins and their mRNAs (FasL, Fas, FADD), but diminished cFLIP expression after treatment with 15 μM 3-OA in a time-dependent manner (Figs. 5A, B). Furthermore, we demonstrated that pretreatement with ZB4 or NOK-1

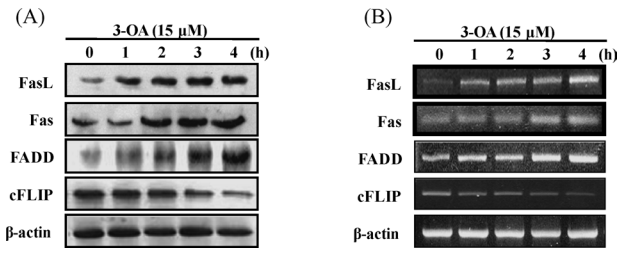

Fig. 5. Expressions of Fas-Related Proteins (A) and Their mRNAs (B) in 3-OA-Induced Apoptosis in HL-60 Cells

(A) Cells were treated with 15 μM 3-OA for the indicated times. Total cellular proteins (40 μg) were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and detected with specific antibodies, as described in Materials and Methods. A representative immunoblot of three separate experiments is shown. The amount of β-actin was measured as an internal control. (B) Samples were treated as described above. Total RNA was prepared for the RT-PCR analysis of the expressions of FasL, Fas, FADD and cFLIP in HL-60 cells treated with indicated times of 15 μM 3-OA. FasL-specific sequences (344 bp), Fas-specific sequences (504 bp), FADD-specific sequences (312 bp) and cFLIP-specific sequences (721 bp) were detected by agarose gel electrophoresis, as described in Materials and Methods. PCR for β-actin was performed to verify that the initial cDNA contents of the samples were similar. The experiment was repeated three times and similar results were obtained.

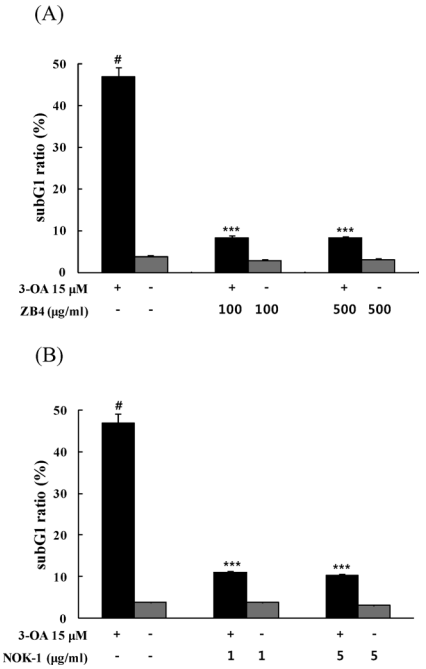

Fig. 6. Effect of Fas/FasL Blocking Antibodies on 3-OA-Induced Apoptosis

After treatment with (A) anti-Fas mAb (ZB4; 100 or 500 μg/ml) or (B) anti-FasL mAb (NOK-1; 1 or 5 μg/ml) for 1 h, HL-60 cells were then treated with 15 μM of 3-OA for 8 h. The apoptotic cells (subG$_1$ population) were measured by PI staining analysis. Data presented are the means±S.D. of results from three independent experiments. $^{\#}p<0.05$ *vs.* control group, $^{***}p<0.001$ *vs.* 3-OA-treated group; significance of difference between treated groups by Student's *t*-test.

markedly inhibited the 3-OA-induced apoptosis. These results further support the involvement of the Fas/FasL pathway in 3-OA-stimulated apoptosis in HL-60 cells.

In mitochondria undergoing mitochondrial permeability transition (MPT), matrix swelling and outer membrane rupture causes the release of mitochondrial cytochrome *c*,[8] once in the cytosol, cytochrome *c* binds to Apaf-1 in a dATP/ATP-dependent manner, an event that triggers the oligomerization of Apaf-1/cytochrome *c* in complexes that activate procaspase-9.[27] The ensuing recruitment and activation of caspase-9 results in the activations of caspase-3, -6, and -7, which function as downstream effectors of the cell death program.[28] Activation of apoptosis effector caspase-3 is essentially required for the apoptosis induced by various stimuli. Caspase-3 is an executioner caspase that can be activated by the following: (i) a mitochondrial pathway involving the activation of caspase-9 caused by the release of cytochrome *c* to the cytosol, which leads to its binding to apoptosis protease activation factor-1 and the subsequent recruitment and activation of procaspase-9, or (ii) a death receptor pathway involving caspase-8.[4] To determine whether caspase-3 was activated by a mitochondrial pathway involving caspase-9 activation or a death receptor pathway involving caspase-8, we treated HL-60 cells with specific inhibitors of caspase-8 or caspase-9 prior to 3-OA treatment. The results obtained pointed towards the involvement of the caspase-8 pathway, but not of the caspase-9 pathway in the execution of 3-OA-induced apoptosis. Furthermore, no dissipation of mitochondrial membrane potential or release of cytochrome *c*, or decrease in Bcl-2 expression was detected in 3-OA treated HL-60 cells (data not shown).

The development of anti-cancer drugs that inhibit abnormal cancer cell proliferation and induce cancer cell death through apoptosis is a fundamental research objective, and many agents have been found to exert anti-cancer effects by inducing cancer cell apoptosis.[29] In the present study, we found that 3-OA, a natural compound present in the underground parts of *Aceriphyllum rossii*, induces the apoptosis of human promyelocytic leukemia and human cervix adenocarcinoma cells. We are currently investigating the feasibility of further developing 3-OA as a chemopreventive agent.

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