Acacetin induces apoptosis in human T cell leukemia Jurkat cells via activation of a caspase cascade

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Abstract. Flavonoids are naturally occurring antioxidants, with several flavonoids shown to have chemopreventive effects on cancer. We investigated the effects of the flavonoid acacetin on human T cell leukemia Jurkat cells. Acacetin inhibited the proliferation of Jurkat cells by inducing apoptosis in a concentration- and time-dependent manner. Acacetin-induced cell death was characterized by changes in nuclear and cell morphology. Treatment of Jurkat cells with acacetin also induced caspase-3, -8 and -9 activities in a time-dependent manner. Acacetin-induced apoptosis was blocked by a broad-spectrum caspase inhibitor, a caspase-3 inhibitor and a caspase-8 inhibitor, but not by a caspase-9 inhibitor. In addition, acacetin promoted the expression of FAF1, phosphor-FADD, Apaf-1 and cytochrome c. Acacetin-induced apoptosis was also accompanied by upregulation of Bax, and downregulation of Bcl-2. Taken together, these results suggest that acacetin may induce apoptosis in T cell leukemia cells, possibly by activating the Fas-mediated pathway. These findings may help in designing cancer therapeutic and chemopreventive agents.

Introduction

Most of the chemotherapeutic agents presently used in cancer treatment were developed by simple screening for cell growth inhibition without knowing their mechanism of action. Since cancer cells frequently become resistant to these drugs, understanding the mechanism of action of chemotherapeutic agents would be helpful in designing drugs for combination chemotherapy; for determining the lowest concentrations that provide efficacy with fewer side-effects; and to assist in the administration of alternative chemotherapeutic agents. Among the mechanisms by which these drugs inhibit the growth of cancer cells are cell cycle arrest, induction of apoptosis, disruption of microtubules, inhibition of angiogenesis, and increasing oxidative damage (1,2).

Apoptosis is a natural process by which cells undergo programmed cell death, controlling cell number and proliferation as part of normal development (3,4). Deregulation of apoptosis may disrupt the delicate balance between cell proliferation and cell death, and is therefore considered a hallmark of most or even all types of cancer (4). In many types of cancer cells, the expression of anti-apoptotic proteins is up-regulated or pro-apoptotic proteins acquire inactivating mutations, resulting in uncontrolled growth of the tumor (4,5).

Two major pathways of apoptosis converge on the effector caspases, the extrinsic and intrinsic cell-death pathways. The extrinsic cell-death pathway can function independently of mitochondria and is activated by cell-surface death receptors, such as Fas and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors, ultimately leading to the recruitment of the adaptor protein Fas-associated death domain (FADD) and subsequent activation of caspase-8, which in turn activates caspase-3. The intrinsic cell-death pathway, also known as the mitochondrial apoptotic pathway, is characterized by a loss of mitochondrial membrane potential and the release from mitochondria of cytochrome c, which interacts with the adaptor protein apoptosis protease-activating factor 1 (Apaf-1), recruiting caspase-9, which activates caspase-3 (6,7). Thus, the key proteins in the apoptotic cascade are the initiator and the effector caspases, which are activated by cleavage early in apoptosis. Indeed many chemotherapeutic agents intervene in apoptotic signaling, via either the intrinsic or extrinsic pathway.

Cancer chemoprevention has been defined as the administration of agents to prevent tumor induction, to inhibit or delay tumor progression, or to inhibit or reverse carcinogenesis at a premalignant stage. Ingestion of flavonoids has been associated with preventive effects against diseases such as cancer, coronary diseases, inflammatory disorders, neurological degeneration, and aging. Flavonoids, which are present in a wide variety of fruits, vegetables, seeds, and medicinal herbs, have been intensively studied in human health, including cancer

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prevention (8). Acacetin (5,7-dihydroxy-4'-methoxyflavone), a flavonoid compound, has been reported to possess anti-peroxidative and anti-inflammatory effects (9,10). Acacetin has been shown to have anti-cancer activities against liver, prostate, lung, stomach, and breast cancer cells. For example, acacetin induced G1 and/or G2-M arrest in prostate cancer DU145 cells and in non-small cell lung cancer A562 cells. In gastric carcinoma cells, acacetin-induced apoptosis was accompanied by caspase activation, reactive oxygen species (ROS) generation and mitochondria-mediated death signaling (11-16).

T cell acute lymphoblastic leukemia (T-ALL) is the most common malignant disease in children, accounting for 70% of all childhood leukemias (17). Approximately 60% of children with T-ALL also have certain chromosomal translocations and constitute a high-risk leukemia subgroup. These patients are particularly resistant to conventional chemotherapy agents, thus requiring alternative treatment strategies (18). The development of a new type of anti-cancer drug for these T-ALL patients, without side effects or the development of drug resistance, is desirable (19). We have assessed the effects of acacetin on the human Jurkat T-cell lymphoblastic leukemia cell line, including on Jurkat cell proliferation and apoptosis.

Materials and methods

Reagents and antibodies. Acacetin was purchased from Sigma Chemical (St. Louis, MO). Other chemicals, of the highest grade available, were purchased from Nacalai Tesque (Kyoto, Japan). Rabbit polyclonal antibodies against phospho-FADD, FAF-1, Apaf-1 and β-actin were purchased from Cell Signaling Technology (Danvers, MA); and mouse anti-cytochrome c monoclonal antibody from Pharmingen (San Diego, CA).

Isolation of peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMCs) were isolated from freshly heparinized blood by Ficoll-Hypaque density gradient centrifugation. The cells were washed in PBS and resuspended at $1x10^6$ cells/ml in RPMI-1640 (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS, endotoxin level <10 EU/ml/ml, Cell Culture Technologies, Herndon, VA), penicillin (100 U/ml), and streptomycin (0.1 mg/ml). PBMCs were cultured in the presence of various concentrations of acacetin.

Cell cultures. All cells were obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan) and cultured in RPMI-1640 medium containing 10% FBS, penicillin (100 U/ml), and streptomycin (0.1 mg/ml) at 37˚C in a humidified 5% CO_2 incubator. Cell viability was measured by counting cells, excluding those stained with 0.2% trypan blue. To maintain exponential growth, cells were seeded at $1x10^6$ cells/ml and passaged every 4-5 days.

Cell survival assay. Inhibition of cell proliferation by acacetin was measured by trypan blue assay. Briefly, cells were seeded in 96-well culture plates ($4x10^4$ cells/well) and incubated in the presence of absence of 20 or 40 μM acacetin for 4, 6, 12, 24, 48 and 72 h. Cell survival (%) was calculated relative to untreated cells.

Table I. Acacetin-induced cytotoxicity in human leukemia cell lines.

Cell line	Characteristics	Cytotoxicity IC_{50} (μM)
Jurkat	T cell leukemia	25.77 (16.22-40.94)
MOLT-4	T cell leukemia	59.07 (47.44-82.22)
HL-60	Promyelocytic leukemia	59.07 (47.57-81.95)
U937	Leukemic monocyte lymphoma	45.71 (25.91-80.65)
K562	Erythroleukemia	≤100
Mononuclear cells	Peripheral blood	≤100

Human leukemia cells and peripheral blood mononuclear cells were incubated for 24 h with acacetin. Cytotoxicity was estimated by counting the cells excluding those stained with 2.0% trypan blue. The comparative cytotoxicity is expressed as the 50% cell growth inhibitory concentration (IC_{50}).

Assessment of apoptosis. Cells were seeded at $2x10^5$ cells/well in 60-mm tissue culture dishes and incubated in the presence or absence of 20 or 40 μM acacetin for 4, 6, 12, 24, 48 and 72 h. To further evaluate the involvement of caspases in acacetin-induced apoptosis, the cells were pretreated with the caspase inhibitors, Z-DEVD-FMK (5 μM), Z-IETD-FMK (5 μM), Z-LEHD-FMK (5 μM) and Z-Asp-CH_2-DCB (100 μM), for 1 h and subsequently with acacetin for 2, 4, 6 and 12 h. To analyze changes in nuclear morphology, cells were collected by centrifugation, washed with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4), incubated for 10 min at room temperature in PBS containing 5 μM bisbenzimide H 33342 fluorochrome trihydrochloride (H 33342) and examined under a model TE 300 fluorescence microscope (Nikon, Tokyo, Japan), with excitation and emission wavelengths of 360 and 420 nm, respectively. Apoptosis was characterized by chromatin condensation followed by partitioning into multiple bodies. At least 300 cells were counted in each experiment, and the percentage of apoptotic cells was calculated.

For flow cytometric analysis, the cells were cultured in triplicate in 35-mm dishes at $4x10^5$ cells/dish. After incubation in the presence or absence of acacetin, the cells were washed with PBS and collected. The cell pellets were suspended in 1 ml of 10 μg/ ml RNase A containing 0.5% Triton-X 100 plus the same volume of 20 μg/ml propidium iodide and incubated in the dark at room temperature for 30 min. The cell suspensions were filtered through a 60-μm mesh filter, and the number of hypodiploid cells (sub-G1 peak) was determined. Data were acquired and analyzed on a FACScan flow cytometer (Becton Dickenson, San Jose, CA).

Detection of caspase activity. The activation of caspases-3, -8 and -9 was assessed using a colorimetric assay kit (MBL,

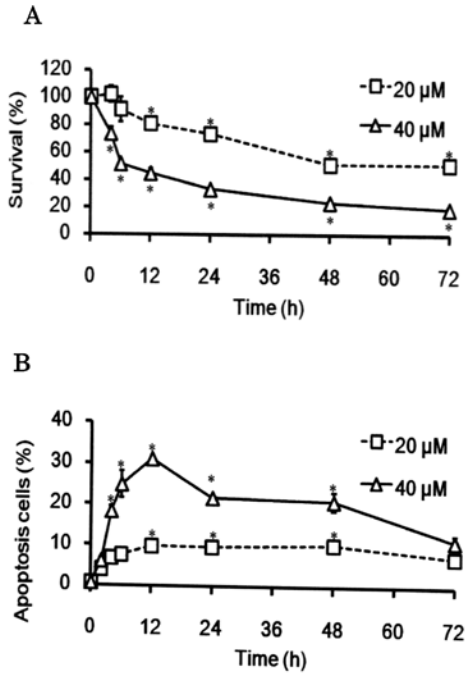

Figure 1. Acacetin induces cytotoxicity and apoptosis in Jurkat cell lines. Jurkat cells were incubated with 20 or 40 μM acacetin for 4-72 h, with (A) cytotoxicity estimated by the trypan blue assay, and (B) apoptosis estimated by H33342 staining, as described in Materials and methods. Each value represents the mean ± SE of three different experiments, each of which included triplicate samples. *p<0.05 compared with the control group.

Nagoya, Japan) according to the manufacturer's instructions. Briefly, 4x10^5 cells were lysed in 100 μl of lysis buffer and incubated at 37˚C for 1 h with the colorimetric caspase substrates DEVD-p-nitroanilide (caspase-3), IETD-p-nitroanilide (caspase-8) or LEHD- p-nitroanilide (caspase-9). The absorbance of each well at 405 nm was measured on an Intermed Model NJ 2300 microplate reader (Tokyo, Japan). The absorbance of acacetin-treated cells was compared with the absorbance of control cells to determine changes in caspase activity.

Western blotting. Cells were washed with PBS and lysed in CelLytic M (Sigma). The lysates were mixed with SDS-PAGE sample buffer and denatured for 5 min at 95˚C, and 30 μg of each sample protein were loaded onto a SDS-polyacrylamide gel (7.5-15%). The resolved proteins were electrotransferred to PVDF membranes using a Trans-Blot SD apparatus (Bio-Rad). The blots were blocked in blocking solution (25 mM Tris-HCl pH 7.4, 137 mM NaCl, 2.68 mM KCl and 5% skim milk), and incubated with antibodies to phospho-FADD (human specific, Ser194), FAF-1, cytochrome c, Apaf-1 and Bc1-2. After washing, the blots were incubated with HRP-conjugated secondary antibody for 1 h and washed, and the levels of protein were analyzed by enhanced chemiluminescence with an ECL plus Western blotting detection system (Amersham, Arlington Heights, IL).

Statistical analysis. Data were expressed as means ± SE of 3 independent experiments and statistical comparisons were made using Fisher's LSD test. p<0.05 was considered statistically significant.

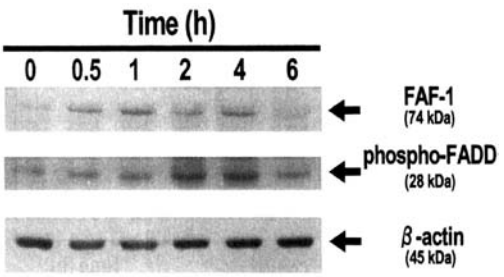

Figure 2. Effect of acacetin on the expression of FAF1 and phosphorylation of FADD in Jurkat cells. Jurkat cells were incubated with 40 μM acacetin for 0.5, 1, 2, 4 or 6 h, and the cell lysates were subjected to 7.5 or 15% SDS-PAGE and transferred to PVDF membranes. The expression of FAF-1 and phospho-FADD was detected by probing with rabbit anti-FAF-1 or anti-phospho-FADD antibody and HRP-conjugated goat anti-rabbit IgG. β-actin as a loading control was detected by reprobing with rabbit anti-β-actin antibody and HRP-conjugated goat anti-rabbit IgG. The results shown are representative of three separate sets of experiments.

Results

Acacetin induces cytotoxicity and apoptosis in human leukemia cell lines. We tested the cytotoxic effect of acacetin on human leukemia cell lines by incubating these cells with acacetin for 24 h (Table I). The cell line most sensitive to acacetin-induced cytotoxicity was the Jurkat T-ALL cell line. We also found that the U937 monocytic cell line, the MOLT-4 T-cell leukemia line and the HL-60 premyelocytic leukemia cell line were susceptible to acacetin. In contrast, the K562 erythroleukemia cell line was almost unaffected by acacetin. Of note, peripheral lymphocytes were not affected by incubation with 100 μM acacetin for 48 h.

We next examined the effects of acacetin on cell morphology, with apoptotic cells detected by the H33342 assay, which measures changes in nuclear morphology. Jurkat cells were incubated with 20 or 40 μM acacetin for 4, 6, 12, 24, 48 and 72 h. We found that the incubation of these cells with 40 μM acacetin for more than 4 h induced considerable cell death (Fig. 1A). Nuclear morphological changes in cells incubated with 40 μM acacetin were observed at 4 h and maximized at 12 h (Fig. 1B). To determine the type of death induced by acacetin, we assessed these cells for apoptotic characteristics, including hypodiploidy (sub-G1 peak), by flow cytometry using PI staining. We found that Jurkat cells exposed to 40 μM acacetin for 4, 6 and 12 h showed sub-G1 peak (data not shown), indicating that the degree of apoptosis induced by acacetin correlated its cytotoxic effects in Jurkat cells.

Effect of acacetin on the expression of FAF1 and phosphorylation of FADD in Jurkat cells. Fas-associated factor 1 (FAF1) is an adaptor protein associated with Fas (20) that has been implicated in Fas-induced apoptosis. The Fas-associated death domain (FADD) was originally identified as an adaptor molecule in Fas-mediated apoptosis, and has since been shown to play an important role in the formation of the death-inducing signaling complex following Fas stimulation (20-22). Using Western blotting, we have tested whether acacetin-induced apoptosis is involved in the Fas-mediated pathway (Fig. 2). We found that acacetin time-dependently enhanced FAF1 expression and the phosphorylation of FADD in Jurkat cells. Moreover, acacetin

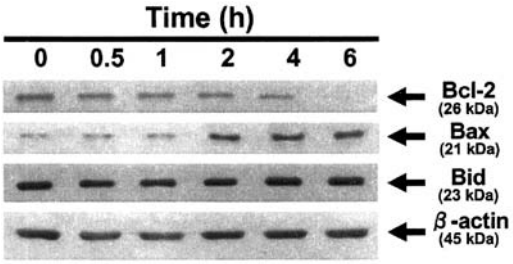

Figure 3. Effect of acacetin on the expression of Bcl-2 family proteins in Jurkat cells. Jurkat cells were incubated with 40 μM acacetin for 0.5, 1, 2, 4 or 6 h, and the cell lysates were subjected to 15% SDS-PAGE and transferred to PVDF membranes. The expression of Bcl-2 and Bax was detected by probing with mouse anti-Bcl-2 or -Bax antibody and HRP-conjugated goat anti-mouse IgG. β-actin as a loading control was detected by reprobing with rabbit anti-β-actin antibody and HRP-conjugated goat anti-rabbit IgG. The results shown are representative of three separate sets of experiments.

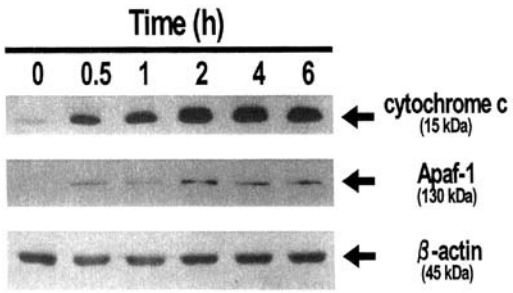

Figure 4. Effect of acacetin on the expression of cytochrome *c* and Apaf-1 in Jurkat cells. Jurkat cells were incubated with 40 μM acacetin for 0.5, 1, 2, 4 or 6 h. The cell lysates were subjected to 7.5 or 15% SDS-PAGE and transferred to PVDF membranes, which were incubated with mouse anti-cytochrome *c* or rabbit anti-Apaf-1 antibody and HRP-conjugated goat anti-mouse or anti-rabbit IgG. β-actin as a loading control was detected by reprobing with rabbit anti-β-actin antibody and HRP-conjugated goat anti-rabbit IgG. The results shown are representative of three separate sets of experiments.

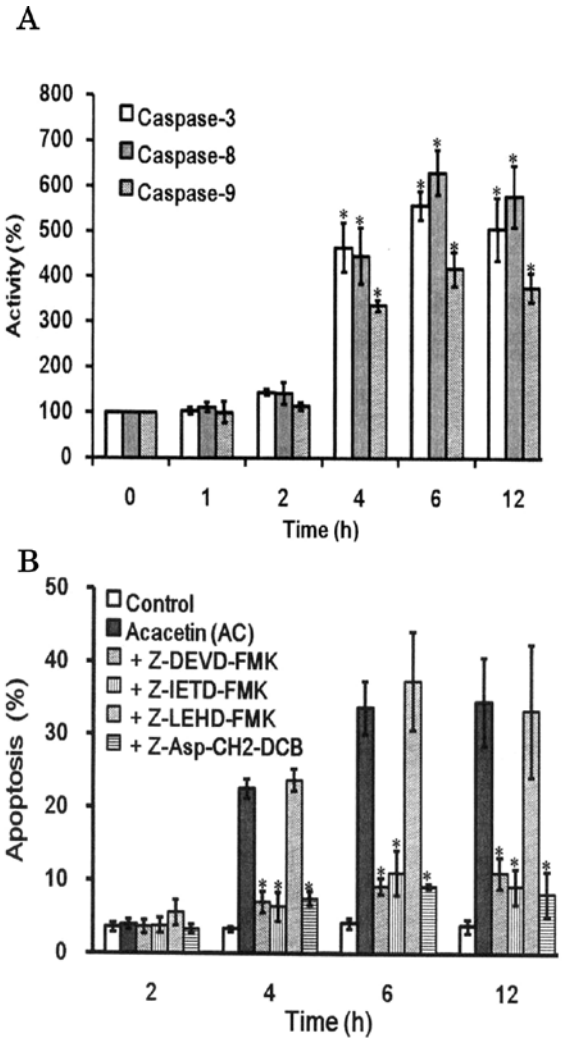

Figure 5. Activation of the caspase family during acacetin-induced apoptosis. Jurkat cells were incubated with 40 μM acacetin for 0.5, 1, 2, 4, 6 or 12 h, harvested and lysed in lysis buffer. (A) Enzymatic activity of caspase-3, -8 and -9 proteases, determined by a colorimetric assay kit using the colorimetric caspase substrates DEVD-p-nitroanilide (caspase-3), IETD-p-nitroanilide (caspase-8) and LEHD- p-nitroanilide (caspase-9), following incubation for 1 h at 30˚C. Each value is presented as the mean ± SE of three independent experiments were performed in triplicate. $^{*}p<0.05$ compared with control group. (B) Jurkat cells were pretreated with the broad-spectrum caspase inhibitor Z-ASP-CH2-DCB, the caspase-3 inhibitor Z-DEVD-FMK, the caspase-8 inhibitor Z-IETD-FMK or the caspase-9 inhibitor Z-LEHD-FMK) for 1 h and subsequently incubated with acacetin for 2, 4, 6 and 12 h. Apoptotic cell death was determined by assaying changes in nuclear morphology. Each value represents the mean ± SE of three separate experiments. $^{*}p<0.05$ compared with acacetin alone.

treatment had no effect on β-actin levels. These findings suggest that acacetin-induced apoptosis in Jurkat cells occurs through FAF1 expression and the phosphorylation of FADD.

Effect of acacetin on the expression of Bcl-2 family proteins in Jurkat cells. Several gene products are important in controlling the apoptotic process, with imbalances in the expression of anti- and pro-apoptotic proteins being one of the major mechanisms affecting the fate of cells during the apoptotic process. One important pro-apoptotic protein is Bax, which is inserted into the outer member of mitochondria and forms a large channel, allowing the release of cytochrome c, a process that can be prevented by Bcl-2 proteins (23). We therefore tested the effects of acacetin on expression of Bax and Bcl-2 in Jurkat cells. We found that acacetin induced a marked increase in Bax protein and time-dependently decreased Bcl-2 expression in Jurkat cells (Fig. 3), suggesting that acacetin-induced apoptosis may cause an imbalance in the expression of anti- and pro-apoptotic proteins.

Effect of acacetin on the expression of cytochrome c and Apaf-1 in Jurkat cells. Induction of apoptosis via the intrinsic pathway, for example by genotoxic stress, is often characterized by the release of cytochrome c from mitochondria and the subsequent ATP-dependent activation of the death regulator Apaf-1, which, in turn, activates caspase-9 (24). When we tested the effects of acacetin on cytochrome c release and activation of Apaf-1, we observed a marked induction of both proteins, which tended to be in a time-dependent manner (Fig. 4). The expression of cytochrome c and Apaf-1 began to increase 0.5 h after treatment with acacetin. These findings suggested that acacetin-induced apoptosis is dependent on the intrinsic pathway of apoptosis, involving the mitochondria.

Activation of the caspase family is involved in acacetin-induced apoptosis. Caspases are members of the cysteine

aspartic acid-specific family of proteases and are activated by a variety of signals, including death receptor ligation, DNA damage, serum starvation and stress (25,26). Caspases from their inactive forms are activated by a cascade of proteolytic reactions. Once activated, a caspase can subsequently cleave its substrates at specific sites. To monitor the enzyme activity of caspases during acacetin-induced apoptosis, we used three substrates labeled with p-nitroanilide. We found that treatment of Jurkat cells with 40 μM acacetin resulted in the time-dependent activation of caspases-3, -8 and -9 (Fig. 5).

Activation of caspase-3 leads to the cleavage of a number of proteins, including the 116 kDa poly (ADP-ribose) polymerase (PARP) to produce an 85 kDa fragment; this reaction is regarded as the hallmark of apoptosis. We found that exposure of Jurkat cells to acacetin resulted in the cleavage of DFF-45 and PARP (data not shown) and that these reactions were associated with caspase activation. To further determine if the activation of caspase-like proteases is necessary for acacetin-induced apoptosis, we preincubated Jurkat cells with several caspase inhibitors, including the broad-spectrum inhibitor Z-ASP-CH_2-DCB, the caspase-3 inhibitor Z-DEVD-FMK, the caspase-8 inhibitor Z-IETD-FMK and the caspase-9 inhibitor Z-LEHD-FMK, to block intercellular proteases, and we assayed acacetin-induced apoptosis by analyzing changes in nuclear morphology. We found that Z-ASP-CH2-DCB, Z-DEVD-FMK and Z-IETD-FMK, but not ZLEHD-FMK, significantly inhibited acacetin-induced apoptosis (Fig. 5B), suggesting that caspase-9 may not be required for acacetin-induced apoptosis.

Discussion

Flavonoids are naturally occurring antioxidants, several of which have been reported to have chemopreventive effects on cancer (27,28). Carcinogens usually cause genomic damage to exposed cells, triggering either apoptosis or proliferation with genomic damage. The latter process can lead to the generation of cancerous cells, which usually exhibit cell cycle abnormalities and are more susceptible to apoptosis-inducing agents. Identifying active compounds in food that induce apoptosis of cell lines is considered a primary mechanism for the chemoprevention of cancer. We therefore investigated whether acacetin can inhibit the growth and proliferation of the various human cancer cell lines. We found that acacetin inhibited the growth of the human T cell leukemia cell lines Jurkat and MOLT-4, the human promyelocytic leukemia cell line HL-60 and the human monocyte cell line U937; suggesting that acacetin showed slight effects on undifferentiated cells.

In contrast, the K562 erythroleukemia cell line showed lower susceptibility to acacetin. K562 has a higher intracellular total glutathione content and lower susceptibly to H_2O_2 than these other cell lines, whereas Jurkat cells have a lower intracellular total glutathione content than the other human cell lines tested (29). Thus, cells with a lower concentration of intracellular glutathione may be more sensitive to acacetin. Oxidative damage to the mitochondrial membrane caused by increased generation of ROS has been shown to play a role in apoptosis (30). In practice, pretreatment with the antioxidants N-acetylcysteine and catalase, which are used as free radical scavengers, increased acacetin-induced cytotoxicity (15). Moreover, intracellular glutathione content has been implicated in the inhibition of proliferation.

We found that acacetin could disrupt mitochondrial function at early stages of apoptosis and could subsequently coordinate caspase-9 activation through the release of cytochrome *c*. In assessing the molecular mechanism by which acacetin triggered apoptosis of Jurkat T cells, we found that treatment with acacetin induced the activities of caspases-3, -8 and -9, along with the cleavage of DFF-45 and PARP, reactions that preceded the onset of apoptosis. Pretreatment with the caspase-3 inhibitor Z-DEVD-FMK or the caspase-8 inhibitor Z-IETD-FMK inhibited acacetin-induced apoptosis, whereas pretreatment with the caspase-9 inhibitor ZLEHD-FMK did not, suggesting that acacetin-induced apoptosis involves a mechanism mediated by caspases-3 and -8.

The receptor-mediated signaling transduction pathway of apoptosis also includes caspase cascades. We found that acacetin treatment of Jurkat cells enhanced the expression of FAF-1 and phosphorylated-FADD. Similarly, acacetin treatment of AGS and HepG2 cells was shown to activate Fas and Fas ligand. Since treatment of Jurkat cells with acacetin for 4 h increased caspase-8 activity, these findings suggest that, in these cells, caspase-8 is involved in cytochrome c-mediated apoptosis and participates in the induction of caspase-3 activity.

The Bcl-2 family of proteins, whose members may be anti-apoptosis or pro-apoptotic, regulate cell death by controlling mitochondrial permeability during apoptosis (31,32). We inferred that, during acacetin-induced apoptosis, the Bcl-2 family of proteins may participate in the events that control changes in mitochondrial membrane potential and trigger cytochrome *c* release. We found that Bcl-2 expression was downregulated and Bax expression was upregulated in acacetin-treated Jurkat cells. Furthermore, we found that acacetin could not induce apoptosis in Bcl-2 transfected Jurkat cells (data not shown), further indicating that acacetin induces apoptosis in Jurkat cells through a pathway involving caspase-8 and Bcl-2.

In conclusion, our observations suggest that the cancer chemopreventive agent acacetin induced Jurkat cell apoptosis in a dose- and time-dependent manner. Analyses of the expression of FAF-1, phosphorylated-FADD and the Bcl-2 family of proteins, the subcellular location of cytochrome c and Apaf-1, and the status of various caspase activities suggested that acacetin-induced apoptosis of Jurkat cells was associated primarily with Fas activation and caspase-8 and -3 activities. These findings may help in designing cancer therapeutic and chemopreventive agents.

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