Induction of Apoptosis in Human Myeloid Leukemic Cells by 1’-Acetoxychavicol Acetate through a Mitochondrial- and Fas-Mediated Dual Mechanism

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ABSTRACT

Purpose: The purpose of this investigation was to determine the antileukemic effects of 1’-acetoxychavicol acetate (ACA) obtained from rhizomes of the commonly used ethno-medicinal plant Languas galanga (Zingiberaceae).

Experimental Design: We evaluated the effects of ACA on various myeloid leukemic cells in vitro and in vivo. We further examined the molecular mechanisms of ACA-induced apoptosis in myeloid leukemic cells.

Results: Low-dose ACA dramatically inhibited cellular growth of leukemic cells by inducing apoptosis. Because NB4 promyelocytic leukemic cells were most sensitive to ACA, we used NB4 cells for further analyses. Production of reactive oxygen species triggered ACA-induced apoptosis. ACA-induced apoptosis in NB4 cells was associated with the loss of mitochondrial transmembrane potential (ΔΨm) and activation of caspase-9, suggesting that ACA-induced death signaling is mediated through a mitochondrial oxygen stress pathway. In addition, ACA activated Fas-mediated apoptosis by inducing of caspase-8 activity. Pretreatment with the thiol antioxidant N-acetyl-L-cysteine (NAC) did not inhibit caspase-8 activation, and the antagonistic anti-Fas antibody ZB4 did not block generation of reactive oxygen species, indicating that both pathways were involved independently in ACA-induced apoptosis. Furthermore, ACA had a survival advantage in vivo in a nonobese diabetic/severe combined immunodeficient mice leukemia model without any toxic effects.

Conclusions: We conclude that ACA induces apoptosis in myeloid leukemic cells via independent dual pathways. In addition, ACA has potential as a novel therapeutic agent for the treatment of myeloid leukemia.

INTRODUCTION

1’-Acetoxychavicol acetate (ACA) is present in seeds and rhizomes of Languas galanga (Zingiberaceae), which is used as a traditional condiment in Thailand and other countries in Southeast Asia (1). Recently, it has been reported that ACA inhibits chemically induced tumor formation and potently suppresses cellular growth of Ehrlich ascites tumor cells (2, 3). Other studies have demonstrated that this compound suppresses tumor promoter-induced EBV activation in vitro (1); ACA has subsequently been shown to inhibit skin tumor promotion in mice and both colonic aberrant crypt foci and adenocarcinoma formation in rats (4–7). ACA is also known to reduce superoxide anion production by inhibiting the xanthine oxidase and NADPH oxidase systems (7, 8), and this activity has been suggested to be partly responsible for its cancer chemopreventive effects (9). However, the mechanism of ACA-induced apoptosis remains unclear, and the effects of ACA on human leukemic cells have never been investigated.

Caspases are believed to play a crucial role in mediating various apoptotic responses. A model has been proposed in which two different caspases, caspase-8 and -9, mediated distinct types of apoptotic stimuli (10, 11). The cascade led by caspase-8 is involved in death receptor-mediated apoptosis such as the one triggered by Fas (also known as APO-1 or CD95). The death receptor, Fas, contains an intracytoplasmic death domain that mediates interactions with the adapter molecule Fas-associated death domain (FADD). FADD associates, in turn, with procaspase-8. Ligation of Fas by Fas ligand results in sequential recruitment of FADD and procaspase-8 to the death domain of Fas to form the death-inducing signaling complex, leading to cleavage of procaspase-8, with the consequent generation of active caspase-8. Caspase-8 then activates downstream effector caspases through cleavage of Bid, committing the cell to apoptosis (12). The mechanisms by which antineoplastic cytotoxic drugs kill leukemic cells are not well understood, although the activation of caspase-3 has been involved in the important signaling pathway of apoptosis (13). Recent observations have resulted in the suggestion that interactions between Fas and Fas ligand may mediate cytotoxic killing of at least some leukemic cell lines (14). On the other hand, the caspase pathway headed by caspase-9 is thought to mediate cytotoxic drug-induced apoptosis. Cytotoxic drugs induce generation of reactive oxygen species (ROS), leading to loss of...
mitochondrial transmembrane potential ($\Delta \Psi_m$) and the release of apoptogenic proteins such as cytochrome $c$ and Smac/DIABLO from the mitochondria into the cytosol. The subsequent interaction of cytochrome $c$ with Apaf-1 protein results in the recruitment of procaspase-9. Activation of procaspase-9 within this multiprotein complex, the apoptosome, results in the processing of caspase-3 and subsequently contributes to apoptotic cell death (15–17). The link between caspase-8 and caspase-9 is provided by caspase-8 cleavage of the proapoptotic Bid protein, whose truncated form is inserted into the mitochondrial outer membrane and promotes cytochrome $c$ release and consequent activation of the apoptosome (18, 19). Anticancer drug-induced apoptosis was generally mediated through either a caspase-8 or caspase-9 pathway.

In the present study, we showed for the first time that a natural product, ACA, dramatically inhibits viability of myeloid leukemic cells via the induction of apoptosis through two different pathways. We further investigated the molecular mechanisms of ACA-induced cell cycle arrest and apoptosis in myeloid leukemic cells in vitro, as well as survival advantage with ACA in vivo.

**MATERIALS AND METHODS**

**Cell Cultures.** The NB4 promyelocytic leukemia cell line was a gift of Dr. M. Lanotte (Hôpital St. Louis, Paris, France; Ref. 20), and the retinoic acid-resistant acute promyelocytic leukemia cell line UF-1 was established in our laboratory from a relapsed patient with acute promyelocytic leukemia who was treated with all-trans-retinoic acid (21). The human leukemic cell lines including HL-60 and K562 were obtained from the Japan Cancer Research Resources Bank (Tokyo, Japan). Bone marrow samples from patients with acute myeloid leukemia (AML) and mononuclear cells from a healthy donor were obtained according to appropriate Human Protection Committee validation at Keio University School of Medicine (Tokyo, Japan) and with written informed consent. Morphology was evaluated from cytopsin slide preparations with Giemsa staining, and viability was assessed by trypsin blue dye exclusion. Cells were maintained in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) with 10% fetal bovine serum (Life Technologies, Inc.), 100 units/ml penicillin, and 100 $\mu$g/ml streptomycin in a humidified atmosphere with 5% CO$_2$. UF-1 cells were grown in RPMI 1640 with 15% fetal bovine serum (Hyclone Laboratory, Logan, UT) under standard culture conditions.

**Reagents.** ACA was synthesized as described previously (1) and dissolved in DMSO at a stock concentration of 20 mM. Synthetic (1$R$,S')-ACA (Fig. 1) has an identical suppressive activity to natural (1'S)-ACA as evaluated by tumor promoter-induced EBV activity (22). The pan-caspase inhibitor Z-VAD-FMK (Calbiochem, La Jolla, CA) and caspase-8 and -9 inhibitors [Z-IETD-FMK and LEHD-CHO, respectively (MBL, Nagoya, Japan)] were dissolved in DMSO (Sigma, St. Louis, MO) at a stock concentration of 100 mM, stored at $-20^\circ$C, and protected from light. The final DMSO concentrations in the medium were not greater than 0.1%. N-acetyl-L-cysteine (NAC) and buthionine sulfoximine were purchased from Sigma.

**Cell Cycle Analysis.** Cells (1 x 10$^6$) were suspended in hypotonic solution [0.1% Triton X-100, 1 mM Tris-HCl (pH 8.0), 3.4 mM sodium citrate, and 0.1 mM EDTA] and stained with 50 $\mu$g/ml propidium iodide. DNA content was analyzed by FACSCalibur (Becton Dickinson, San Jose, CA). The population of cells in each cell cycle phase was determined using Cell ModFIT software (Becton Dickinson).

**Assays for Apoptosis.** Apoptosis was determined based on morphological change. Apoptotic cells were quantified by annexin V-FITC and propidium iodide double staining using a staining kit purchased from PharMingen (San Diego, CA). Induction of apoptosis was also detected by DNA fragmentation assay. The $\Delta \Psi_m$ was determined by flow cytometry using rhodamine 123 (Sigma). Briefly, cells were washed twice with PBS and incubated with 1 $\mu$g/ml rhodamine 123 at 37$^\circ$C for 30 min. Rhodamine 123 intensity was determined by flow cytometry.

**Caspase Assays.** Caspase-related protease activity was determined by using a commercially available kit (PharMingen) according to the manufacturer’s instructions. Briefly, cells were fixed and permeabilized using the Cytofix/Cytoperm for 20 min at 4$^\circ$C, pelleted, and washed with Perm/Wash buffer (PharMingen). Cells (1 x 10$^6$) were then stained with polyclonal antibody against the active form of caspase-3, -9, and -8 (0.25 mg/liter; PharMingen) for 20 min at room temperature; washed in Perm/Wash buffer; stained with goat antirabbit FITC (Super Techs, Bethesda, MD); and analyzed via flow cytometry. For caspase inhibitor assay, cells were pretreated with a synthetic pan-caspase inhibitor (20 mM Z-VAD-FMK) and caspase-8 and -9 inhibitors (50 mM Z-IETD-FMK and LEHD-CHO, respectively) for 1 h before the addition of ACA.

**Measurement of ROS Production.** To assess the generation of ROS, control and ACA-treated cells were incubated with 5 $\mu$M dehydroxy ethidium (Molecular Probes, Eugene, OR), which is rapidly oxidized to a fluorescent intercalator, ethidium, by cellular oxidants. Cells (1 x 10$^6$) were stained with polyclonal antibody against the active form of caspase-3, -9, and -8 (50 mM Z-IETD-FMK and LEHD-CHO, respectively) for 15 min at 37$^\circ$C and then washed and resuspended in PBS. The oxidative conversion of dehydroxy ethidium to ethidium was analyzed by flow cytometry. To measure intracellular glutathione (GSH) levels, cells (1 x 10$^6$) were stained with 20 $\mu$M 5-chloromethyl fluorescein diacetate (Molecular Probes) for 30 min at 37$^\circ$C and then analyzed by flow cytometry.

**Cell Lysate Preparation and Western Blotting.** Cells were collected by centrifugation at 700 x g for 10 min, and then the pellets were resuspended in lysis buffer [1% NP40, 1 mM phenylmethylsulfonyl fluoride, 40 mM Tris-HCl (pH 8.0), and 150 mM NaCl] at 4$^\circ$C for 15 min. Mitochondrial and cytosolic fractions were prepared with digitonin-nagarse treatment. Protein concentrations were determined using a DC protein assay system (Bio-Rad, Richmond, CA). Cell lysates (15 $\mu$g protein/
lane) were fractionated in 12.5% or 7.5% SDS-polyacrylamide gels before being transferred to Immobilon-P membrane (Millipore, Bedford, MA) according to a standard protocol. Antibody binding was detected by using the enhanced chemiluminescence kit with hyper-enhanced chemiluminescence film (Amersham, Buckinghamshire, United Kingdom). β-Actin was used as an indicator for equality of lane loading. The following antibodies were used in this study: anti-caspase-3, anti-caspase-8, anti-cytochrome c, and anti-Rb (PharMingen); anti-cyclin B (Cell Signaling Technology, Inc., Beverly, MA); anti-Fas and anti-Bid (MBL); and anti-Bcl-2, anti-BAX, anti-p21cip1/waf1, anti-p27kip1, anti-poly(ADP-ribose) polymerase, and anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA).

**Assays for Fas.** We analyzed the expression of Fas by incubating cells with monoclonal anti-Fas-FITC antibody (UB2; Immunotech, Nottingham, United Kingdom) or control mouse IgG1 FITC antibody for 30 min at 4°C and then analyzing them by flow cytometry. For Fas inhibitor assay, antagonistic ZB4 monoclonal antibody (MBL) was added at 250 ng/ml 1 h before treatment with 10 μM ACA or 200 ng/ml agonistic anti-Fas antibody (CH11; MBL).

**Immunoprecipitation.** After treatment with ACA, cells (1.5 × 10⁶) were washed twice in PBS and incubated with the cleavable cross-linker 3,3'-dithiobis[sulfsuccinimidyl-propionate] (Pierce Chemical Co., Rockford, IL) for 10 min at 4°C. The reaction was stopped by a 5-min incubation in PBS containing 10 mM ammonium acetate at 4°C. Cells were washed twice in PBS and lysed in lysis buffer for 15 min on ice. After centrifugation at 17,800 × g for 15 min, an anti-human FADD antibody (MBL) was added and reacted at 4°C overnight. Immune complexes were precipitated using protein A-Sepharose (Pharmacia Co., Orsay, France) and washed three times in lysis buffer. The precipitate was resuspended in Laemmli buffer containing 2.5% β-mercaptoethanol and boiled for 5 min. Samples were fractionated on 12.5% SDS-polyacrylamide gels before being transferred to Immobilon-P membranes (Millipore) according to a standard protocol. Caspase-8 content was analyzed by using antihuman caspase-8 antibody (PharMingen). To monitor loading of protein samples, the same membranes were reprobed with an anti-FADD antibody.

**Animal Model and Experimental Design.** We have established a human all-trans-retinoic acid-sensitive acute promyelocytic leukemia cell line, NB4, which is sensitive to 1′-Acetoxychavicol acetate (ACA). We have used this cell line to study the effects of ACA on cell growth and apoptosis. We have shown that ACA induces G0-G1 cell cycle arrest followed by apoptosis. The cell viability was assessed by trypan blue dye exclusion. Results are expressed as the mean of three duplicate experiments, and the SD was within 5% of the mean. Cell cycle analysis was performed using flow cytometry. DNA content was analyzed by means of flow cytometry. G0-G1, G2-M, and S indicate the cell phase, and sub-G1 DNA content refers to the portion of apoptotic cells. Each phase was calculated using the ModFit program. Three duplicate experiments were performed with similar results. DNA content was analyzed by means of flow cytometry. G0-G1, G2-M, and S indicate the cell phase, and sub-G1 DNA content refers to the portion of apoptotic cells. Each phase was calculated using the ModFit program. Three duplicate experiments were performed with similar results. DNA content was analyzed by means of flow cytometry. G0-G1, G2-M, and S indicate the cell phase, and sub-G1 DNA content refers to the portion of apoptotic cells. Each phase was calculated using the ModFit program. Three duplicate experiments were performed with similar results.
elocytic leukemia model in a nonobese diabetic (NOD)/severe combined immunodeficient mice system using NB4 cells (23). Briefly, NOD/severe combined immunodeficient mice (The Jackson Laboratory, Bar Harbor, ME) were pretreated with 3 Gy of total body irradiation, which is a sublethal dose that was expected to enhance the acceptance of xenografts. Subsequently, NB4 cells (10^7) in their logarithmic growth phase were inoculated s.c. into NOD/severe combined immunodeficient mice. Inoculated NB4 cells formed s.c. tumors at the injection site, and cells grew rapidly. Fourteen days after implantation of the cells, mice with the transplanted cells were randomly assigned to be injected with PBS (n = 15) or 3 mg/kg ACA (n = 15) via i.p. injection for 3 days. The study was approved by the Animal Care and Use Committee at the Keio University School of Medicine. Mice were routinely monitored to determine their general condition, and survival times were used to determine therapeutic efficacy. When the mice showed severe wasting, or when observations were finished, mice were sacrificed according to the United Kingdom Coordinating Committee on Cancer Research guidelines, and the day of sacrifice was recorded (24).

Results

Effects of ACA on Cellular Proliferation of Various Leukemia Cells. We first investigated the effects of ACA on cellular proliferation in four myeloid leukemic cell lines [NB4, UF-1, HL-60, and K562]. ACA (10 μM) inhibited cellular proliferation in various myeloid leukemia cell lines. Fig. 3 Effects of 1'-acetoxychavicol acetate (ACA) on caspase activation. A, cells were incubated with 10 μM ACA for 3 h and analyzed for activities of various caspases by fluorescence-activated cell-sorting analysis and colorimetric assay. ACA treatment (■) significantly increased caspases-3, -8, and -9 activities. Combination of ACA and each caspase inhibitor (□) blocked caspase activities. B, time course of caspase-3, -8, and -9 cleavage in ACA-treated NB4 cells. Cells were cultured with 10 μM ACA for the indicated times and analyzed by immunoblotting with anti-caspase-3, -8, and -9 antibodies. C, effects of caspase inhibitors on ACA-treated NB4 cells. Inhibition of ACA-induced apoptosis of NB4 cells was estimated in coculture with a series of caspase inhibitors. Cells were preincubated with each caspase inhibitor [20 μM Z-VAD-FMK (pan-caspase inhibitor), 50 μM Z-IETD-FMK (caspase-8 inhibitor), and 50 μM LEHD-CHO (caspase-9 inhibitor)] for 1 h before the addition of 10 μM ACA. For A and C, values represent the mean ± SD for three separate experiments performed in triplicate. D, effects of caspase inhibitors on ACA-induced caspase cleavage. Cells were preincubated with 50 μM Z-IETD-FMK and 50 μM LEHD-CHO for 1 h and then cultured with 10 μM ACA for 6 h. Protein lysates were separated with 10% SDS-PAGE and analyzed by blotting with anti-caspase-8 and -9 or β-actin antibodies.
growth of all myeloid leukemic cells, but not normal bone marrow mononuclear cells from a healthy donor, in a dose- and time-dependent manner (Fig. 2, A and B). Interestingly, ACA decreased cellular growth of NB4 cells with the lowest IC₅₀ (4.72 μM). Therefore, we used NB4 cells for subsequent investigations. Cultivation with ACA rapidly increased the population of cells in the G₀-G₁ phase with a reduction of cells in S phase, and a strong induction of apoptosis was shown by the appearance of a hypodiploid DNA peak with sub-G₁ DNA content 6 h after treatment (Fig. 2C). Apoptosis was assessed in terms of both morphological changes and DNA ladder formation. DNA fragmentation was confirmed by electrophoresis of genomic DNA extracted from NB4 cells treated with 10 μM ACA for 4 h (Fig. 2D). These results indicate that ACA led to cell cycle arrest at the G₀-G₁ phase followed by apoptosis.

**Effects of ACA on Caspase Activity.** Treatment with ACA for 3 h significantly induced caspase-3, -8, and -9 activities in NB4 cells (Fig. 3A). Various caspase inhibitors blocked caspase activities (Fig. 3A). We also found that ACA induced caspase-3, -8, and -9 activation as assessed by cleavage of proforms into the active cleaved forms using polyclonal anti-human caspase-3, -8, and -9 antibodies (Fig. 3B). Interestingly, ACA-induced caspase-8 activation was rapid, occurring within 3 h of treatment and before activation of caspase-3 and -9 (Fig. 3B). NB4 cells were treated with 10 μM ACA for 24 h, either alone or in combination with 20 μM Z-VAD-FMK (pan-caspase inhibitor), 50 μM Z-IETD-FMK (caspase-8 inhibitor), or 50 μM LEHD-CHO (caspase-9 inhibitor). Each caspase inhibitor alone did not have any effect on the proliferation of NB4 cells. ACA-induced apoptosis was almost completely blocked by treatment with Z-VAD-FMK, although Z-IETD-FMK and LEHD-CHO partially inhibited ACA-induced apoptosis in NB4 cells (Fig. 3C). Z-IETD-FMK and LEHD-CHO inhibited ACA-induced caspase-8 and -9 cleavage, respectively (Fig. 3D). These results suggest that ACA-induced apoptosis is associated with the activation of caspase-3, -9, and -8.

**ACA-Induced Death Signaling Is Mediated through the Mitochondrial Pathway.** After treatment with 10 μM ACA for 3 h, low rhodamine 123 staining in NB4 cells indicated an increase in the loss of ΔΨm in a time-dependent manner (Fig. 4A). ACA also induced a substantial release of cytochrome c and Smac/DIABLO from the mitochondria into the cytosol (Fig. 4B). In addition, ACA induced a translocation of Bax from the cytosol to the mitochondria within 3 h, whereas the protein accumulated in the mitochondria (Fig. 4B). These results suggest that the mitochondrial-dependent pathway plays an important role in ACA-induced apoptosis.

**Expression of Cell Cycle- and Apoptosis-Associated Proteins in ACA-Treated NB4 Cells.** To characterize the molecular mechanism of ACA-induced cell cycle arrest followed by apoptosis in NB4 cells, we examined the expression of cell cycle- and apoptosis-associated proteins during treatment with
ACA. Expression of p21\textsuperscript{WAF1/CIP1}, p27\textsuperscript{KIP1}, and Fas was increased in a time-dependent manner with dephosphorylation of Rb, reduction of cyclin D1 and B1 protein expression, and cleavage of poly(ADP-ribose) polymerase (Fig. 5). In contrast, ACA did not affect the expression of Bcl-2 or Bid protein (Fig. 5).

Effects of ACA on Intracellular ROS Generation. Within 0.5 h, NB4 cells treated with 10 \mu M ACA showed an increase in intracellular ROS compared with control cells (Fig. 6A), corresponding to the reduction of intracellular GSH (Fig. 6B); 1 mM buthionine sulfoximine, a specific inhibitor of \gamma-glutamylcysteine synthetase, induced GSH depletion and synergically enhanced ACA-induced apoptosis (Fig. 6C). Treatment with a thiol antioxidant, NAC, an excellent supplier of GSH, attenuated but did not completely inhibit ACA-induced apoptosis with complete inhibition of ROS generation in NB4 cells (Fig. 6D). These results indicate that ROS generation plays a partial role in ACA-induced apoptosis, but another pathway may also contribute to the ACA-induced apoptotic pathway in NB4 cells.

ACA Activates Fas/Fas Ligand-Mediated Apoptosis in NB4 Cells. ACA rapidly activated caspase-8 and -9, and each specific inhibitor partially blocked ACA-induced apoptosis in NB4 cells, respectively (Fig. 3B). For that reason, we investigated whether the Fas-mediated pathway was involved in ACA-induced apoptosis. Suppression of Fas by 12-h exposure to antagonistic anti-Fas antibody ZB4 dramatically inhibited ACA-induced apoptosis, similar to that of agonistic anti-Fas antibody (CH11) in NB4 cells (Fig. 7, A and B). However, longer exposure (24 h) to ZB4 partially blocked ACA-induced cell proliferation (Fig. 7A). CH11-induced apoptosis in NB4 cells was completely blocked by ZB4 (Fig. 7, A and B). Corresponding to these results, expression of Fas on the plasma membrane was significantly increased immediately after ACA treatment (Fig. 7C) with induction of Fas ligand (data not shown). The death-inducing signaling complex was also formed in NB4 cells treated with ACA (Fig. 7D). These results indicate that the apoptotic

Fig. 6 Effect of 1′-acetoxychavicol acetate (ACA) on generation of reactive oxygen species. A, NB4 cells were treated with 10 \mu M ACA for the indicated times, after which cells were labeled with an oxidative-sensitive dye (dehydroxy ethidium) and analyzed by flow cytometry to determine the percentage of cells displaying an increase in production of reactive oxygen species. B, generation of glutathione in ACA-treated NB4 cells. Cells were treated with 20 \mu M 5-chloromethyl fluorescein acetate for 30 min and then analyzed by flow cytometry. C, effects of buthionine sulfoximine on ACA-treated NB4 cells. Cells were treated with 5 or 10 \mu M ACA and then incubated without (■) or with (□) 1 mM buthionine sulfoximine for 12 h. Cell viability was assessed by trypan blue dye exclusion. D, cells were pretreated with 100 \mu M N-acetyl-L-cysteine and then treated with 10 \mu M ACA for 12 h, and induction of apoptosis was examined by annexin V/propidium iodide double staining.
pathway related to Fas/Fas ligand also seems to be involved in ACA-induced apoptosis.

ACA-Induced Apoptosis in NB4 Cells Mediated through Dual Pathways. Because Bid protein, which is known to link caspases-8 and -9, was not cleaved during treatment with ACA (Fig. 5), we hypothesized that Fas/Fas ligand- and ROS-dependent pathways are independently activated during ACA-induced cell death. We thus investigated the effects of antagonistic anti-Fas antibody ZB4 on ROS generation and the effects of NAC on the enzymatic activity of caspase-8. Inhibition of the Fas pathway by ZB4 did not affect ROS generation, and the activity of caspase 8 was not altered during NAC treatment (Fig. 8, A and B). In addition, costimulation of ZB4 and NAC additively inhibited ACA-induced apoptosis (Fig. 8C), indicating that ROS generation was completely independent of the Fas/Fas ligand-mediated pathway.

Effects of ACA on Primary Samples from Patients with AML. We examined the effects of ACA on primary AML cells from 10 patients (Table 1). Representative cases of ACA-induced apoptosis and generation of ROS are shown in Fig. 9, A

Fig. 7 1'-Acetoxychavicol acetate (ACA)-induced apoptosis via the Fas pathway. A, antagonistic anti-Fas antibody blocks ACA-induced apoptosis in NB4 cells. NB4 cells were preincubated for 1 h in the presence of 500 ng/ml ZB4 antagonistic anti-Fas-antibody for 12 h or 24 h and then treated with 10 μM ACA and 250 ng/ml Fas-IgG (CH11), after which cell viability was assessed by trypan blue dye exclusion. Results are expressed as the means ± SD of three duplicate experiments. B, induction of apoptosis was examined by annexin V/propidium iodide double staining. These data represent three separate experiments. C, flow cytometric analysis of Fas expression on the plasma membrane of tumor cells. NB4 cells were treated with 10 μM ACA for 1 h, and Fas expression was determined by flow cytometry using anti-Fas IgG FITC antibody (UB2). We used antimouse IgG1 and IgG2a FITC antibody as the negative control. Results are representative of three duplicate experiments. A representative case is shown in the inset. D, ACA-induced death-inducing signaling complex formation. NB4 cells were stimulated with 10 μM ACA for the indicated times. Proteins were extracted and immunoprecipitated with an antibody directed against Fas-associated death domain. After blotting to nitrocellulose membranes, Western blot analysis was performed using an antibody directed against caspase-8.

Fig. 8. A, antagonistic anti-Fas antibody blocks ACA-induced apoptosis in NB4 cells. NB4 cells were preincubated for 1 h in the presence of 500 ng/ml ZB4 antagonistic anti-Fas-antibody for 12 h or 24 h and then treated with 10 μM ACA and 250 ng/ml Fas-IgG (CH11), after which cell viability was assessed by trypan blue dye exclusion. Results are expressed as the means ± SD of three duplicate experiments. B, induction of apoptosis was examined by annexin V/propidium iodide double staining. These data represent three separate experiments. C, flow cytometric analysis of Fas expression on the plasma membrane of tumor cells. NB4 cells were treated with 10 μM ACA for 1 h, and Fas expression was determined by flow cytometry using anti-Fas IgG FITC antibody (UB2). We used antimouse IgG1 and IgG2a FITC antibody as the negative control. Results are representative of three duplicate experiments. A representative case is shown in the inset. D, ACA-induced death-inducing signaling complex formation. NB4 cells were stimulated with 10 μM ACA for the indicated times. Proteins were extracted and immunoprecipitated with an antibody directed against Fas-associated death domain. After blotting to nitrocellulose membranes, Western blot analysis was performed using an antibody directed against caspase-8.
and B. ACA remarkably inhibited cellular growth of freshly isolated cells from patients with AML by induction of apoptosis corresponding to induction of ROS generation (Fig. 9, A and B) and Fas and Fas ligand expression (data not shown). In contrast, ACA did not affect cellular growth of bone marrow mononuclear cells from healthy volunteers (Fig. 2, A and B). These results strongly supported the finding that induction of both Fas expression and ROS generation plays a crucial role in the effects of ACA on fresh myeloid leukemic cells, and ACA has potential as a treatment for patients with AML.

**Effects of ACA in Vivo.** Our in vitro data prompted us to examine whether the effects of ACA are equally valid in vivo. To evaluate the effect on survival, NB4-transplanted NOD/severe combined immunodeficient mice were treated with ACA (Fig. 10). The survival curves of mice in both groups are shown in Fig. 10, and the difference in survival between the two groups was statistically significant by log-rank test analysis ($P < 0.005$; Fig. 10). As shown in Fig. 10, ACA markedly improved overall survival. During the treatment, ACA-treated mice appeared healthy. In addition, pathological analysis at autopsy revealed no capsaicin-induced tissue changes in any of the organs (data not shown). These results suggest that ACA had no toxic effects on mice during this treatment.

**DISCUSSION**

Natural products have been the mainstay of cancer chemotherapy for the past 30 years (25). For example, the main constituent of the plant *Catharanthus roseus* was the forerunner of the anticancer agents known as the Vinca alkaloids, vinblastine and vincristine. Both drugs were introduced to the clinical field in the late 1960s and have contributed to long-term remissions and cures of testicular teratoma, malignant lymphoma, lymphoblastic leukemia, and many other cancers. Natural products or their structural relatives now comprise about 50% of the drugs that are used for cancer chemotherapy. Therefore, it is an important project to discover novel anticancer agents from natural products through a routine examination of terrestrial plants and microorganisms. There have been a series of studies reporting that ACA, a natural product from edible plants in southeast-
ACA Induces Apoptosis of Leukemic Cells

ACA, 1'-acetoxychavicol acetate; AML, acute myeloid leukemia.

Diagnosis was based on French-American-British classification.

Induction of apoptosis and Fas expression were expressed as fold increase of the percentage of control cells.

Table 1  Baseline characteristics of patients and effect of ACA on primary AML cells

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<th>Patient no.</th>
<th>Age (yrs)</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Blast (%)</th>
<th>Annexin V-positive cells (%)</th>
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Patient characteristics of patients and effect of ACA on primary AML cells.

Induction of apoptosis and Fas expression were expressed as fold increase of the percentage of control cells.

Recently, it has been reported that superoxide dismutases are target molecules of estrogen-induced apoptosis in leukemic cells identified by cDNA microarray assay and that inhibition of superoxide dismutase causes an accumulation of ROS and leads to the release of cytochrome c from the mitochondria (33). Most ACA-sensitive NB4 cells among the various leukemic cells used in this study were reported to have weak activities of antioxidant enzymes including glutathione peroxidase, catalase, and glutathione S-transferase (34, 35). These results suggest that ACA-induced apoptosis in leukemic cells is modulated by the cellular GSH redox system.

The role of membrane death receptor-mediated apoptosis is known to be one of the pathways to caspase-8 activation. On activation of death receptors by their ligation, the death receptors recruit the adapter molecule FADD by the death domain that is also present on FADD, followed by activation of caspase-8. We demonstrated that Fas ligand levels were increased in NB4 cells during treatment with ACA. NB4 cells were sensitive to Fas-induced apoptosis because agonistic anti-Fas antibody CH11 induced apoptosis, and antagonistic anti-Fas antibody ZB4 inhibited the induction of apoptosis in NB4 cells. In addition, ZB4 induced the expression of cell surface Fas in association with reduction in apoptotic cells. Moreover, we demonstrated that up-regulation of Fas ligand expression and formation of death-inducing signaling complex during treatment with ACA corresponded to activation of caspase-8. These results indicate that signaling by the Fas/Fas ligand system plays an important role in the apoptotic killing of NB4 cells by ACA.

The time-response curve of caspase-8 in ACA-treated NB4 cells was similar to that of caspase-9, suggesting that both proteases are active in this apoptotic pathway. Caspase-8 directly activates the downstream caspase, caspase-3. Caspase-9, which is activated by cytochrome c from mitochondria, can also activate caspase-3 (36, 37). In certain cells, the mitochondrial activation-mediated pathway has been shown to be required for Fas-mediated apoptosis (28). In these cells, Bid mediates the release of cytochrome c from mitochondria initiated by caspase-8 activation (18, 38). Therefore, Bid interconnects the extrinsic apoptotic pathway initiated by death receptors to the intrinsic...
pathway of mitochondria-mediated apoptosis (39). In our study, the antioxidant NAC did not affect activation of caspase-8, and antagonistic anti-Fas antibody ZB4 did not inhibit ROS generation. Also, Bid protein was not cleaved by treatment of NB4 cells with ACA. ACA-induced apoptosis was reduced in an additive way when NB4 cells were pretreated with ACA or ZB4. From these results, we conclude that there are two different pathways (for mitochondrial oxidative stress-mediated and Fas-mediated cell death signaling) in ACA-treated NB4 cells.

Acute leukemia is a hematological neoplastic disorder and generally shows aggressive clinical manifestations with poor prognosis in the clinical setting. The therapeutic approach to acute leukemia is basically chemotherapy for achieving complete remission, but side effects and complications such as serious infection due to anticancer drugs are severe problems. In particular, side effects of anticancer drugs might be fatal in older patients or immunocompromised patients. In addition, repeated episodes of relapse of the disease may lead to refractory or chemotherapy-resistant leukemia. Therefore, novel effective therapeutic strategies are actively being sought in the world. A component of a traditional Thai condiment, ACA, is a natural compound, and it appears to be safer than current chemotherapeutic drugs. ACA remarkably inhibited cellular growth of primary cells from patients by the induction of apoptosis, whereas the same dose of ACA did not affect the cellular growth of bone marrow mononuclear cells from healthy volunteers, indicating that the effects of ACA are specific to neoplastic cells. In this study, we demonstrated the anticancer effects of ACA both in vitro and in vivo. Fas receptor is known to be constitutively expressed in the liver, indicating that the liver is very sensitive to Fas-induced apoptosis (40), and mice treated with an agonistic anti-Fas antibody died from hepatic failure caused by generalized apoptosis of hepatocytes (41). However, in our study, we could not observe any organ damage in vivo, suggesting that ACA had no toxic effects on mice during this treatment.

We conclude that ACA might be developed as a new, potent anticancer agent for the management of hematological malignancies. In addition, ACA has potential as a novel therapeutic agent to replace the more cytotoxic agents currently used to treat patients with myeloid leukemia.

REFERENCES

2130 ACA Induces Apoptosis of Leukemic Cells


Induction of Apoptosis in Human Myeloid Leukemic Cells by 1'-Acetoxychavicol Acetate through a Mitochondrial- and Fas-Mediated Dual Mechanism

Keisuke Ito, Tomonori Nakazato, Akira Murakami, et al.