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Apicidin, a Histone Deacetylase Inhibitor, Induces Apoptosis and Fas/Fas Ligand Expression in Human Acute Promyelocytic Leukemia Cells*

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We previously reported that apicidin arrested human cancer cell growth through selective induction of $p21^{WAF1/Cip1}$. In this study, the apoptotic potential of apicidin and its mechanism in HL60 cells was investigated. Treatment of HL60 cells with apicidin caused a decrease in viable cell number in a dose-dependent manner and an increase in DNA fragmentation, nuclear morphological change, and apoptotic body formation, concomitant with progressive accumulation of hyperacetylated histone H4. In addition, apicidin converted the procaspase-3 form to catalytically active effector protease, resulting in subsequent cleavages of poly(ADP-ribose) polymerase and $p21^{WAF1/Cip1}$. Incubation of HL60 cells with z-DEVD-fmk, a caspase-3 inhibitor, almost completely abrogated apicidin-induced activation of caspase-3, DNA fragmentation, and cleavages of poly(ADP-ribose) polymerase and $p21^{WAF1/Cip1}$. Moreover, these effects were preceded by an increase in translocation of Bax into the mitochondria, resulting in the release of cytochrome *c* and cleavage of procaspase-9. The addition of cycloheximide greatly inhibited activation of caspase-3 by apicidin by interfering with cleavage of procaspase-3 and DNA fragmentation, suggesting that apicidin-induced apoptosis was dependent on *de novo* protein synthesis. Consistent with these results, apicidin transiently increased the expressions of both Fas and Fas ligand. Preincubation with NOK-1 monoclonal antibody, which prevents the Fas-Fas ligand interaction and is inhibitory to Fas signaling, interfered with apicidin-induced translocation of Bax, cytochrome *c* release, cleavage of procaspase-3, and DNA fragmentation. Taken together, the results suggest that apicidin might induce apoptosis through selective induction of Fas/Fas ligand, resulting in the release of cytochrome *c* from the mitochondria to the cytosol and subsequent activation of caspase-9 and caspase-3.

Histone acetylation, which is regulated by a balance between histone acetylases and deacetylases (HDACs)[1] (1), has been suggested to play an important role in gene expression by changing the dynamics of chromatin folding during gene expression (2). The hypothesis is supported by the fact that hyperacetylation of lysine residues in the N-terminal tails of histone correlates with active gene loci, and their hypoacetylation, in contrast, occurs at silence or heterochromatic chromosomal regions (3). Furthermore, accumulating evidences indicate that histone acetylases and HDACs act as transcriptional coactivators and transcriptional corepressors, respectively (3–6). Therefore, deregulation of histone acetylase and HDAC has been suggested to play a causative role in the generation of cancer by changing expression pattern of various genes.

Various HDAC inhibitors such as sodium *n*-butyrate (7), trapoxin (8), trichostatin A (9), depudecin (10), FR901228 (11), oxamflatin (12), and MS-27–275 (13), which accumulate acetylated histones in the nucleus, have been repeatedly demonstrated to arrest cell growth and reverse neoplastic characteristics in cultured cells. Recent accumulating evidences suggest that induction of histone hyperacetylation by HDAC inhibitors is responsible for the antiproliferative activity and reversal of neoplastic characteristics through selective induction of genes, which play important roles in the cell cycle and cell morphology (14). In addition to cell cycle arrest and differentiation, HDAC inhibitors have been shown to induce apoptosis (15–17), which is characterized by typical structural changes including cell shrinkage, membrane blebbing, chromatin condensation, and nuclear DNA fragmentation.

The signaling pathways of apoptosis can be divided into two compartments involving either the plasma membrane, where death receptors such as tumor necrosis factor receptor 1, Fas, DR-3, DR-4, or DR-5 reside, or the mitochondria, which contain several proteins that regulate apoptosis. One pathway is initiated by engagement of the tumor necrosis factor or Fas receptors and thereby activate caspase-8 (18, 19). In turn, caspase-8 cleaves Bid and induces cytochrome *c* release (20, 21) or can also directly activate caspase-3 (22). In the second pathway, other signals that are important but not well undefined converge to induce the release of cytochrome *c* (23, 24). Cytosolic cytochrome *c* binds to Apaf-1, induces autoprocessing of caspase-9, and subsequently activates caspase-3 (21, 25). The caspase proteolytic cascade is a central component of the cellular machinery of the apoptotic process. The early mitochondrial changes, *i.e.* the release of apoptogenic factors, especially

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[1] The abbreviations used are: HDAC, histone deacetylase; PARP, poly(ADP-ribose) polymerase; z-DEVD-fmk, benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone; Ac-DEVD-pNA, acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide; PBS, phosphate-buffered saline; mAb, monoclonal antibody.

cytochrome *c*, function as a powerful trigger for the activation of the caspase cascade. The caspase family of aspartate-specific cysteine proteases has been demonstrated to be critical mediators in the cell death pathway (26). These enzymes can be divided into two groups; they are initiator caspases such as caspase-8 and caspase-9, whose main function is to activate downstream caspases, and executor caspases such as caspases-3, -6, and -7, which are responsible for dismantling cellular proteins. A number of caspase-3 substrates have now been identified, including protein kinases, the retinoblastoma protein, cytoskeletal proteins, and DNA fragmentation-inducing proteins. Cleavage of these substrates may either activate or inactivate their essential functions or produce the cleavage products with altered activities.

Apicidin, a histone deacetylase inhibitor, is a novel cyclic tetrapeptide with a potent broad spectrum of antiproliferative activity against various cancer cell lines (14) and antiprotozoal activity against Apicomplexan parasites (27). Although HDAC inhibitors have been shown to induce apoptosis, it is unclear which apoptotic signaling pathway is responsible for the induction of apoptosis or whether *de novo* protein synthesis is required. In this study, we evaluated the apoptotic potential of apicidin in human acute promyeolcytic leukemia cells HL60 and investigated the mechanism of apicidin-induced apoptosis by analyzing the signaling pathway of apoptosis. Here, we provide evidence that apicidin induces apoptosis via a mitochondrial/cytochrome *c*-dependent pathway, which requires *de novo* protein synthesis of Fas/Fas ligand, resulting in cytochrome *c* release from the mitochondria to the cytosol and subsequent activation of caspase-9 and caspase-3.

EXPERIMENTAL PROCEDURES

Materials—Apicidin (cyclo(*N*-*O*-methyl-L-tryptophanyl-L-isoleucinyl-D-pipecolinyl-L-2-amino-8-oxodecanoyl)) known as an antiprotozoal agent, was prepared from *Fusarium* sp. Strain KCTC 16677 according to the method previously described (28).

Cell Culture—The human promyelocytic leukemia cell line HL60 was grown in suspension in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT), 1% penicillin/streptomycin, and 1% L-glutamine (Invitrogen).

Cell Viability—HL60 cells were seeded at 5×10^5 cells/well in 6-well plate. Cell viability was determined by hemocytometer at 0, 3, 6, 12, 24, 48, and 72 h by trypan blue exclusion (0.4% w/v, Sigma) by counting at least 100 cells from each individual culture.

DNA Fragmentation Assay—The cells were rinsed with ice-cold PBS and harvested by pipetting. The cell pellets were resuspended and incubated in 100 mM Tris-HCl (pH 8.0), 25 mM EDTA, 0.5% SDS, and 0.1 μg/ml proteinase K at 60 °C overnight. The digested cells were extracted for DNA with phenol/chloroform (1:1) and chloroform/isoamyl alcohol (1:24). The extracted DNA was precipitated and digested in 10 mM Tris-HCl (pH 5.0) containing 1 mM EDTA and 10 μg/RNase for 1 h at 37 °C. Ten micrograms of DNA per sample was resolved by electrophoresis in a 1.8% agarose gel impregnated with ethidium bromide (0.5 μg/ml), and the DNA pattern was examined by ultraviolet transillumination.

Cell Morphology—After treatment of cells with drugs for a certain period of time, cells were pipetted and collected followed by washing once with ice-cold PBS. Cells were attached to the slide by cytospin (500 rpm, 5 min), air-dried, fixed, and stained with the Hoechst 33258 (10 μg/ml). The stained cells were examined by fluorescence microscopy (Magnification ×400).

Flow Cytometry—To analyze an effect of apicidin on the cell cycle progression and apoptosis, the DNA content profile of a given population was determined by flow cytometry according to the method described by Noguchi and Browne (29). Thus HL60 cells were treated with an increasing concentration of drugs for the indicated times. Briefly, after fixing the treated cells with 70% ethanol and treating with 0.25 μg/ml RNase, nuclei were stained with 50 μg/ml propidium iodide, and the relative DNA content was measured by BRYTE HS system (Bio-Rad) and ModFit LT (Verity Software House, Inc., Topsham, ME).

Preparation of Mitochondria and Cytosolic Fractions—HL60 cells treated with apicidin were harvested and rinsed with ice-cold PBS, and cell pellets were resuspended in 300 μl of buffer A (20 mM Hepes-KOH (pH 7.5), 10 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol) containing 250 mM sucrose and proteinase inhibitors. In some experiments, cells were pretreated with 100 ng/ml NOK-1 mAb (Pharmingen) for 1 h before the addition of apicidin. After homogenization, unbroken cells, piece of large plasma membrane, and nuclei were removed by centrifugation at 1,000 × g for 10 min at 4 °C. The supernatant was centrifuged at 10,000 × g for 20 min at 4 °C. The pellet fraction containing the mitochondria was dissolved in 50 μl of TNC buffer (10 mM Tris acetate (pH 8.0), 0.5% Nonidet-40, 5 mM $CaCl_2$). The supernatant was further centrifuged at 50,000 × g for 2 h to obtain cytosol.

Preparation of Cell Extracts and Western Blotting Analysis—HL60 cells were rinsed twice with ice-cold wash buffer solution containing 50 mM Tris-HCl (pH 7.5), 120 mM NaCl, 20 mM NaF, 1 mM EDTA, 5 mM EGTA, 15 mM sodium pyrophosphate, 30 mM *p*-nitrophenyl phosphate, 1 mM benzamidine, and 0.1 mM phenylmethylsulfonyl fluoride and were then extracted in the same buffer containing 1% Nonidet P-40. Cells were collected by pipetting, homogenized, and centrifuged at 15,000 × g for 15 min at 4 °C. Protein concentration was measured by the method of Bradford with bovine serum albumin as the standard (30). Aliquots of the supernatant were stored at −70 °C until use. For the determination of apoptotic proteins, cell lysates containing 50 μg of total protein were subjected to SDS-PAGE on 7.5–15% slab gels, and proteins were transferred to polyvinylidene difluoride membranes. Membranes were blocked for 30 min in PBS containing 0.1% Tween 20 and 5% (w/v) dry skim milk powder and incubated overnight with anti-Bax (1:1000), anti-Bcl-2 (1:500), anti-caspase-3 (1:1000), anti-caspase-9 (1:1000), anti-cytochrome *c* (0.5 μg/ml), anti-Fas (1:1000), anti-Fas ligand (0.5 μg/ml), anti-HDAC1 (1:2000), anti-PARP (0.5 μg/ml), or anti-$p21^{WAF1/Cip1}$ (1:1000). The membranes were then washed with PBS containing 0.1% Tween 20 and incubated for 2 h with an anti-rabbit secondary antibody or anti-mouse secondary antibody conjugated to alkaline phosphatase, and bound antibodies were detected with the enhanced amplified alkaline phosphatase immunoblot system (Bio-Rad).

Histone Isolation and Immunodetection of Acetylated Histone H4—HL60 cells were incubated with apicidin or 0.1% Me_2SO in culture medium, cells were harvested after the indicated times, and histones were isolated by the established techniques (14). Samples were resuspended in Laemmli sample buffer for 3 min and subjected to 15% SDS-PAGE for the determination of acetylated histone H4. Acetylated histone H4 was detected with anti-acetyl histone H4 antiserum (Upstate Biotechnology, Inc.).

Analysis of Acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pNA) Protease Activity—The protease assay mixture included 240 μl of reaction buffer (100 mM HEPES, (pH 7.5), 20% (v/v) glycerol, 5 mM dithiothreitol, and 0.5 mM EDTA), 30 μl of 1 mM acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide in Me_2SO (100 μM final concentration; Biomol), and 30 μl of cell lysates. Samples were incubated at 37 °C for 1 h, and enzyme-catalyzed release of *p*-nitroanilide was monitored at 405 nm in an ultra-microplate reader (Bio-Tek instruments, Inc.). The percentage increase of absorbance in the induced samples relative to control samples indicates the percentage increase of the caspase-3 activity in these samples (31, 32).

RESULTS AND DISCUSSION

Effect of Apicidin on Growth and Histone H4 Acetylation of Human Acute Promyelocytic Leukemia Cells HL60—Apicidin, a fungal metabolite, has been shown to exhibit antiparasitic activity by inhibiting HDAC in *Plasmodium falciparum* (27). Recent studies further demonstrated that apicidin has a broad spectrum of antiproliferative activity against various cancer cell lines. Treatment of HeLa cells with apicidin induced accumulation of hyperacetylated histone H4, morphological changes, and cell cycle arrest at G_1 phase, which were accompanied by increased expression of gelsolin and $p21^{WAF1/Cip}$, suggesting that histone hyperacetylation induced by apicidin was responsible for the antiproliferative activity through selective induction of genes that play important roles in the cell cycle and cell morphology (14). Therefore, to further evaluate the apoptotic potential of apicidin, we first examined the effect of apicidin on the growth of HL60 cells, a human acute promyelocytic leukemia cell line, using trypan blue exclusion assay. As shown in Fig. 1*A*, treatment of HL60 cells with up to 0.1 μg/ml apicidin for 72 h caused only a slight decrease of viable cells; however, increasing concentrations of apicidin (0.5–2 μg/

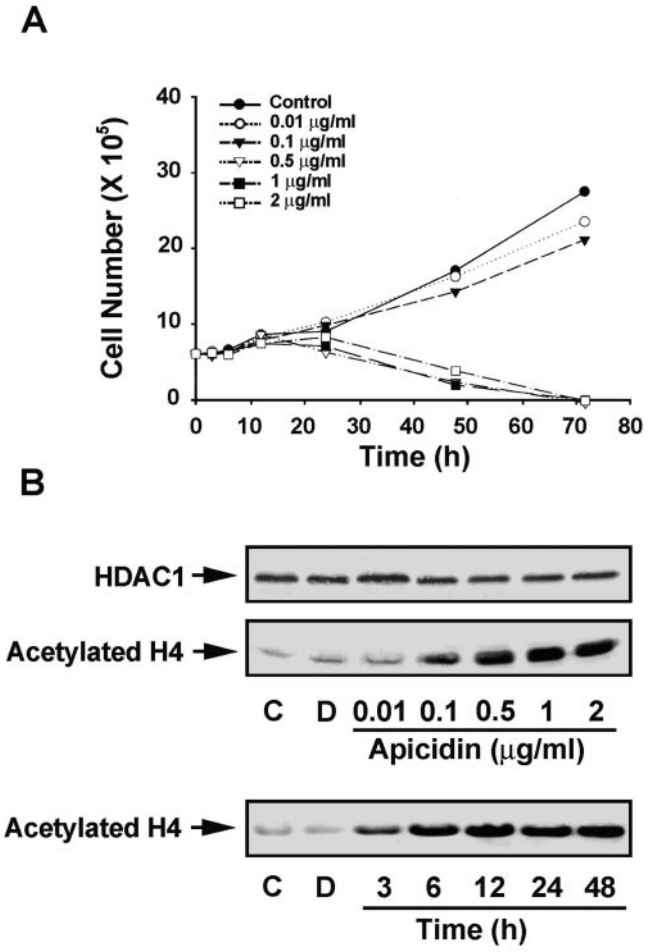

FIG. 1. **Effect of apicidin on growth and acetylation of histone H4 of HL60 cells.** *A*, HL60 cells (seeded at a density of 5×10^5/well in 6-well plates) were treated with various concentrations of apicidin for the indicated times, and then viable cells were counted after staining with 0.4% tryphan blue solution. *B*, proliferating HL60 cells were treated with various concentrations of apicidin for 24 h or treated with 1 μg/ml apicidin for the indicated times. Histone fractions were isolated, and 50 μg of protein were run on 15% SDS-PAGE gel, blotted, and probed with antibody against acetylated histone H4. Also, the expression level of HDAC1 in cell lysate was examined by immunoblot analysis with anti-HDAC1 antibody. *C*, control group; *D*, 0.1% Me_2SO-treated group.

ml) for 48–72 h resulted in a sharp decline in viable cell number, indicating extensive cell death within 48 h.

The level of histone acetylation *in vivo* is mainly maintained by the balance between histone acetylase and HDAC. Human HDAC, one of the regulators of histone acetylation, was recently purified and cloned by using trapoxin-based affinity matrix (33). Thus, to further examine the relationship between cell death and histone acetylation of HL60 cells, we next analyzed the effect of apicidin on the intracellular level of histone H4 in HL60 cells using specific antibody against acetylated histone H4; HL60 cells were treated with various concentrations of apicidin for 24 h, and histones extracted from nuclei were subjected to SDS-PAGE and immunoblot analysis. As shown in Fig. 1*B*, acetylation of histone H4 increased in a time- and dose-dependent manner, reaching a maximum level at 0.5 μg/ml apicidin with no further induction observed at higher concentrations, whereas acetylated histone H4 in proliferating HL60 cells was hardly detected in the absence of apicidin. This effect appears not to be due to alteration of expression level of HDAC, since apicidin did not cause any change in the amount of HDAC1 (Fig. 1*B*). Although transient histone H4 acetylation was observed in human keratinocyte by HDAC inhibitors including sodium *n*-butyrate and trichostatin A (34), histone hyperacetylation in HL60 cells by apicidin was persistent up to 48 h, similar to that observed in HeLa cells (14). Taken together, the *in vivo* effect of apicidin on the level of nuclear histone acetylation was very closely correlated with the effect of apicidin on cell death, indicating that cell death by apicidin was most likely due to inhibition of HDAC.

Induction of Apoptosis by Apicidin—Recently various HDAC inhibitors including depudecin, FR901228, trapoxin, oxamflatin, and trichostatin A have been shown to induce apoptosis of v-*ras*-transformed NIH3T3 cells (10, 11, 34) or HeLa cells (9, 12). These observations prompted us to examine whether the effect of apicidin on cell death was attributable to the induction of apoptosis. Because cells undergoing apoptosis show a characteristic cleavage of DNA into oligonucleosome fragments manifested as "DNA laddering," a hallmark of apoptosis (35), we exposed HL60 cells to different concentrations of apicidin for 24 h and determined apoptosis levels. As shown in Fig. 2*A*, whereas concentrations of up to 0.1 μg/ml apicidin induced little if any DNA fragmentation, increasing concentration of apicidin (0.2–2 μg/ml) resulted in an appearance of internucleosomal DNA ladder, as observed in HL60 cells treated with camptothecin, which is well known to induce typical apoptosis. To further clarify the nature of DNA fragmentation, we next examined the effects of apicidin on nuclear morphological change, another hallmark of apoptosis. As shown in Fig. 2*B*, treatment of HL60 cells with apicidin induced chromatin condensation and nuclear fragmentation in a dose-dependent manner, judged by Hoechst 33258 staining of the cells. It revealed a peculiar feature of nuclei, having an equal distribution of much more intensive fluorescent and smaller diameter than the control-like nuclei. We then performed fluorescence-activated cell sorter analysis of propidium iodide-stained nuclei to assess the cells for the presence of a hypodiploid or sub-G_1 fraction resulting from DNA fragmentation. Fig. 2, *B* and *C*, shows that the number of HL60 cells in the sub-G_1 fraction increased in a dose- and time-dependent manner in response to apicidin. Although treatment of HL60 cells with concentrations of up to 0.1 μg/ml apicidin for 24 h resulted in a slight increase in accumulation of sub-G_1 fraction, increasing concentrations of apicidin (0.5–1 μg/ml) led to a progressive accumulation of sub-G_1 fraction. Consistent with the increased accumulation of sub-G_1 fraction, G_0/G_1 fractions markedly decreased in a dose-dependent fashion, indicating extensive induction of apoptosis. These results very closely correlated with the induction of internucleosomal DNA ladder and nuclear morphological change. The detection of sub-G_1 fractions together with the presence of DNA ladders and nuclear morphological changes suggest that apicidin triggers an apoptotic pathway in HL60 cells.

Activation of Caspase-3 and Cleavage of PARP and $p21^{WAF1/Cip1}$ *by Apicidin*—A family of aspartate-specific cysteinyl proteases (caspases) plays a pivotal role in the execution of programmed cell death (36). Thus, to gain insights into the mechanism by which apicidin induces apoptosis, we investigated the effects of apicidin on caspases. Of the many caspases involved in apoptosis, we first examined the *in vivo* effect of apicidin on caspase-3, which is activated by a number of apoptotic signals. As shown in Fig. 3*A*, incubation of HL60 cells with apicidin resulted in the activation of capsase-3, evidenced by conversion of the proenzyme form of caspase-3 (p32) to the catalytically active effector protease (p17). Activation of caspase-3 protease during apicidin-induced apoptosis was also confirmed by examining PARP, a known endogenous substrate for caspase-3 (37); caspase-3 protease activation was accompa-

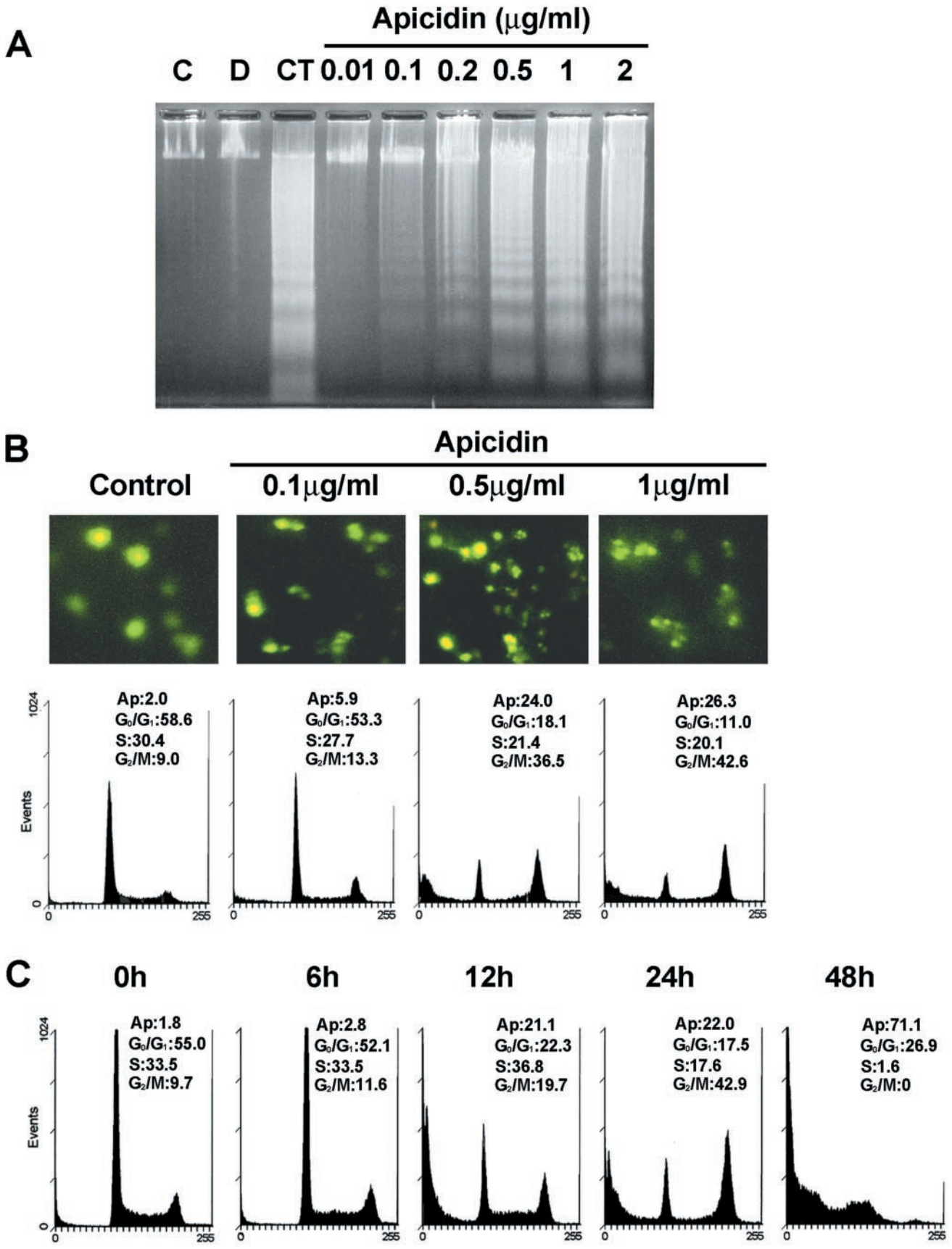

FIG. 2. **Effect of apicidin on DNA fragmentation, morphological changes, and apoptotic DNA contents in HL60 cells.** *A*, HL60 cells were treated with various concentrations of apicidin for 24 h, and total genomic DNA was extracted and resolved on 1.8% agarose gel. Apoptotic DNA fragmentation was visualized by ethidium bromide staining. *C*, control group; *D*, 0.1% Me_2SO-treated group; *CT*, 1 μM camtothecin-treated group. *B*, after treatment with the indicated concentrations of apicidin for 24 h, morphological changes and apoptotic DNA contents were analyzed by fluorescence microscopy and flow cytometry, respectively. Ap, sub-G_1 fraction; S, S phase. *C*, at the indicated time point after treatment with 1 μg/ml apicidin, cells were harvested and fixed in 70% ethanol. After staining with propidium iodide, apoptotic DNA contents were analyzed by flow cytometry.

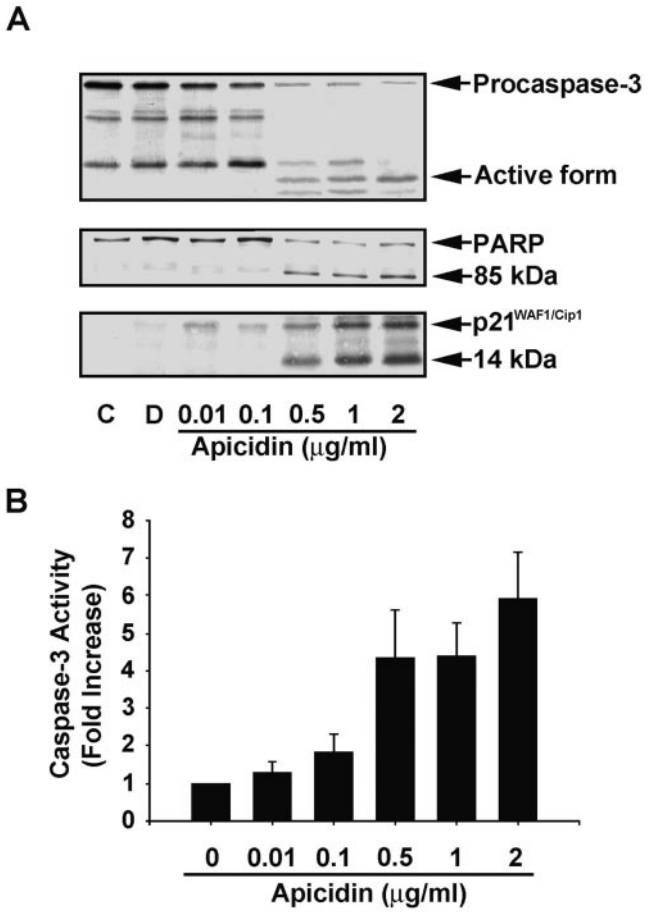

FIG. 3. **Activation of caspase-3 and proteolytic cleavage of p21$^{WAF1/Cip1}$ and PARP induced by apicidin.** *A*, after treatment with the indicated concentrations of apicidin for 48 h, the cleavage of procaspase-3, PARP, and p21$^{WAF1/Cip1}$ were examined by Western blot analysis. *C*, control group; *D*, 0.1% Me_2SO-treated group. *B*, the cell lysates prepared from the experiments in *A* were incubated with Ac-DEVD-pNA, a substrate for caspase-3 *in vitro*, for 1 h at 37 °C. Caspase-3 activity was calculated relative to the activity in 0.1% Me_2SO-treated cell lysate. Data are expressed as mean ± S.D. from three separate experiments in duplicate.

nied by the cleavage of PARP (116 kDa) into an 85-kDa C-terminal fragment and a 28-kDa N-terminal fragment (Fig. 3*A*). Because p21$^{WAF1/Cip1}$ has also been shown to be one of the substrates for caspase-3-like proteases (38, 39), we examined whether p21$^{WAF1/Cip1}$ could be cleaved in HL60 cells after apicidin treatment and found that apicidin treatment induced the expression of p21$^{WAF1/Cip1}$, and p21$^{WAF1/Cip1}$ was cleaved to a 14-kDa fragment (Fig. 3*A*). We next attempted to directly determine caspase-3 activity in apicidin-treated cell extracts by monitoring the release of *p*-nitroanilide from Ac-DEVD-pNA, a substrate of caspase-3. Lysates from HL60 cells treated with apicidin exhibited an increased peptide cleavage activity in a dose-dependent manner (Fig. 3*B*). The kinetics of Ac-DEVD-pNA cleavage corresponded temporally with apicidin-induced internucleosomal DNA fragmentation, morphological change, and apoptotic body formation (data not shown). These results suggested that caspase-3 was involved in the induction of apoptosis by apicidin. To further confirm the involvement of caspase-3 in apicidin-induced apoptosis, we examined the effect of a cell-permeable caspase inhibitor, benzyloxycarbonyl-Asp-Glu-Val-Asp-fmk (z-DEVD-fmk), on apicidin-induced apoptosis. As shown in Fig. 4*A*, cells treated with either z-DEVD-fmk alone or control cells without treatment of apicidin showed no cleavage products (data not shown), whereas treatment with 100 μM z-DEVD-fmk completely inhibited apicidin-induced caspase-3 activity. Consistent with these results, cleavage of PARP and p21$^{WAF1/Cip1}$ (Fig. 4*B*) and DNA ladder (Fig. 4*C*) induced by apicidin was also almost completely abrogated by z-DEVD-fmk. Taken together, these results indicated that apicidin-induced apoptosis required activation of caspase-3.

Effects of Apicidin on Cleavage of Procaspase-9—To understand the mechanism by which caspase-3 is activated by apicidin, we further investigated the effect of apicidin on the cleavage of caspase-9, an upstream activator of caspase-3 (40). Therefore, HL60 cells were incubated with apicidin (1 μg/ml) for various times, and cell lysates were subjected to SDS-PAGE and immunoblot analysis using a specific antibody. As shown in Fig. 5, treatment of HL60 cells with apicidin for up to 6 h caused a slight reduction of procaspase-9; however, incubation for a longer time (from 12 to 24 h) led to an almost complete disappearance of procaspase-9, with progressive accumulation of cleavage product of procaspase-9, indicating activation of caspase-9. Although procaspase-3 was also cleaved in a time-dependent manner similar to that of caspage-9 cleavage, the kinetics of proteolysis of procaspase-3 was slower than that of procaspase-9, indicating that maximal procaspase-9 cleavage preceded maximal procaspase-3 proteolytic processing. These results indicated that apicidin-induced caspase-3 activation was associated with caspase-9 activation.

Effect of Apicidin on Bax Translocation and Cytochrome c Release—Previous studies with several models of apoptosis have shown that Bax translocates from the cytosol to the mitochondria when overexpressed or in response to certain cell death stimuli. Moreover, translocation of Bax to the mitochondria in a number of systems has been suggested to be responsible for the release of cytochrome *c* from the mitochondria to the cytosol and the activation of apoptosis (41, 42). To examine whether this pathway was activated by apicidin, we investigated the effect of apicidin on Bax translocation and cytochrome *c* release. Thus, apicidin-treated HL60 cells were collected and fractionated into cytosolic and mitochondrial fractions, and the distribution of Bax and cytochrome *c* in these fractions was examined by Western blot analysis using an anti-Bax polyclonal and anti-cytochrome *c* monoclonal antibody. As shown in Fig. 6*A*, increasing amounts of Bax were detected in the mitochondria in a time-dependent manner after the addition of apicidin to HL60 cells, concomitant with the decrease of Bax in the cytosol fraction. At the same time, cytochrome *c* in the cytosol fraction markedly increased in a time-dependent manner, correspondent with the decrease of cytochrome *c* in the mitochondrial fraction (Fig. 6*B*). The time course of cytochrome *c* release into the cytosol correlated well with the appearance of apoptotic cells and the activation of caspase-3. These results suggested that apicidin induced translocation of Bax to the mitochondria, subsequently releasing cytochrome *c* preferentially into the cytosol of HL60 cells. Although previous studies have also shown that alternations in the ratio between proapoptotic and antiapoptotic members of the Bcl-2 family rather than the absolute expression levels of any single Bcl-2 family members can determine apoptotic sensitivity (43, 44), apicidin failed to alter the ratio as well as change absolute expression levels of Bcl-2 and Bax protein (data not shown).

Induction of Fas and Fas Ligand by Apicidin and Its Effect on Cytochrome c Release—To assess a possibility that synthesis of new proteins was required for apicidin-induced apoptosis, HL60 cells were preincubated for 1 h with 1 μg/ml cycloheximide, protein synthesis inhibitor, followed by the addition of apicidin for 24 h. Cycloheximide almost completely inhibited apicidin-induced conversion of procaspase-3 (p32) to the cata-

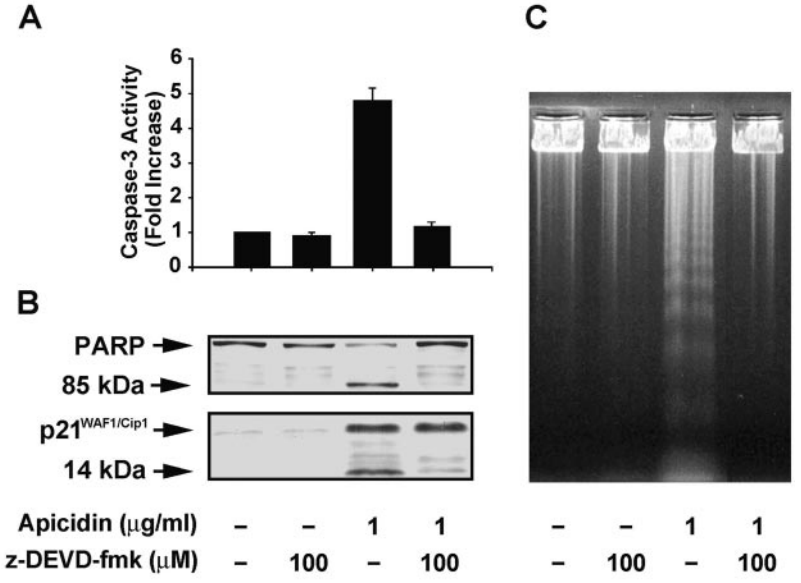

FIG. 4. **Effect of caspase-3 inhibitor on cleavage of PARP and p21$^{WAF1/Cip1}$, and DNA fragmentation.** *A*, HL60 cells were pretreated with 100 μM z-DEVD-fmk for 2 h and then treated with 1 μg/ml apicidin for 48 h. The cell lysates were incubated with Ac-DEVD-pNA for 1 h at 37 °C. Caspase-3 activity was calculated relative to the activity in 0.1% Me_2SO-treated cell lysates. Data are expressed as mean ± S.D. from three separate experiments in duplicate. *B*, the cleavages of PARP and p21$^{WAF1/Cip1}$ were examined by Western blot analysis using the cell lysates prepared from the experiments in *A*. *C*, HL60 cells were pretreated with 100 μM z-DEVD-fmk for 2 h and then treated with 1 μg/ml apicidin for 48 h. Apoptotic DNA fragmentation was visualized by ethidium bromide staining.

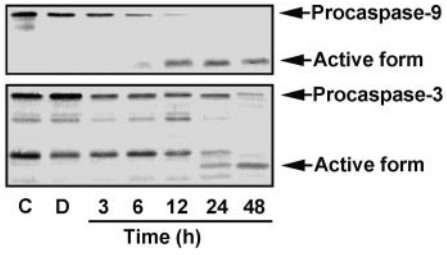

FIG. 5. **Time course analysis of caspase activation by cleavage of procaspase-9 and procaspase-3.** At indicated time point after treatment with 1 μg/ml apicidin, cells were lysed, and 50 μg of protein were subjected to SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed with anti-caspase-9 antibody and anti-caspase-3 antibody. *C*, control group; *D*, 0.1% Me_2SO-treated group.

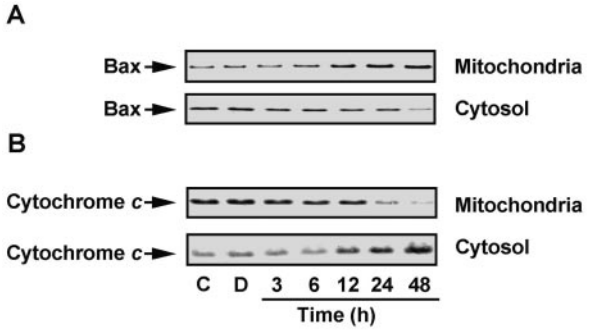

FIG. 6. **Effect of apicidin on Bax translocation and cytochrome *c* release.** HL60 cells were treated with 1 μg/ml apicidin for the time intervals indicated. Cytosolic and mitochondrial fractions were prepared as described under "Experimental Procedures." *A*, translocation of Bax from the cytosol to the mitochondria was analyzed by Western blotting with anti-Bax antibody. *B*, cytochrome *c* release from the mitochondria to the cytosol was analyzed with anti-cytochrome *c* antibodies. *C*, control group; *D*, 0.1% Me_2SO-treated group.

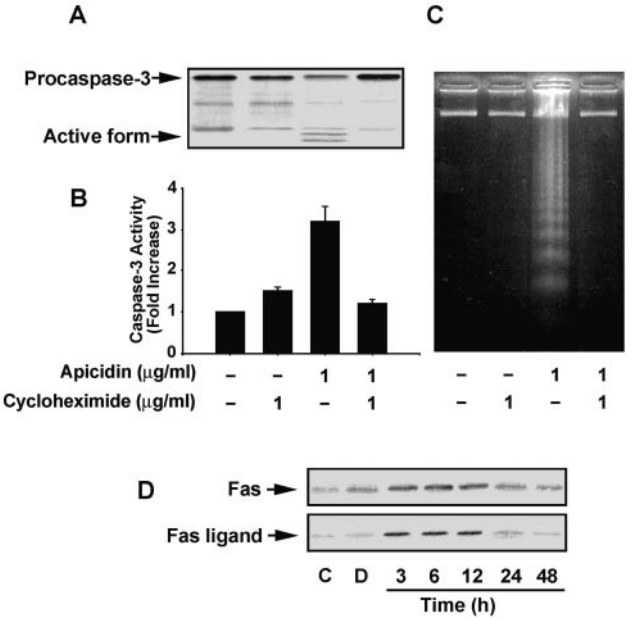

FIG. 7. **Induction of Fas and Fas ligand gene expression by apicidin.** *A*, HL60 cells were pretreated with 1 μg/ml cycloheximide for 1 h followed by treatment with 1 μg/ml apicidin for 48 h. Cleavage of procaspase-3 was analyzed by Western blotting. *B*, caspase-3 activity was directly assayed using Ac-DEVD-pNA, a substrate for caspase-3 *in vitro*. Data are expressed as the means ± S.D. from three separate experiments in duplicate. *C*, after pretreatment with cycloheximide for 1 h, apicidin-induced DNA fragmentation was visualized by ethidium bromide staining. *D*, at the indicated time point after treatment with 1 μg/ml apicidin, cells were lysed, and 50 μg of protein was run on a 15% SDS-PAGE gel, blotted, and proved with an antibody against anti-Fas and anti-Fas ligand. *C*, control group; *D*, 0.1% Me_2SO-treated group.

lytically active caspase-3 (p17) (Fig. 7*A*), apicidin-induced caspase-3 activity (Fig. 7*B*), and DNA fragmentation (Fig. 7*C*), indicating that *de novo* protein synthesis was required for apicidin-induced apoptosis.

Fas is a 45-kDa member of tumor necrosis factor receptor superfamily. This cell surface receptor together with its cognate ligand, Fas ligand, forms an important system of apoptotic initiation (45). To further evaluate *de novo* protein synthesis required for apicidin-induced apoptosis, we investigated Fas/Fas ligand, the best characterized death receptor/death ligand with respect to its signal transduction pathway (46, 47). Treatment of HL60 cells with apicidin caused transient expression of Fas and Fas ligand, with maximal expression at 6 h, and slow decrease in the expression levels of Fas and Fas ligand ensued within 48 h (Fig. 7*D*). Thus, we hypothesized that the secretion and autocrine/paracrine engagement of Fas ligand with Fas at the cell surface are associated with the induction of apoptosis via cytochrome *c* release by apicidin. To test this possibility, before the addition of apicidin, the cells were incubated with the NOK-1 mAb that interferes with the Fas-Fas ligand inter-

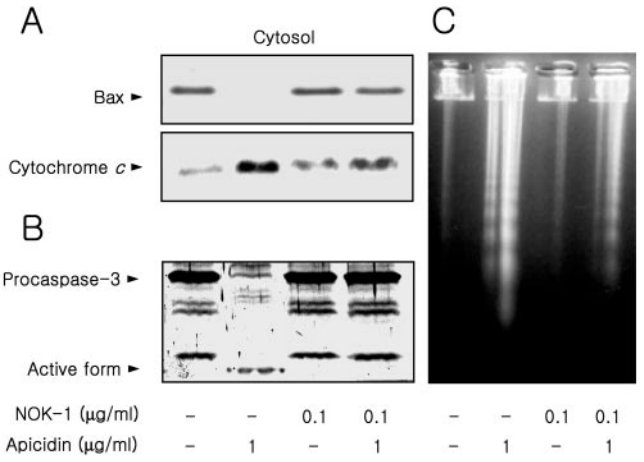

FIG. 8. **Involvement of Fas/Fas ligand in apicidin-induced apoptosis.** *A*, after pretreatment with 0.1 μg/ml NOK-1 mAb for 1 h, HL60 cells were treated with 1 μg/ml apicidin for 48 h. Cytosolic fractions were prepared as described under "Experimental Procedures." Bax translocation and cytochrome *c* release were analyzed with anti-Bax and anti-cytochrome *c* antibodies, respectively. *B*, also, cleavage of procaspase-3 in cell lysate was analyzed by Western blotting. *C*, HL60 cells were pretreated with 0.1 μg/ml NOK-1 mAb for 1 h and then treated with 1 μg/ml apicidin for 48 h. Apoptotic DNA fragmentation was visualized by ethidium bromide staining.

action and is inhibitory to Fas signaling (48). Preincubation with NOK-1 mAb blocked apicidin-induced translocation of Bax from the cytosol to the mitochondria, cytochrome *c* release from the mitochondria to the cytosol, cleavage of procaspase-3, and DNA fragmentation (Fig. 8). In contrast, NOK-1 mAb alone had no effect. Taken together, the results suggested that apicidin might induce apoptosis through selective induction of Fas/Fas ligand, resulting in cytochrome *c* release from the mitochondria to the cytosol and subsequent activation of caspase-9 and caspase-3. Consistent with these results, m-carboxycinnamic acid bishydroxamide, another HDAC inhibitor, has been shown to induce Fas and Fas ligand in human neuroblastomas, resulting in apoptosis (17).

In summary, we demonstrated here that apicidin, a HDAC inhibitor, was a potent apoptotic agent in HL60 cells. Apicidin treatment caused cell death, probably due to the induction of apoptosis, which was paralleled with *in vivo* accumulation of histone H4. This was further supported by the observation that cell death by apicidin was accompanied by DNA fragmentation, nuclear morphological change, and accumulation of sub-G_1 fraction. Apicidin-induced apoptosis of HL60 cells appeared to be through the activation of caspase-3 by cleavage of procaspase-3 and subsequent cleavage of PARP and $p21^{WAF1/Cip1}$, which play an important role in apoptosis (38, 39, 49, 50).

Various HDAC inhibitors including depudecin (10), FR901228 (11), trapoxin (8), oxamflatin (12), and trichostatin A (9) have been shown to induce selective expression of various genes through hyperacetylation of histone. Among them, $p21^{WAF1/Cip1}$ has been shown to be responsible for the cell cycle arrest of many cancer cells (12–14). In addition, it has recently been shown that caspase-3-mediated cleavage of $p21^{WAF1/Cip1}$ could convert cancer cells from growth arrest to undergoing apoptosis (15–17). As observed in HeLa cells (14), apicidin treatment of HL60 cells up-regulated the expression of $p21^{WAF1/Cip1}$, and the induced $p21^{WAF1/Cip1}$ was subsequently cleaved to a 14-kDa fragment, therefore suggesting that selective induction of $p21^{WAF1/Cip1}$ and its subsequent cleavage by apicidin could, in part, contribute to the induction of apoptosis in HL60 cells. Furthermore, apicidin treatment led to an increased translocation of Bax into the mitochondria, thereby leading to the release of cytochrome *c* from the mitochondria to the cytosol and cleavage of procaspase-9. The time course of Bax translocation and cytochrome *c* release correlated well with the cleavage of procaspase-9 and procaspase-3, indicating the involvement of the mitochondrial compartment in apicidin-induced apoptosis.

In addition to the induction of $p21^{WAF1/Cip1}$, apicidin appeared to require the expression of other proteins for the induction of apoptosis, since cycloheximide treatment greatly inhibited apicidin activation of caspase-3 by interfering with cleavage of procaspase-3 and DNA fragmentation. Indeed, apicidin transiently increased the expression levels of both Fas and Fas ligand, which can initiate the apoptotic signaling pathway. Furthermore, apicidin-induced translocation of Bax, cytochrome *c* release, cleavage of procaspase-3, and DNA fragmentation were antagonized by pretreatment with NOK-1 mAb, suggesting that the expression of Fas and Fas ligand is involved in the induction of apoptosis via cytochrome *c* release by apicidin. However, NOK-1 mAb could not completely suppress DNA fragmentation by apicidin, indicating the possible involvement of Fas/Fas ligand-independent pathway. Consistent with these results, the previous study has shown that m-carboxycinnamic acid bishydroxamide, a HDAC inhibitor, can induce Fas and Fas ligand (17). Therefore, these results suggest that inhibition of HDAC activity by apicidin is closely associated with inductions of $p21^{WAF1/Cip1}$ and Fas and Fas ligand, cytochrome *c* release into the cytosol, and activation of caspase-9 and caspase-3, thereby leading to induction of apoptosis. To our knowledge, the results we present here are the first study elucidating the possible link between apicidin-induced apoptosis and *de novo* protein synthesis of Fas/Fas ligand. Thus, apicidin appears to be a potential therapeutic agent for the treatment of human acute promyelocytic leukemia. Its therapeutic potential could be further supported by a recent study that a patient with recurrent retinoid-resistant acute promyelocytic leukemia achieved complete clinical and cytogenetic remission through the addition of a HDAC inhibitor to all-*trans*-retinoic acid therapy (51). Taken together, our results provide an insight into the mechanism of apoptosis via a mitochondrial/cytochrome *c*-dependent pathway resulting from *de novo* protein synthesis by apicidin.

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