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Acacetin Induces Apoptosis in Human Gastric Carcinoma Cells Accompanied by Activation of Caspase Cascades and Production of Reactive Oxygen Species

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Acacetin (5,7-dihydrocy-4′-methoxy flavone), which is a flavonoid compound, possesses anti-peroxidative and anti-inflammatory effects. The effects of acacetin on cell viability in human gastric carcinoma AGS cells were investigated. This study demonstrated that acacetin was able to inhibit cell proliferation and induce apoptosis in a concentration- and time-dependent manner. Acacetin-induced cell death was characterized with changes in nuclear morphology, DNA fragmentation, and cell morphology. The molecular mechanism of acacetin-induced apoptosis was also investigated. Treatment with acacetin caused induction of caspase-3 activity in a time-dependent manner, but not caspase-1 activity, and induced the degradation of DNA fragmentation factor (DFF-45) and poly-(ADP-riobse) polymerase. Cell death was completely prevented by a pancaspase inhibitor, Z-Val-Ala-Asp-fluoromethyl ketone. Furthermore, treatment with acacetin caused a rapid loss of mitochondrial transmembrane potential, stimulation of reactive oxygen species (ROS), release of mitochondrial cytochrome *c* into cytosol, and subsequent induction of procaspase-9 processing. Antioxidants such as *N*-acetylcysteine and catalase, but not superoxide dismutase, allopurinol, or pyrrolidine dithiocarbamate, significantly inhibited acacetin-induced cell death. In addition, it was found that acacetin promoted the up-regulation of Fas and FasL prior to the processing and activation of pro-caspase-8 and cleavage of Bid, suggesting the involvement of a Fas-mediated pathway in acacetin-induced apoptosis. On the other hand, the results showed that acacetin-induced apoptosis was accompanied by up-regulation of Bax and p53, down-regulation of Bcl-2, and cleavage of Bad. Taken together, these results suggest that ROS production and a certain intimate link might exist between receptor- and mitochondria-mediated death signalings that committed to acacetin-induced apoptosis in AGS cells. The induction of apoptosis by acacetin may provide a pivotal mechanism for its cancer chemopreventive action.

KEYWORDS: Acacetin; reactive oxygen species; apoptosis; cytochrome *c*; caspase-9; caspase-3; caspase-8; poly(ADP-ribose) polymerase; DNA fragmentation factor; caspase-activated deoxyribonuclease; Bax; Bad; Bcl-2; Bcl-X_L

INTRODUCTION

Gastric adenocarcinoma is a global health problem and the second-ranked cause of cancer-related death in the world (*1*). Studies have shown that a high intake of smoked, salted, nitrated foods and a high intake of carbohydrates, but a low intake of vegetables, fruits, and milk, are linked to cancer incidence. These diets have been shown to significantly increase the risk for stomach cancer (*2*). Epidemiological studies have provided convincing evidence that dietary factors can modify the processes of carcinogenesis, including initiation, promotion, and progression of several types of human cancer (*3*). The occurrence of gastrointestinal (GI) cancers has increased strikingly during the past decade. For instance, colorectal cancer is the second leading cause of cancer mortality in Western societies (*4*) and one of the world's most common malignancies (*5*, *6*). Consequently, the fight against GI cancer is an important global issue.

Flavonoids are widespread in fruits, vegetables, seeds, and medicinal herbs. All of them might be one type of polyphenolic flavonoid, which have the diphenylpropane (C6C3C6) skeleton, including monomeric flavanols, flavones, flavanols, and flavanones, and have been intensively studied for their role in

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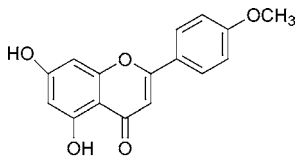

Acacetin

Figure 1. Chemical structure of acacetin.

human health, including cancer prevention. Previous studies have shown that many flavonoids exhibit potent antitumor activity against several rodent and human cancer cell lines (*7*−*10*). The antitumor properties of some flavonoids have been studied with respect to apoptosis and cell cycle arrest. The molecular mechanisms of apoptosis by flavonoids remain largely unclear, but appear to involve modulation of multiple apoptotic regulatory proteins. Acacetin (5,7-dihydroxy-4′-methoxy flavone) (**Figure 1**) has been reported to possess anti-peroxidative, anti-inflammatory, anti-plasmodial (*11*−*13*), and anti-proliferative activities (*14*, *15*). Although a broad range of biological and pharmacological activities of acacetin have been reported, the mechanism by which acacetin induces apoptosis is not yet known.

Apoptosis is a defined type of cell death and differs from traditional cell death, necrosis. Many recent studies have indicated that anticancer drugs or cancer chemopreventive agents act through the induction of apoptosis to prevent tumor promotion, progression, and the occurrence of cellular inflammatory responses other than necrosis (*16*, *17*). Apoptosis is also a gene-directed form of cell death with well-characterized morphological and biochemical features (*18*). Initiation of apoptosis appears to be a common mechanism of many cytotoxic agents used in chemotherapy. The process of apoptosis is orchestrated by the activation of "executioner" caspases, a family of cytosolic proteases, stored in most cells as zymogens. Proteolytic cleavage activates the initiating zymogen, which in turn triggers sequential proteolytic activation of each successive procaspase in the apoptosis cascade (*19*, *20*). To date, at least 10 distinct caspases in mammalian cells have been identified (*21*). There are two main pathways involving apoptotic cell death. First, it is the interaction of the cell surface receptor, such as Fas, TNFR, DR3, DR4, and DR5, with their ligands. Activation of death receptors (Fas) by cross-linking with their natural ligands (Fas ligand) induces receptor clustering and formation of a death-inducing signaling complex (DISC). The complex recruits, via the adaptor molecule Fas-associated death domain protein (FADD), multiple procaspase-8, resulting in the activation of caspase-8, and activated caspase-8 directly cleaves and activates caspase-3, which in turn cleaves other caspases (*22*−*24*). Second, it is involved in the participation of mitochondria, for most forms of apoptosis in response to cellular stress, loss of survival factors, and developmental cues (*20*, *25*). Moreover, the mitochondrial pathway was regulated by the Bcl-2 family of proteins, including anti-apoptoic proteins such as Bcl-2 and Bcl-X_L and pro-apoptotic proteins such as Bad, Bid, Bim, Bax, and Bak (*26*). In many cells, survival or death depends on the altered expression level of death inhibitor to death promoter, respectively. Furthermore, it appears that a range of molecular affinities exist, which control the interactions between family members, such as Bcl-2 (or Bcl-X_L) for Bax to promote cell survival or Bax homodimer formation to promote cell death (*27*, *28*). Current evidence suggests that Bcl-2 acts upstream of caspse-3 activation, at the level of cytochrome *c* release, to prevent apoptosis (*29*). It has been shown that the Bcl-2 and Bcl-X_L of mammals can be converted into potent pro-apoptotic molecules when they are cleaved by caspases, resulting in accelerated cell apoptosis (*30*, *31*).

Our previous studies demonstrate that polyphenols exhibit antitumor activity against human cancer cell lines (*32*, *33*). In the current study, we first examined the antiproliferative effects of acacetin and structurally related flavonoids on human gastric carcinoma cells. Our results clearly demonstrate that acacetin can induce apoptosis in a dose-dependent manner in AGS cells. We further evaluated the molecular mechanisms of apoptotic effects induced by acacetin. To elucidate the anticancer mechanism of acacetin, we investigated the production of ROS, the change of the Bcl-2 protein family, and caspases in acacetin-induced apoptosis in human AGS cancer cells.

MATERIALS AND METHODS

Cell Culture and Chemicals. The human AGS gastric carcinoma cell lines (CCRC 60102) were obtained from Food Industry Research and Development Institute (Hsinchu, Taiwan) and cultured in Dulbecco's modified Eagle's medium/nutrient mixture F-12 containing 10% heat-inactivated fetal bovine serum (GIBCO BRL, Grand Island, NY), 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 2 mM L-glutamine (GIBCO BRL) and were kept at 37 °C in a humidified 5% CO_2 incubator. The inhibitors of caspase-3 (Z-Val-Ala-Asp-fluoromethyl ketone, Z-VAD-FMK) and caspase-1 (acetyl-Tyr-Val-Ala-Asp-aldehyde, Ac-YVAD-CHO) were purchased from Calbiochem (La Jolla, CA). Propidium iodide was obtained from Sigma Chemical Co. (St. Louis, MO). Acacetin was purchased form Fluka (St. Gallen, Switzerland), and its purity was >99%.

Cell Survival Assay. Cell viability was assayed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Briefly, AGS cells were plated at a density of 1×10^5 cells/mL into 24-well plates. After overnight growth, cells were pretreated with a series of concentrations of acacetin for 24 h. The final concentrations of dimethyl sulfoxide in the culture medium were <0.1%. At the end of treatment, 30 μL of MTT was added, and cells were incubated for a further 4 h. Cell viability was determined by scanning with an ELISA reader with a 570-nm filter.

Determination of Cell Growth Curve. The AGS cancer cells (5×10^4) were plated in 35-mm Petri dishes. The next day, the medium was changed and various flavonoids were added. Control cells were treated with DMSO to a final concentration of 0.05% (v/v). At the end of incubation, cells were harvested for cell count using a hemocytometer (*34*).

DNA Extraction and Electrophoresis Analysis. The AGS human cancer cells were harvested, washed with phosphate-buffered saline (PBS), and then lysed with digestion buffer containing 0.5% sarkosyl, 0.5 mg/mL proteinase K, 50 mM tris(hydroxymethyl)aminomethane (pH 8.0), and 10 mM EDTA at 56 °C overnight and treated with RNase A (0.5 μg/mL) for 3 h at 56 °C. The DNA was extracted by phenol/chloroform/isoamyl (25:24:1) before loading and was analyzed by 2% agarose gel electrophoresis. The agarose gels were run at 50 V for 120 min in Tris-borate/EDTA electrophoresis buffer (TBE). Approximately 20 μg of DNA was loaded in each well, visualized under UV light, and photographed (*35*).

Acridine Orange Staining Assay. Cells (5×10^5) were seeded into 60-mm Petri dishes and incubated at 37 °C for 24 h. The cells were harvested after treatment for 24 h, and 5 μL of cell suspension was mixed on a slide with an equal volume of acridine orange solution (10 μg/mL in PBS). Green fluorescence was detected between 500 and 525 nm by using an Olympus microscope (Olympus America, Inc., Lake Success, NY). Bright-staining condensed chromatin was detected in apoptotic cells.

Scanning Electron Microscopy (SEM) Analysis. SEM was used to examine the surface topography of the acacetin-treated AGS cells (Hitachi S-3500N). Cells were seeded at a density of 2×10^5 cells/mL onto six-well tissue culture plates. After overnight growth, cells were treated with 60 μM acacetin, and the final concentrations of DMSO in the culture medium were <0.1%. Following 24 h of incubation with 60 μM acacetin, the cell morphology was assayed by SEM. Briefly,

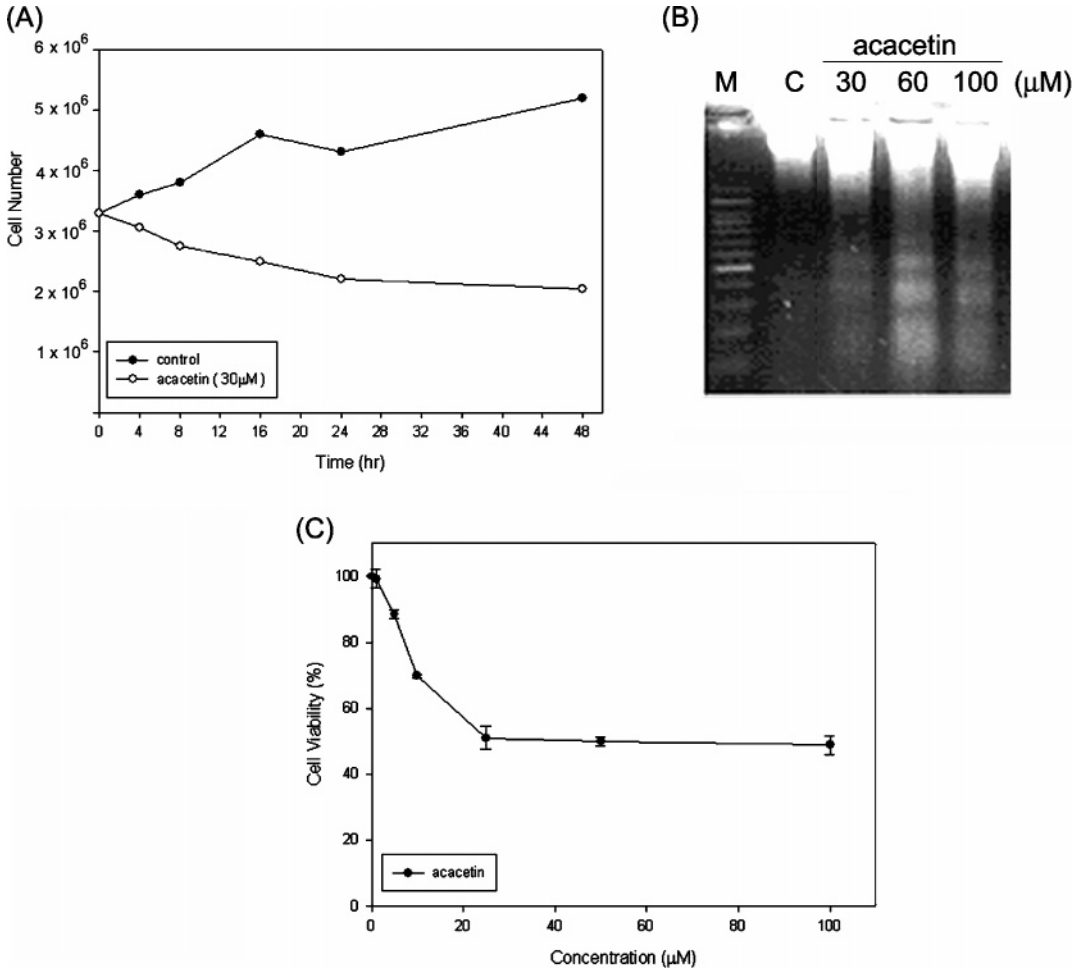

Figure 2. Analysis of the cell viability and DNA integrity of acacetin-treated AGS cells as determined by MTT assay, DNA electrophoresis, and trypan blue exclusion assay. (**A**) AGS cells were treated with different concentrations of the indicated compound for 24 h. Viability of the cells was determined by MTT assay. (**B**) AGS cells were treated with 30 μM acacetin for 0, 4, 8, 12, 24, and 48 h. The cells were harvested and detected by cell counter. (**C**) AGS cells were treated with different concentrations of various flavonoids for 24 h, and the DNA fragmentation was analyzed by 2% agarose electrophoresis, as described under Materials and Methods. Cells were treated with 0.1% DMSO as vehicle control. Data were represented as means ± SE for three determinations. M, 100 bp DNA ladder size marker.

cells were rinsed three times with PBS and then fixed with 3% paraformaldehyde for 10 min. After a final rinse with PBS, a contrast treatment in 1% osmium tetroxide (Alfa) for 1 h was performed, followed by extensive rinsing in PBS and dehydration through a graded series of ethanol from 30, 50, 70, 90, and 100%. After air-drying, surfaces were thinly sputter coated with platinum (Ion Sputter, E-1010, Hitachi; with 15 mA for ~5 min). Cells on polished titanium disks and on glass cover slips were used as controls.

Flow Cytometry. AGS cells (2×10^5) were cultured in 60-mm Petri dishes and incubated for 24 h. The cells were then harvested, washed with PBS, resuspended in 200 μL of PBS, and fixed in 800 μL of iced 100% ethanol at 20 °C. After being left to stand overnight, the cell pellets were collected by centrifugation, resuspended in 1 mL of hypotonic buffer (0.5% Triton X-100 in PBS and 0.5 μg/mL RNase), and incubated at 37 °C for 30 min. Next, 1 mL of propidium iodide solution (50 μg/mL) was added, and the mixture was allowed to stand on ice for 30 min. Fluorescence emitted from the propidium iodide−DNA complex was quantitated after excitation of the fluorescent dye by FACScan cytometry (Becton Dickinson, San Jose, CA).

ROS Production Determination. ROS production was monitored by flow cytometry using DCFH-DA. This dye is a stable nonpolar compound that readily diffuses into cells and is hydrolyzed by intracellular esterase to yield DCFH, which is trapped within the cells. Hydrogen peroxide or low molecular weight peroxides produced by the cells oxidize DCFH to the highly fluorescent compound 2′,7′-dichlorofluorescein (DCF). Thus, the fluorescence intensity is proportional to the amount of peroxide produced by the cells. Cells were treated with acacetin (60 μM) for different time periods, and DCFH-DA (30 μM) was added into the medium for a further 30 min at 37 °C.

Analysis of Mitochondrial Transmembrane Potential. The change of the mitochondrial transmembrane potential was monitored by flow cytometry. Briefly, AGS cells were exposed to acacetin (60 μM) for different time periods and the mitochondrial transmembrane potential was measured directly using 40 nM 3,3′-dihexyloxacarbocyanine [DiOC6(3)] (Molecular Probes, Eugene, OR). Fluorescence was measured after staining of the cells for 30 min at 37 °C. Histograms were analyzed using Cell Quest software and were compared with histograms of control untreated cells.

Western Blotting. The nuclear and cytosolic proteins were isolated from AGS cells after treatment with 60 μM for 0, 3, 6, 9, 12, and 24 h. The total proteins were extracted via the addition of 200 μL of gold lysis buffer (50 mM Tris-HCl, pH 7.4; 1 mM NaF; 150 mM NaCl; 1 mM EGTA; 1 mM phenylmethanesulfonyl fluoride; 1% NP-40; and 10 μg/mL leupeptin) to the cell pellets on ice for 30 min, followed by centrifugation at 10000*g* for 30 min at 4 °C. The cytosolic fraction (supernatant) proteins were measured by Bio-Rad protein assay (Bio-Rad Laboratories, Munich, Germany). The samples (50 μg of protein) were mixed with 5× sample buffer containing 0.3 M Tris-HCl (pH 6.8), 25% 2-mercaptoethanol, 12% sodium dodecyl sulfate (SDS), 25 mM EDTA, 20% glycerol, and 0.1% bromophenol blue. The mixtures were boiled at 100 °C for 5 min and were subjected to 12% SDS−polyacrylamide minigels at a constant current of 20 mA. Subsequently, electrophoresis was ordinarily carried out on SDS−polyacrylamide gels. For electrophoresis, proteins on the gel were electrotransferred onto

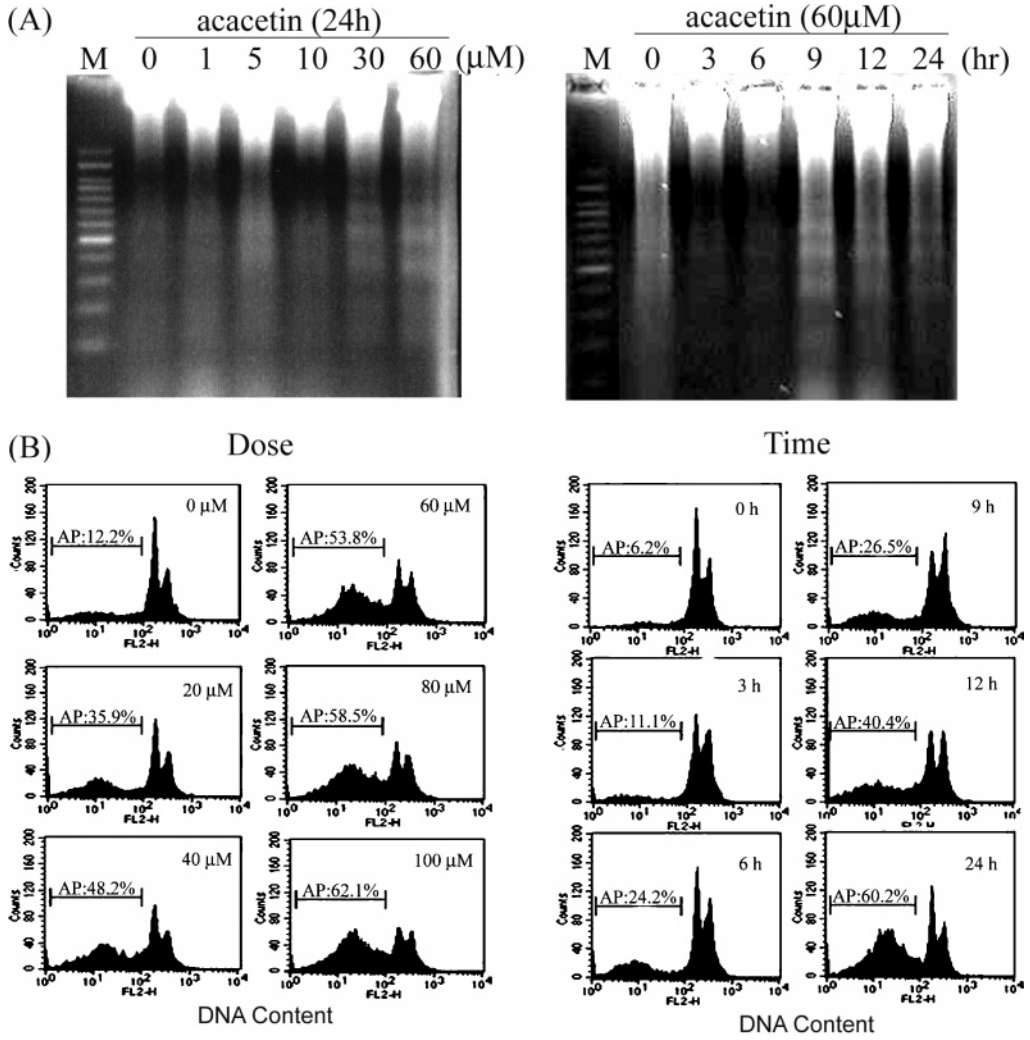

Figure 3. Induction of DNA fragmentation in AGS cells by acacetin. (**A**) AGS cells were treated with increasing doses of acacetin for 24 h or treated with 60 μM for the indicated time, and internucleosomal DNA fragmentation was analyzed by agarose gel electrophoresis. M, 100 bp DNA ladder size marker. Data shown are representative of three independent experiments. (**B**) Sub-G1 cells in acacetin-treated AGS cells were determined by flow cytometry. The method of flow cytometry used is descried under Materials and Methods. AP (apoptotic peak) represents apoptotic cells with a lower DNA content. The data presented are representative of three independent experiments.

an immobile membrane (PVDF; Millipore Corp., Bedford, MA) with transfer buffer composed of 25 mM Tris-HCl (pH 8.9), 192 mM glycine, and 20% methanol. The membranes were blocked with blocking solution containing 20 mM Tris-HCl and then immunoblotted with primary antibodies including anti-Bcl-2, anti-Bcl-X_L, anti-Bad, anti-Bax, anti-α-tubulin (Santa Cruz Biotech.), anti-PARP (UBI, Inc., Lake Placid, NY), anti-Bid, and anti-p21 (Transduction Laboratory, Lexington, KY), and anti-DFF45/ inhibitor of caspase-activated DNase (ICAD) antibody (MBL, Naka-Ku, Nagoya, Japan) at room temperature for 1 h. Detection was achieved by measuring the chemiluminescence of blotting agent (ECL, Amersham Corp., Arlington Heights, IL), after exposure of the filters to Kodak X-Omat films. The mitochondria and cytosolic fractions isolated from cells were used for immunoblot analysis of cytochrome *c* as described (*32*). The cytochrome *c* protein was detected by using anti-cytochrome *c* antibody (Research Diagnostic Inc., Flanders, NJ).

Activity of Caspase. Cells were collected and washed with PBS and suspended in 25 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 5 mM EDTA, 5 mM dithiothione, 2 mM phenylmethanesulfonyl fluoride, 10 μg/mL pepstatin A, and 10 μg/mL leupeptin after treatment. Cell lysates were clarified by centrifugation at 12000*g* for 20 min at 4 °C. Caspase activity in the supernatant was determined by a fluorogenic assay (Promeaga's CaspACE Assay System Corp., Madison, WI). Briefly, 50 μg of total protein, as determined by Bio-Rad protein assay (Bio-Rad Laboratories), was incubated with 50 μM substrate Ac-Try-Val-Ala-Asp-AMC (Ac-YVAD) (caspase-1-specific substrate), Ac-Asp-Glu-Val-Asp-AMC (Ac-DEVD-AMC) (caspase-3-specific substrate), Ac-Ile-Glu-Thr-Asp-AMC (Ac-IETD-AMC) (caspase-8-specific substrate), or Ac-Leu-Glu-His-Asp-AMC (Ac-LEHD-AMC) (caspase-9-specific substrate) at 30 °C for 1 h. The release of methylcoumaryl-7-amine (AMC) was measured by excitation at 360 and emission at 460 nm using a fluorescence spectrophotometer (Hitachi, F2000).

RESULTS

Acacetin Induces Apoptosis in Human Gastric Carcinoma Cells. Previous studies have shown that flavonoids are potent antiproliferation and anticancer agents (*34*, *36*−*38*). Here we investigated the cytotoxicity of acacetin. The structure of acacetin is illustrated in **Figure 1**. To assess the inhibitory effect of acacetin on the growth of human gastric carcinoma cells, we first determined the growth rates of AGS cells. Exponentially growing cultures of AGS cells were continuously cultured in the absence or presence of different concentrations of acacetin. After 24 h of treatment, the cell growth rates were determined by MTT assay. As shown in **Figure 2A**, acacetin induced significant growth inhibition of AGS cells. Exponentially growing AGS cultures rapidly underwent growth inhibition with the addition of 30 μM acacetin, as evidenced by a decrease of

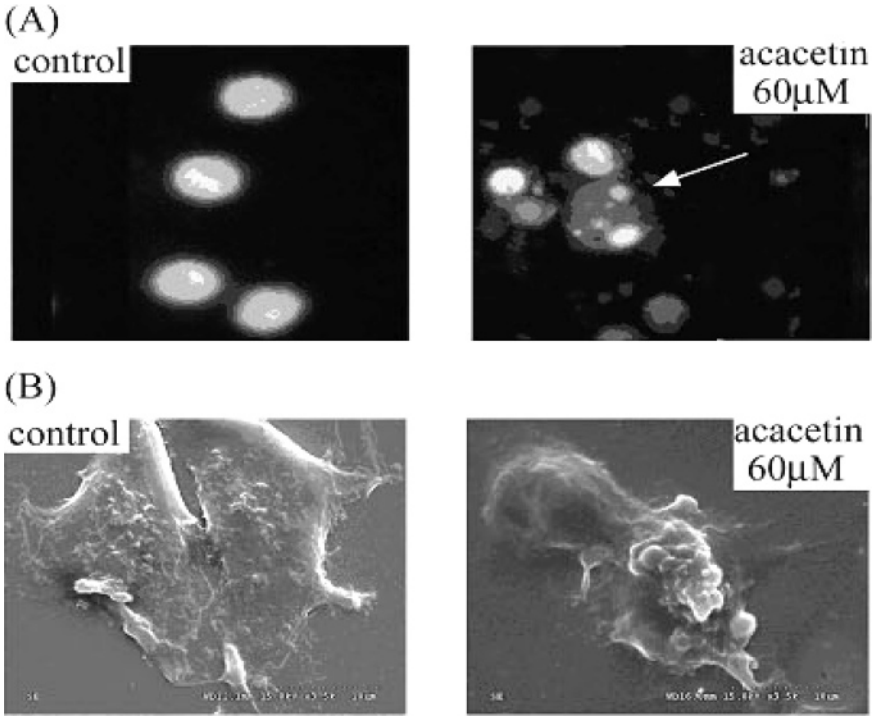

Figure 4. Chromatin condensation and morphological changes induced by acacetin in AGS cells. (**A**) AGS cells were treated with 0.05% DMSO as vehicle control or with 60 μM acacetin for 24 h, and cells were harvested and washed with PBS following by staining with acridine orange. The nuclear staining was examined by fluorescence microscopy. (**B**) After treatment with 60 μM acacetin for 24 h, the morphological changes were determined by scanning electron microscopy (3500×). The data presented are representative of three independent experiments.

cell proliferation over the experimental period (**Figure 2B**). As described previously (*39*), the apoptotic cell is characterized by specific changes in the cell including DNA fragmentation and the presence of a subdiploid peak. As shown in **Figure 2C**, the induction of DNA fragmentation was demonstrated by incubating AGS cells with different concentrations of acacetin for 24 h, and then the genomic DNA from cells was subjected to agarose gel electrophoresis. A clear DNA fragmentation was does-dependent (**Figure 3A**). At 60 μM acacetin, digested genomic DNA was evident at 12 h (**Figure 3B**). To investigate the induction of a sub-G1 cell population, the DNA content of AGS cells treated with acacetin for various periods and concentrations was analyzed by flow cytometry (**Figure 3C**). Cells were treated with acacetin (60 μM) and stained with propidum iodide. As seen in **Figure 3C**, the percentages of apoptotic AGS cells were 6.2, 11.1, 24.2, 26.5, 40.1, and 60.2% after 0, 3, 6, 9, 12, and 24 h of incubation with acacetin, respectively, and 12.2, 35.9, 48.2, 53.8, 58.5, and 62.1% after incubation with 0, 20, 40, 60, 80, and 100 μM acacetin, respectively. To characterize the cell death induced by acacetin, we examined the nuclear morphology of dying cells with a fluorescent DNA-binding agent, acridine orange. Within 24 h of treatment with 60 μM acacetin, cells clearly exhibited significant morphological changes and chromosomal condensation, which is indicative of apoptotic cell death. Such results imply that the cytotoxic action of acacetin was due to its ability to induce apoptosis.

Activation of Caspase-3, Not Caspase-1, Is Involved in Acacetin-Induced Apoptosis. We then asked whether caspases were involved in the cell death response induced by acacetin. Caspases are activated in a sequential cascade of cleavages from their inactive forms (*40*). Once activated, caspases can subsequently cleave their substrates at specific sites. For example, caspase-3 cleaves preferentially after a DXXD↓X, whereas caspase-1 cleaves at YXXD↓X. To monitor the enzyme activity of caspases during acacetin-induced apoptosis, we used two fluorogenic peptide substrates. Ac-EDVD-AMC is a specific substrate for caspase-3, whereas Ac-YVAD-AMC detects caspase-1 activity. As illustrated in **Figure 5A**, acacetin (60 μM) induced a dramatic increase in DEVE-specific caspase activity in treated AGS cells. In contrast to the increase in DEVD-specific activity, negligible YVAD-specific activity was observed. Activation of caspase-3 leads to the cleavage of a number of proteins, one of which is poly(ADP-ribose) polymerase (PARP). The cleavage of PARP is the hallmark of apoptosis. PARP (116-kDa) is cleaved to produce an 85-kDa fragmentation during apoptosis (*41*). As was already described, ICAD is a mouse homologue of human DFF-45. Caspase-3 cleaves DFF-45, and, once caspase-activated deoxyribonuclease (CAD) is released, it can enter the nucleus, where it degrades chromosomal DNA to produce interchromosomal DNA fragmentation (*42*, *43*). **Figure 5B** shows that exposure of AGS cells to acacetin causes the cleavage of DFF-45 and degradation of 116-kDa PARP to 85-kDa fragments. The protein cleavages were associated with activation of caspase-3. To further determine if the activation of a caspase-3-like protease is necessary for apoptosis induced by acacetin, caspase inhibitors including the caspase-3-like protease inhibitor Z-VAD-FMK and caspase-1-like protease inhibitor Ac-YVAD-FMK were used to block intracellular protease, and acacetin-induced cell viability was analyzed by MTT assay. Results in **Figure 5C** show that the caspase-3-like inhibitor, Z-VAD-FMK, but not the caspase-1 inhibitor, Ac-YVAD-FMK, significantly inhibited acacetin-induced apoptosis.

Involvement of Mitochondrial Dysfunction, ROS Production, Release of Cytochrme *c* from Mitochondria to Cytosol, and Caspase-9 Activation in Acacetin-Induced Apoptosis. Recently, it has become clear that apoptosis involves a disruption of mitochondrial membrane integrity that is decisive for the cell death process. We next evaluated the effects of acacetin on the

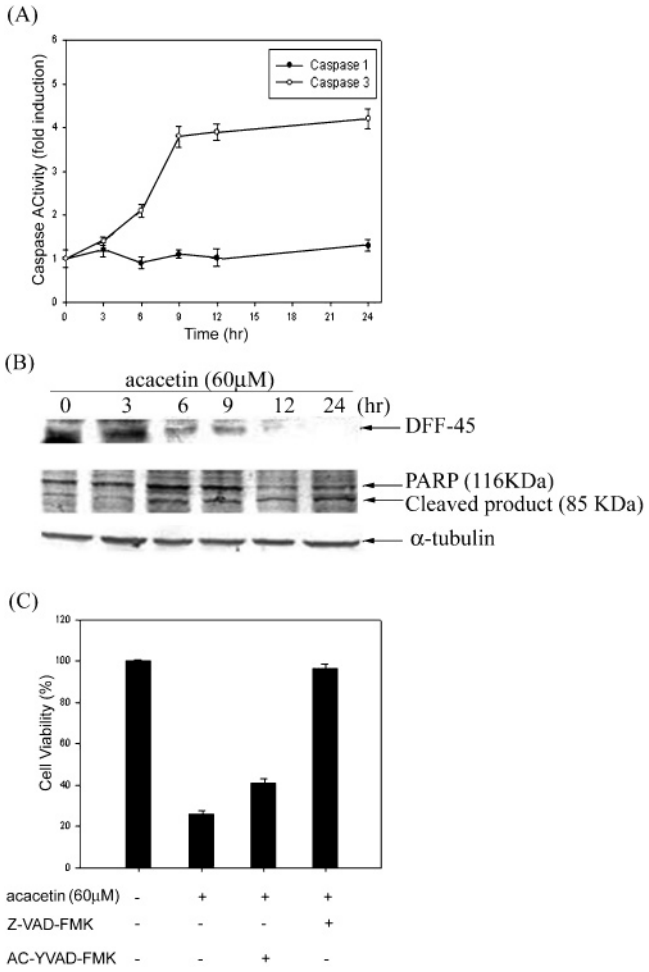

Figure 5. Specific induction of caspase-3 activities resulted in the processing of caspase-3 substrates during acacetin-induced apoptosis. AGS cells were treated with 60 μM acacetin for 0, 3, 6, 9, 12, and 24 h. Cells were harvested and lysed in lysis buffer. (**A**) Enzymatic activity of caspase-3 or caspase-1 was determined by incubation of 50 μg of total protein with fluorogenic substrate, Ac-DEVD-AMC or Ac-YVAD-AMC, respectively, for 1 h at 30 °C. The release of AMC was monitored spectrofluorometrically (excitation = 360 nm; emission = 460 nm). Data represent means ± SE for three determinations. (**B**) Cleavage of PARP and DFF-45 induced by acacetin was time-dependent. AGS cells were treated as indicated and analyzed by western blotting as described under Materials and Methods. (**C**) AGS cells were pretreated with either inhibitor, Z-VAD-FMK, or Ac-YVAD-FMK, for 1 h followed by acacetin for another 24 h. Cell viability was examined by MTT assay. Each value is presented as the mean ± SE of three independent experiments.

mitochondrial transmembrane potential ($\Delta\Psi_m$) and the release of mitochondrial cytochrome *c* into cytosol. We measured $\Delta\Psi_m$ using the fluorescent probe DiOC6(3) fluorescence and monitored it via flow cytometry. As shown in **Figure 6A**, which compares AGS cells exposed to acacetin and control cells, the DiOC6(3) fluorescence intensity shifted to the left from 199.46 to 61.54 in acacetin-induced apoptotic AGS cells at 30 min. These results demonstrate that acacetin causes a decrease in mitochondrial transmembrane potential in AGS cells. Furthermore, we studied the loss of mitochondrial transmembrane potential resulting in the generation of ROS by assessing ROS generation using the fluorescent probe DCFH-DA and monitoring by flow cytometry. AGS cells were treated with 60 μM acacetin for 1 h. An increase of intracellular peroxide levels by acacetin was detected for 1 h. These data indicated that the increment of ROS might play a role as an early mediator in acacetin-induced apoptosis. The findings point to an effect of acacetin on mitochondrial function and accumulation of ROS. The features are cues for the induction of apoptosis. Caspase-9 binds to Apaf-1 in a cytochrome *c*- and dATP-dependent fashion to become active and, in turn, cleaves and activates caspase-3 (*44*). As shown in **Figure 6C**, the release of mitochondrial cytochrome *c* into the cytosol was detected at 3 h in acacetin-treated AGS cells. Furthermore, we examined the activation of caspase-9 by fluorogenic peptide substrate: Ac-LEHD-AMC is a specific substrate for caspase-9. Acacetin induced a significant increase in LEHD-specific caspase-9 activity in AGS cells, approximately 3.8- and 7.0-fold those in the control group at 3 and 9 h, but the activity decreased after 12 h. In contrast to the increase in caspase-9 activity, negligible caspase-1 activity was observed.

ROS Production Involves in Acacetin-Induced Apoptosis. Recently, growing evidence has indicated that ROS played an important role in the induction of apoptosis. Therefore, antioxidants such as *N*-acetylcysteine (NAC), catalase (CAT), superoxide dismutase (SOD), allopurinol (ALL), and pyrrolidine dithiocarbamate (PDTC) were in the present study examined as to whether ROS production is an essential event for acacetin-induced apoptosis. As shown in **Figure 7**, pretreatment with NAC and CAT, but not SOD, ALL, and PDTC, protects AGS cells form acacetin-induced cytotoxicity in a dose-dependent manner.

Activation of Fas-Mediated Apoptosis Pathway by Acacetin, Resulting in Activation of Caspase-8 and Cleavage of Bid. To assess whether acacetin promoted apoptosis via receptor-mediated pathway, the Fas and Fas ligand (FasL) protein levels were determined by western blotting. The result showed that acacetin could stimulate the expression of Fas and FasL after treatment with acacetin. Maximum FasL was detected at 9 and 6 h, respectively (**Figure 8A**). Engagement of Fas and FasL results in the clustering of intracellular death domains of Fas and of Fas-associated death domain (FADD) and procaspase-8 into the death-inducing signal complex (DISC), where caspase-8 is activated (*24*). To verify whether the activation of caspase-8 was associated with Fas and FasL production in response to acacetin treatment, the activation of caspase-8 was detected after treatment of AGS cells with 60 μM acacetin at the indicated time points. As shown in **Figure 8B**, the gradual increase of caspase-8 activity showed a time-dependent manner, indicating that acacetin could activate the Fas-mediated pathway to lead to the apoptosis in AGS cells.

We next measured the pro-apoptosis protein, Bid, which upon cleavage by caspase-8, produced the truncated Bid fragment (tBid), and tBid targets mitochondria, causing mitochondria damage and amplifying apoptotic signals by activating the mitochondria pathway (*45*, *46*). The results showed that Bid cleavage occurred at 12 h (**Figure 8C**). These data suggested that the cleavage of Bid by active caspase-8 may be one of the mechanisms that contributed to the activation of mitochondrial pathway during acacetin-induced apoptosis.

Effect of Acacetin on the Expression of Bcl-2 Family, $p21^{Cip1/WAF1}$, and p53 Protein in AGS Cells. Several gene products are known to be important in controlling the apoptotic process. The imbalance of expression of anti- and pro-apoptotic protein after the stimulus is one of the major mechanisms underlying the ultimate fate of cells in apoptotic process. We examined the expression of pro-apoptotic protein, Bax, which inserts into the outer member of mitochondria and forms a large channel, allowing the release of cytochrome *c*; this process can

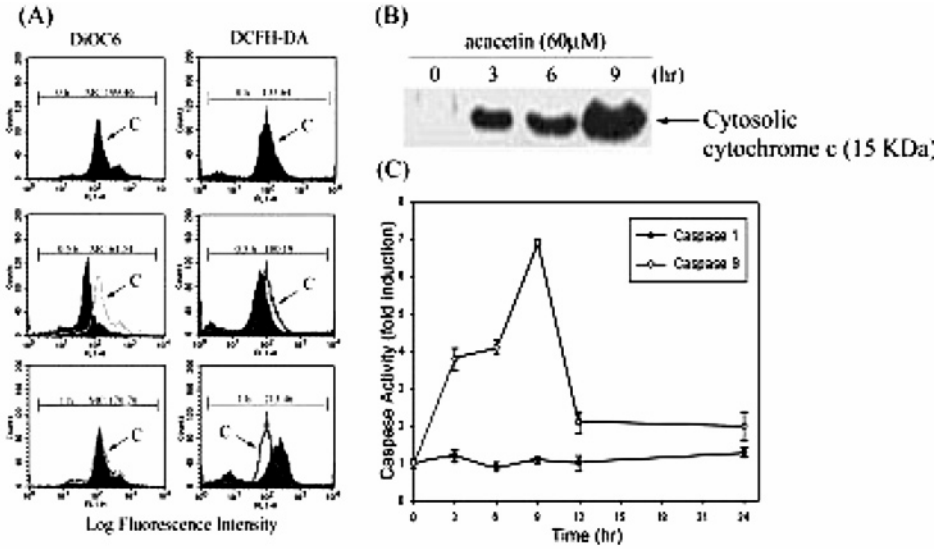

Figure 6. Induction of mitochondrial dysfunction, ROS generation, and cytochrome *c* release following caspase-9 activation in acacetin-induced apoptosis. (**A**) AGS cells were treated with 60 μM acacetin for indicated times and were then incubated with 40 nM 3,3′-dihexyloxacarbocyanine and DCFH-DA (20 μM), respectively, and analyzed by flow cytometry. Data are presented as log fluorescence intensity. C, control. (**B**) AGS cells were treated with 60 μM acacetin at indicated periods. Subcellular fractions were prepared as described under Materials and Methods, and cytosolic cytochrome *c* was detected by cytochrome *c* antibody. (**C**) To determine the kinetics of caspase-9 activation, cells were treated with 60 μM acacetin for different time periods or treated with 0.05% DMSO as vehicle control. Cells were harvested and lysed in lysis buffer. Enzymatic activity of caspase-9 and caspase-1 proteases was determined by incubation of 50 μg of total protein with fluorogenic substrates, Ac-LEHD-AMC or Ac-YVAD-AMC, respectively, for 1 h for 30 °C. The release of AMC was monitored (excitation = 360; emission = 460). Data represent means ± SE for three determinations.

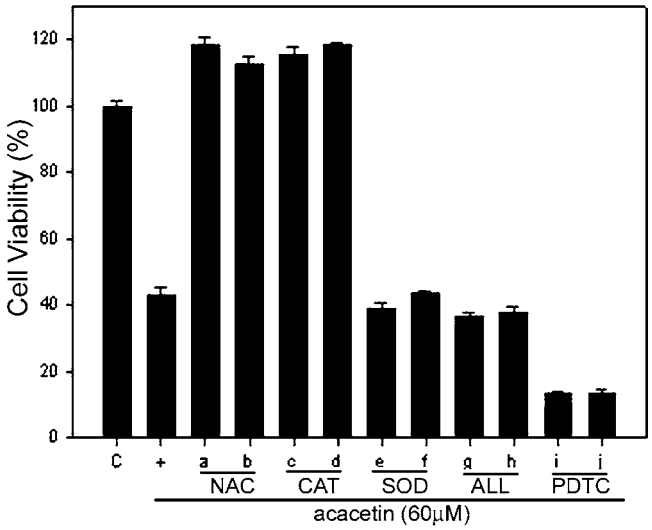

Figure 7. Effects of NAC, CAT, SOD, ALL, and PDTC on acacetin-treated AGS cells. AGS cells were treated with different concentrations of NAC, CAT, SOD, ALL, and PDTC for 1 h followed by acacetin (60 μM) treatment for another 24 h. The cell viability was determined by MTT assay. Each value is presented as the mean ± SE of three independent experiments. NAC (a, 2.5 mM; b, 5 mM), CAT (c, 200 units/mL; d, 400 units/mL); SOD (e, 100 μg/mL; f, 200 μg/mL); ALL (g, 50 μM; h, 100 μM); PDTC (i, 20 μM; j, 40 μM).

be prevented by Bcl-2 or Bcl-X_L (*47*). **Figure 9A** (upper panel) shows that for a marked dose increase of Bax protein its cleaved product in acacetin-treated AGS cells was observed. We next examined the expression of anti-apoptotic proteins, Bcl-X_L and Bcl-2, for various concentrations after 24 h of acacetin treatment. The dose decrease of Bcl-2 (**Figure 9A**, lower panel) and dose increase of the cleaved product of Bcl-X_L were observed in acacetin-treated cells (**Figure 9A**, middle panel). The marked cleavage of Bad and a significant change in the expression of Bcl-2 observed at 9 h (**Figure 9B**) were also observed in acacetin-treated cells. Previous studies demonstrated that p53 protein is a potent transcription factor, activated and accumulated in response to DNA-damaging agents (*48*), leading to cell cycle arrest or apoptosis (*49*, *50*). **Figure 9C** shows that the p53 protein level was elevated significantly at 3 h after 60 μM acacetin treatment in AGS cells. Such results implied that the p53 might be playing some important roles in acacetin-induced apoptosis observed in the AGS cells.

DISCUSSION

Flavonoids are naturally occurring plant polyphenols found in abundance in diets rich in fruits, vegetables, and plant-derived beverages such as tea. Epidemiological studies have shown that the intake of certain vegetables, fruits, and tea in the daily diet provides effective cancer prevention (*51*). Carcinogens usually cause genomic damage in exposed cells. As a consequence, the damaged cells may be triggered either to undergo apoptosis or to proliferate with genomic damage, leading to the formation of cancerous cells that usually exhibit cell cycle abnormalities and which are more susceptible to various apoptosis-inducing agents (*52*, *53*). Therefore, identifying active compounds from food with apoptosis-inducing activity against cell lines is considered to be a primary mechanism for the chemoprevention of cancer.

Acacetin has been reported to exhibit many biological effects including anticancer activity (*14*, *15*), but their anticancer mechanism is still elusive. In this study, we clarified the molecular mechanism by which acacetin triggered human gastric carcinoma AGS cells undergoing apoptosis. The present results demonstrate for the first time that acacetin can inhibit the proliferation of human gastric carcinoma cells through triggering apoptosis. As shown in **Figure 2**, acacetin was the potent inhibitor of cell viability and caused the potent and rapid induction of apoptosis, concurrent with DNA ladders, sub-G1 peak appearance, chromatin condensation, and apoptotic appearance in AGS cells. This induction of apoptosis occurred

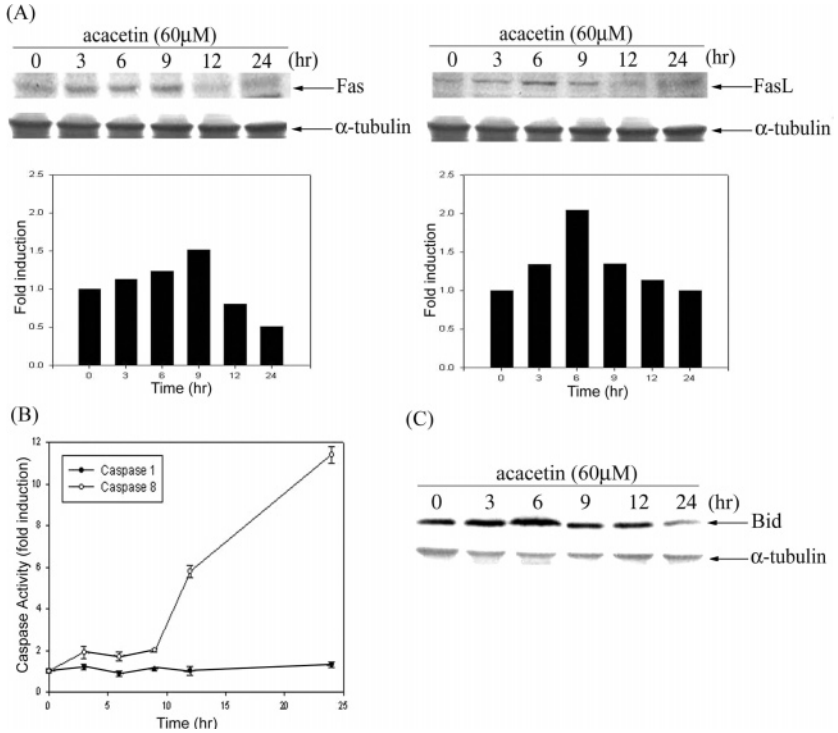

Figure 8. Increase in the expression of Fas and FasL leading activation of caspase-8 and decrease of Bid protein in acacetin-treated AGS cells. (**A**) AGS cells were treated with 60 μM acacetin for 0, 3, 6, 9, 12, and 24 h, and the expression of Fas, FasL, and Bid proteins (**C**) was analyzed by western blotting as described under Materials and Methods. This experiment was repeated three times with similar results. (**B**) To determine the kinetics of caspase-8 activation, cells were treated with 60 μM acacetin for different time periods or treated with 0.05% DMSO as vehicle control. Cells were harvested and lysed in lysis buffer. Enzymatic activity of caspase-8 and caspase-1 proteases was determined by incubation of 50 μg of total protein with fluorogenic substrates, Ac-IETD-AMC or Ac-YVAD-AMC, respectively, for 1 h for 30 °C. The release of AMC was monitored (excitation = 360; emission = 460). Each value is presented as the mean ± SE of three independent experiments.

within hours, consistent with the view that acacetin induced apoptosis by activating preexisting apoptosis machinery. Indeed, treatment with acacetin caused an induction of caspase-3, but not caspase-1, associated with the degradation of DFF-45 and PARP, which preceded the onset of apoptosis. Pretreatment with the caspase-3 inhibitor Z-VAD-FMK inhibited acacetin-induced apoptosis, suggesting that apoptosis induced by acacetin involves a caspase-3-mediated mechanism. Mitochondrial transmembrane potential ($\Delta\Psi_m$) is often employed as an indicator of cellular viability, and its disruption has been implicated in a variety of apoptosis phenomena (*54*). Mitochondria have also been implicated as a source of ROS during apoptosis. Reduced mitochondria membrane potential has recently been shown to lead to increased generation of ROS and apoptosis (*55*). Herein, we demonstrated that acacetin could disrupt the functions of mitochondria at the early stages of apoptosis and subsequently coordinate caspase-9 activation, but not caspase-1, through the release of cytochrome *c*. AGS cells showed increasing ROS production after acacetin treatment (**Figure 6**). The increase in ROS was probably due to the affected mitochondria cycling dioxygen through the electron transport assembly, and generating ROS by one-electron-transfer mitochondria could be a main target of nonspecific damage through oxidative stress at the level of the outer and inner membranes (*56*, *57*). As a consequence of oxidative membrane damage, membrane potential and permeability-barrier function are impaired, leading to further mitochondrial damage. Recently, oxidative damage to the mitochondrial membrane due to increased generation of ROS has been shown to play a role in apoptosis (*58*). Therefore, we speculated that intracellular generation of ROS could be an important factor in acacetin-induced apoptosis. To verify this, we performed experiments to confirm the effects of antioxidant on acacetin-induced apoptosis. Pretreatment with the antioxidants NAC and CAT, which are used as free radical scavengers, caused a significantly increased viability against acacetin-induced cytotoxicity.

The receptor-mediated signaling transduction pathway of apoptosis is another major pathway in activating caspase cascades. In our study, we observed the enhancement expression of Fas and FasL in acacetin-treated AGS cells (**Figure 8**). The result is in agreement with reports of Fas ligands, mFasL, and sFasL increasing in acacetin-treated Hep G2 cells. Acacetin induced a marked increase in caspase-8 activity to ~11-fold during 24 h. Recent studies have shown that caspase-3 can activate caspase-8 (*59*, *60*). Thus, we propose that caspase-8 is involved in cytochrome *c*-mediated apoptosis and participates in a feedback amplification loop involving caspase-3 in acacetin-treated AGS cells.

The Bcl-2 family of proteins, whose members may be anti-apoptotic or pro-apoptotic, regulate cell death by controlling the mitochondria membrane permeability during apoptosis (*61*, *62*). We, therefore, inferred that Bcl-2 family proteins might participate in the event that controlled the change in mitochondrial membrane potential and trigger cytochrome *c* release during apoptosis induced by acacetin. In our study, we found the down-regulation of the Bcl-2 expression and the cleavage of the Bad in acacetin-treated cells (**Figure 9**). We also found the up-regulation of Bax expression and the cleavage of Bcl-X_L during acacetin-induced apoptosis in AGS cells (**Figure 9**). However, the ratio between Bcl-2 and Bax and Bcl-X_L cleavage determines cell survival or death. The p53 tumor suppressor is a predominantly nuclear transcription factor, activated by various

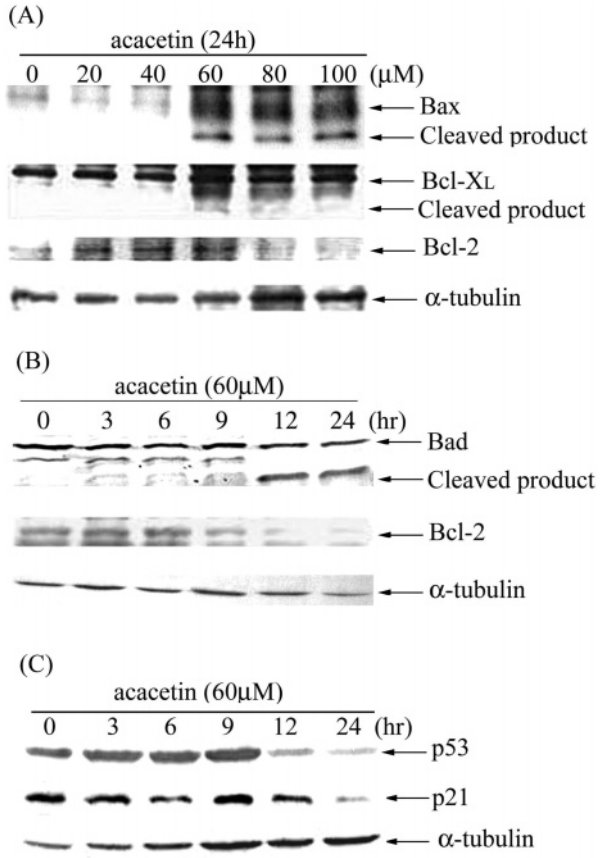

Figure 9. Effect of acacetin on Bcl-2 protein family, p21, and p53 expression in acacetin-treated AGS cells. AGS cells were treated with (**A**) various concentrations of acacetin for 24 h or (**B**, **C**) 60 μM acacetin for the indicated time point. Expression of Bax, Bcl-X_L, Bcl-2, Bad, p21, and p53 was analyzed by western blotting as described under Materials and Methods. Alternative expression of Bcl-2 family and p53 proteins was found in treated AGS cells. This experiment was repeated three times with similar results.

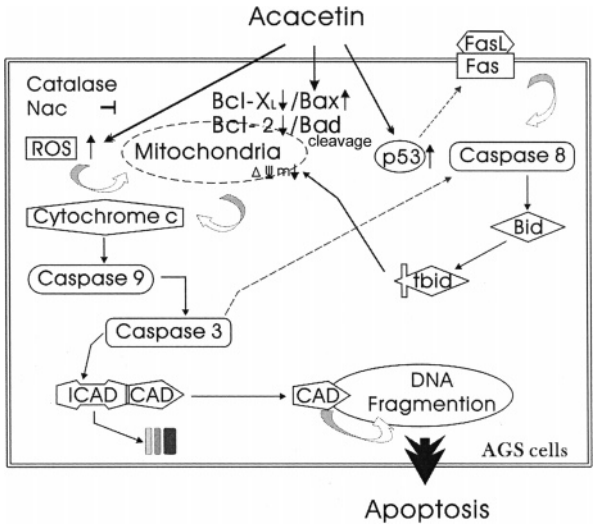

Figure 10. Schematic representation of action mechanism by which acacetin induced apoptosis in AGS cells. The initial event induced by acacetin may be to induce ROS production and coordinative modulation of the Bcl-2 family, further dissipate $\Delta\Psi_m$, result in cytochrome *c* release, and lead to consequent activation of caspase-9, caspase-3, and caspase-8. Alternatively, p53 further induced Fas and FasL expression that drove activation of caspase-8. Caspase-8 might indirectly disturb $\Delta\Psi_m$ by cleaving Bid and cause the release of cytochrome *c*. Active caspase-3 further cleaves inhibitor of caspase-activated DNase (ICAD), allows the caspase-activated DNase (CAD) to enter the nucleus, and degrades chromosomal DNA.

stresses including chemopreventive agents (*63*). Normal p53 function acts as tumor suppressor inducing both growth arrest and apoptosis. p53 activates the Fas gene in response to DNA damage by anticancer drugs (*64*). Treatment of the AGS cells with acacetin results in an increase in the level of p53 protein (**Figure 9**). The results presented herein account for acacetin-triggered apoptosis might increase the expression of Bax and Fas protein dependent on the p53 protein that affects mitochondrial function, raising the possibility that the expression of Bax or Fas could be transcriptionally regulated in response to acacetin treatment, but this issue should be elucidated. The ability to penetrate the cell membrane of flavonoids was reported as an important issue in antioxidant activity: the more lipophilic a compound, the better it can penetrate the lipid membrane and lower the antioxidant activity in cells (*65*). Although acacetin could change the integrity of the mitochondrial membrane by regulating the expression of Bcl-2 family proteins, we did not rule out the possibility that acacetin could penetrate into cells and directly target mitochondria to increase membrane permeability and decrease $\Delta\Psi_m$ accompanied by ROS production. On the basis of these data, we propose a possible apoptotic mechanism induced by acacetin (**Figure 10**). The initial event induced by acacetin may be to induce ROS, because NAC and CAT prevented apoptosis.

In summary, our results clearly demonstrate that the cancer chemopreventive agent acacetin triggered apoptosis in a dose- and time-dependent manner in AGS cells. Analyses of expression of the p53, Fas, FasL, and Bcl-2 family proteins, generation of ROS, subcellular location of cytochrome *c*, and the status of various caspases activities suggested that apoptosis induced by acacetin in AGS cells was mainly associated with ROS production, mitochondrial dysfunction, and Fas activation. Therefore, we speculate that the induction of apoptosis observed in this study may provide a distinct mechanism for the cancer therapeutic and chemopreventive functions of acacetin.

ABBREVIATIONS USED

DFF, DNA fragmentation factor; PARP, poly(ADP-ribose) polymerase; Apaf-1, apoptotic protease activating factor 1; ICAD, inhibitor of caspase-3-activated DNase; DCHF-DA, dichlorodihydrofluorescein diacetate; ROS, reactive oxygen species; NAC, *N*-acetylcysteine; CAT, catalase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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