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Apigenin Induces Apoptosis through Mitochondrial Dysfunction in U-2 OS Human Osteosarcoma Cells and Inhibits Osteosarcoma Xenograft Tumor Growth in Vivo

Chin-Chung Lin,[†,‡] Ya-Ju Chuang,[§] Chien-Chih Yu,[§] Jai-Sing Yang,[∥] Chi-Cheng Lu,[⊥] Jo-Hua Chiang,[⊥] Jing-Pin Lin,[#] Nou-Ying Tang,[#] An-Cheng Huang,*[,▽] and Jing-Gung Chung[○,◆,*]

[†]Department of Chinese Medicine, Fong-Yuan Hospital, Department of Health, Executive Yuan, Taichung 420, Taiwan

[‡]Department of Nursing, College of Medicine and Nursing, Hung Kuang University, Taichung 433, Taiwan

[§]School of Pharmacy, China Medical University, Taichung 404, Taiwan

[∥]Department of Pharmacology, China Medical University, Taichung 404, Taiwan

[⊥]Department of Life Sciences, National Chung Hsing University, Taichung 402, Taiwan

[#]School of Chinese Medicine, China Medical University, Taichung 404, Taiwan

[▽]Department of Nursing, St. Mary's Medicine Nursing and Management College, no. 100, Lane 265, Sec. 2, San-Sing Road, San-Sing Township, Yilan 266, Taiwan

[○]Department of Biological Science and Technology, China Medical University, no. 91, Hsueh-Shih Road, Taichung 40402, Taiwan

[◆]Department of Biotechnology, Asia University, Taichung 413, Taiwan

ABSTRACT: The cytostatic drug from natural products has acted as a chemotherapeutic agent used in treatment of a wide variety of cancers. Apigenin, a type of flavonoid, exhibits anticancer actions, but there is no report to show that apigenin induced apoptosis in osteosarcoma cells. The aim of this study was to investigate the effects of apigenin on U-2 OS human osteosarcoma cells and clarify that the apigenin-induced apoptosis-associated signals. The cytotoxic effects of apigenin were examined by culturing U-2 OS cells with or without apigenin. The percentage of viable cells via PI staining, apoptotic cells, productions of ROS and Ca^{2+}, and the level of mitochondrial membrane potential ($\Delta\Psi m$) were assayed by flow cytometry. The levels of apoptosis-related proteins were measured by immunoblotting. Results indicated that apigenin significantly decreased cell viability. Apigenin effectively induced apoptosis through the activations of caspase-3, -8, -9, and BAX and promoted the release of AIF in U-2 OS cells. In nude mice bearing U-2 OS xenograft tumors, apigenin inhibited tumor growth. In conclusion, apigenin has anticancer properties for induction of cell apoptosis in U-2 OS cells and suppresses the xenograft tumor growth. These findings offer novel information that apigenin possibly possesses anticancer activity in human osteosarcoma.

KEYWORDS: *Apigenin, apoptosis, mitochondrial dysfunction, U-2 OS human osteosarcoma cells, xenograft tumor*

INTRODUCTION

In human bone tumors, osteosarcoma is the most common primary malignancy; approximately 20% of all primary sarcomas is in bone [1] and occurs in adolescents and young adults.[2] The treatments of osteosarcoma include surgery, radiotherapy, and chemotherapy but are still unsatisfying. Although new therapies have been used in chemotherapy, the induction of drug-resistant and unwanted side effects are involved in chemotherapy which remain serious problems.[3] It was reported that currently used chemotherapeutic agents for cancer patients possess cytotoxic effects in normal cells, and they may induce DNA mutations that lead to secondary cancers.[4] Thus, numerous studies have focused on how to improve the effect of current chemotherapy or to find a new compound from natural products.[3−5]

Apoptosis, a programmed cell death type I, is a genetically mediated mechanism which involve the signal transduction,[5] cell shrinkage, nuclei condensation,[6,7] and the degradation of cellular protein and DNA.[8] The BCL-2 family of proteins are involved in apoptotic death and play a regulator of apoptosis, including pro-apoptotic protein such as BAX and antiapoptotic protein BCL-2[9,10] and both proteins affect the mitochondrial membrane potential of mitochondria before leading to apoptosis via caspase-dependent and/or caspase-independent pathways.[11] Thus, the ratio of BAX and BCL-2 could determine the fate of cells, and the occurrence of the change of the ratio could impair the normal apoptotic program which may lead to the formation of various apoptosis-related diseases.[12,13]

Apigenin (4′,5,7-trihydroxyflavone), a type of flavonoid, is widely contained in many fruits and vegetables such as oranges, tea, chamomile, onions, and wheat sprouts. It has been used as a dietary supplement due to its antitumor properties.[14] Apigenin has anticancer activity in many human cancer cells such as prostate cancer, colon carcinoma, and breast cancer, with low cytotoxicity and no mutagenic activity.[15−17] Furthermore, apigenin enhances the intracellular accumulation of reactive

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oxygen species (ROS) and antioxidant enzyme expression[18] and decreases SOD activity in lung cancer cells.[19]

Although many studies have shown the effect of apigenin in many human cancer cells, the exact mechanism of apigenein-induced apoptosis in human osteosarcoma cells is still unclear. In the current study, our primary aim was to find a possible molecular mechanism of apigenin-induced apoptosis in human U-2 OS osteosarcoma cells and investigate if apigenin inhibits U-2 OS xenografts tumor growth in vivo.

MATERIALS AND METHODS

Chemicals and Reagents. Apigenin, dimethyl sulfoxide (DMSO), propidium iodide (PI), RNase A, Triton X-100, and antiactin were obtained from Sigma Aldrich Corp. (St. Louis, MO, USA). McCoy's 5a medium, penicillin−streptomycin, trypsin−EDTA, fetal bovine serum (FBS), and L-glutamine were obtained from Gibco/Life Technologies (Carlsbad, CA, USA). 2,7-Dichlorodihydrofluorescein diacetate (H_2DCFDA), 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$), and Fluo-3/AM were obtained from Molecular Probes/Life Technologies (Eugene, OR, USA). Anti-BAX (catalogue no. 04-434) was purchased from Merck Millipore (Billerica, MA, USA). The other primary antibodies and secondary antibody-conjugated horseradish peroxidase (HRP) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

U-2 OS Cell Line. The human osteosarcoma cell line (U-2 OS) was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were maintained in 75 cm^2 tissue culture flasks with 90% McCoy's 5a medium supplemented with 10% FBS, 2 mM L-glutamine, 100 Units/mL penicillin, and 100 mg/mL streptomycin and grown under a humidified 5% CO_2 atmosphere at 37 °C.[20]

Assessments of Cell Morphological Changes and Determination of Percentage of Viability. U-2 OS cells at the density of 2×10^5 cells/well were placed onto 12-well plates and maintained for 24 h at 37 °C before being treated with 0, 50, 75, 100, 150 and 200 μM of apigenin for 24 and 48 h. The concentration of 0.5% DMSO (solvent) was used for the control regimen. After incubation, cells in each treatment were examined and photographed under a phase-contrast microscope for the cell morphological changes. The total 2×10^5 cells per sample from each treatment were centrifuged at 1000*g* for 5 min before cell pellets were suspended in 0.5 mL of PBS containing 5 μg/mL PI, and total viable cells were determined by using a flow cytometer (BD Biosciences, FACSCalibur, San Jose, CA, USA) as previously described.[21,22]

4,6-Diamidino-2-phenylindole Dihydrochloride (DAPI) Staining for Apoptotic Cells. U-2 OS cells at a density of 2×10^5 cells/well were placed onto 6-well plates and treated with apigenin at the final concentration (0, 50, 100, and 150 μM) for 24 h. The cells were then washed with PBS, permeabilized in 0.1% Triton X-100 for 10 min at room temperature, and stained with DAPI (2 μg/mL, Molecular Probes/Life Technologies) for 15 min at 37 °C. Finally, the stained cells were observed and photographed by fluorescence microscopy as previously described.[21,22] Subsequently, the mean fluorescence intensity (MFI) from apoptotic cells was quantified using a Metamorph imaging system (Universal Imaging Corp., Downingtown, PA, USA) in three random fields from each well.[23] DNA damage was subjected to comet assay assessment as described elsewhere.[21,22]

Determinations of the Reactive Oxygen Species (ROS) Productions, Intracellular Ca^{2+} Release, and the Levels of Mitochondrial Membrane Potential ($\Delta\Psi$m) in U-2 OS Cells. U-2 OS cells at a density of 2×10^5 cells/well were maintained in 12-well plates and treated with 75 μM apigenin for 0, 0.25, 0.5, 3, 6, 12, and 24 h before cells from each treatment were harvested and then resuspended in 500 μL of 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA) (10 μM) for ROS (H_2O_2) measurement, resuspended in 500 μL of Fluo-3/AM (2.5 μg/mL) for intracellular Ca^{2+} concentrations, and suspended in 500 μL of the mitochondrial membrane potential indicator 3, 3′-dihexyloxacarbocyanine iodide ($DiOC_6$) (100 nmol/L) for $\Delta\Psi$m. All samples were incubated at 37 °C for 30 min and then were analyzed by flow cytometry as described elsewhere.[21,22]

Assays of Caspase-3 and -8 Activities. U-2 OS cells at the density of 2×10^5 cells/well were placed in a 12-well plate before being treated with 75 μM apigenin and incubated for 0, 12, 24, and 48 h. The activity of caspase-3 and -8 were assessed according to manufacturer's instructions (OncoImmunin, Inc., Gaithersburg, MD, USA) of caspases activity assay kits. Cells in each well were harvested, and substrates (PhiPhiLux-G_1D_2 for caspase-3 and CaspaLux 8-L_1D_2 for caspase-8, respectively) were individually added to each cells and were incubated for 60 min and then underwent assay for the caspases activity as previously described.[20−22]

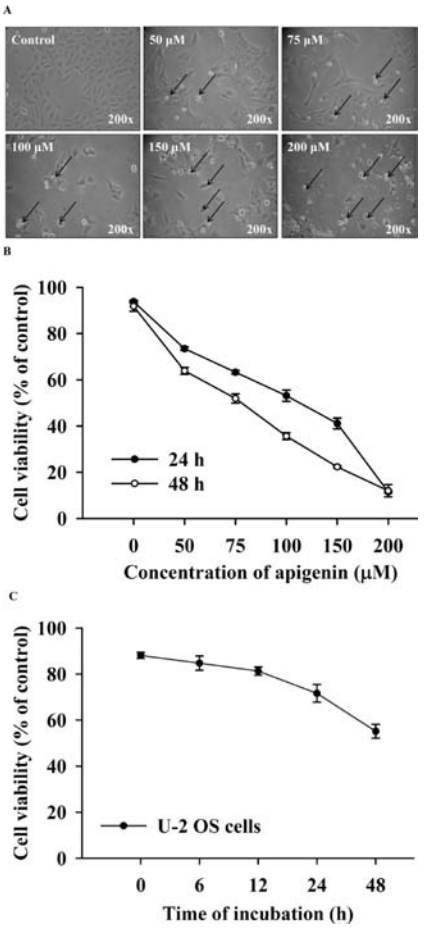

Figure 1. Apigenin induces cell's morphological changes and decreased the percentage of viable U-2 OS cells. Cells at 2×10^5 cells/well were plated onto 90% McCoy's 5a medium +10% FBS with various concentrations of apigenin for 24 and 48 h. Cells in each well were examined and photographed for the morphological changes under phase-contrast microscope (A). The total percentages of viable cells (B,C) were measure by flow cytometry as described in Materials and Methods. Each point is mean ± SD of three experiments, $*p < 0.05$ was significantly different from the control.

Western Blotting Analysis. U-2 OS cells at the density of 1×10^6 cells/well in a 6-well plate were exposed to 75 μM apigenin and incubated for 0, 6, 12, 24, 48, and 72 h. After that, cells were harvested from each treatment and extracted into the PRO-PREP protein extraction solution (iNtRON Biotechnology, Seongnam-si, Gyeonggi-do, Korea). The concentration of total proteins was determined utilizing Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA) for Western blotting as previously described.[20−22] At the end of electrophoresis, the proteins in the gels were transferred to the Immobilon-P transfer membrane (Millipore, Bedford, MA, USA), which was blocked with 5% nonfat milk in TBST buffer for 1 h. The membranes were hybridized with primary antibodies such as BAX, caspase-9, pro-caspase-3, AIF, caspase-8, PARP, p53, GRP78, caspase-12, and ATF4 and were thereafter stained by horseradish peroxidase (HRP)-conjugated secondary antibody. Blots were detected and the band densities were quantified using the NIH ImageJ 1.45 program (Bethesda, MI, USA) as

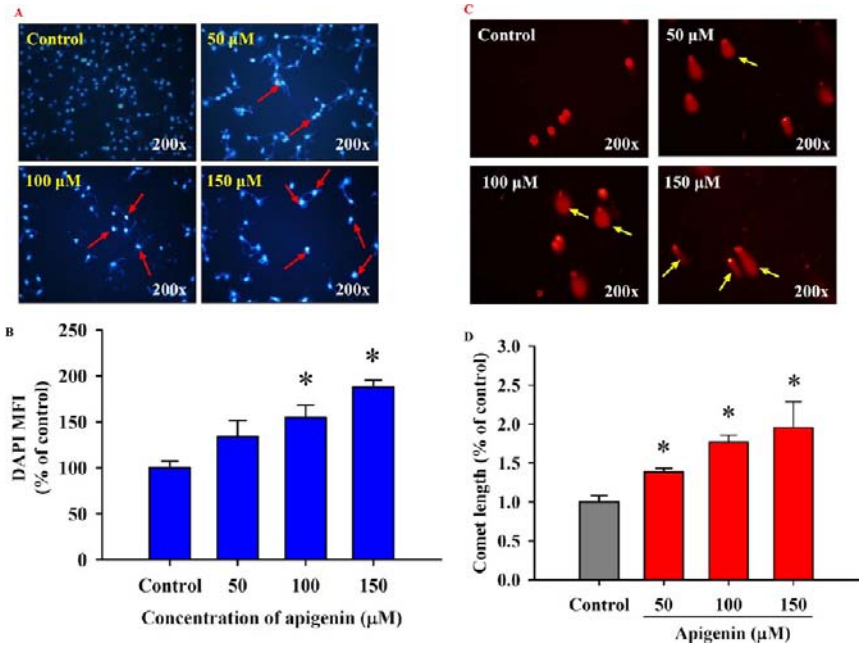

Figure 2. Apigenin triggers apoptosis in U-2 OS cells. Cells at 2×10^5 cells/well were incubated with 0, 50, 100, and 150 μM of apigenin for 24 h. Cells then were harvested for apoptosis and DNA damage by DAPI staining and comet assay and photographed by fluorescence microscopy as described in Materials and Methods. Each experiment was done with triple sets, $^*p < 0.05$ was significantly different from the control.

described previously.[20−22] The anti-β-actin antibody was used for equal protein loading.

Confocal Laser Scanning Microscopy for Examining the Translocation of AIF and GADD153 Proteins in U-2 OS Cells. U-2 OS cells were cultured on 4-well chamber slides at a density of 5×10^4 cells/well and then were treated without or with 75 μM apigenin for 24 h before cells were fixed directly in 4% formaldehyde in PBS for 15 min and then followed by permeabiliztion with 0.3% Triton-X 100 in PBS for 1 h. Then all samples were stained by using primary antibodies to AIF (1:100 dilution) and GADD153 (1:100 dilution) overnight. Then cells on the slides were washed twice with PBS and were stained with secondary antibody (FITC-conjugated goat antimouse IgG at 1:100 dilution) (green fluorescence). Finally, all samples were stained with PI (red fluorescence) for DNA as described previously.[20−22] All samples were photomicrographed and were obtained using a Leica TCS SP2 confocal spectral microscope.

Assays for mRNA Levels of Caspase-3, -9, and GADD153. U-2 OS cells were treated with 75 μM apigenin for 0, 24, and 48 h, and total RNA was extracted using the Qiagen RNeasy Mini Kit as previously described.[20−22] RNA samples were reverse transcribed with a High Capacity cDNA Reverse Transcription Kit at 42 °C for 30 min according to the standard protocol of the supplier (Applied Biosystems, Foster City, CA, USA). Quantitative PCR was performed using the following conditions: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s at 95 °C, 1 min at 60 °C using 1 μL of the cDNA reverse-transcribed as described above, 2X SYBR Green PCR Master Mix (Applied Biosystems), and 200 nM of forward and reverse primers (Caspase-3: F, CAGTGGAGGCCGACTTCTTG; R, TGGCACAAAGCGACTGGAT. Caspase-9: F, TGTCCTACTCTACTTTCCCAGGTTTT; R, GTGAGCCCACTGCTCAAAGAT. GADD153: F, TGCACCAAGCATGAACAATTG; R, TTGGCACTAGTGAGAGGGTAGTCA. GAPDH: F, ACACCCACTCCTCCACCTTT; R, TAGCCAAATTCGTTGTCATACC.). Each assay was run on an Applied Biosystems 7300 real-time PCR system in triplicates, and expression fold-changes were derived using the comparative C_T method.

Tumor Xenograft Model. Twenty male BALB/c nude mice (4−6 weeks of age) were obtained from the Laboratory Animal Center of National Applied Research Laboratories (Taipei, Taiwan). All mice were fed commercial diet and water ad libitum. U-2 OS cells were resuspended in serum-free McCoy's 5a medium with Matrigel basement membrane matrix (BD Biosciences) at a 5:1 ratio. Cell suspension (1×10^7 cells) was subcutaneously injected into the flanks of mice and incubated for 21 days to form xenofraft tumors when it reached to 100 mm^3. Animals with tumors were randomly assigned to one of two treatment groups (vehicle control and injected with 2 mg/kg apigenin every 3 days). At the end of the study for 30 days administration, animals were sacrificed and body weight was determined. Tumors were removed, measured, and weighed individually as previously described.[20,24] Our animal study was approved by the Institutional Animal Care and Use Committee (IACUC) of China Medical University (Taichung, Taiwan).

Statistical Analysis. The quantitative data are shown as means ± SD and the statistical differences between the apigenin-treated and control samples were calculated by one-way ANOVA followed by Dunnett's test. A value of $^*p < 0.05$ was considered significant. The results from the in vitro studies are representative of at least three independent experiments.

RESULTS

Apigenin Induces Cell Morphological Changes and Decreases the Percentage of Viable U-2 OS Cell. After U-2 OS cells were treated with various concentrations of apigenin for various time periods, cells were examined for cell morphological changes and we determined the percentage of viable cells. Results are shown in Figure 1. Figure 1A indicates that apigenin induced cell morphological changes and the hallmark of the apoptotic mode, including rounding and shrinkage in a concentration-dependent manner. Parts B and C of Figure 1 show that apigenin induced cell death in a dose- and time-dependent manner.

Apigenin-Induced Apoptosis and DNA Damage in U-2 OS Cells Is Confirmed by DAPI Staining and Comet Assay. Detection of apoptotic cells and DNA damage was accomplished by fluorescence staining. U-2 OS cells were treated with various concentrations of apigenin, and apoptotic cells were performed by DAPI staining and comet assay. As shown in Figure 2A,B, apigenin treatment in U-2 OS cells showed the cells increase of

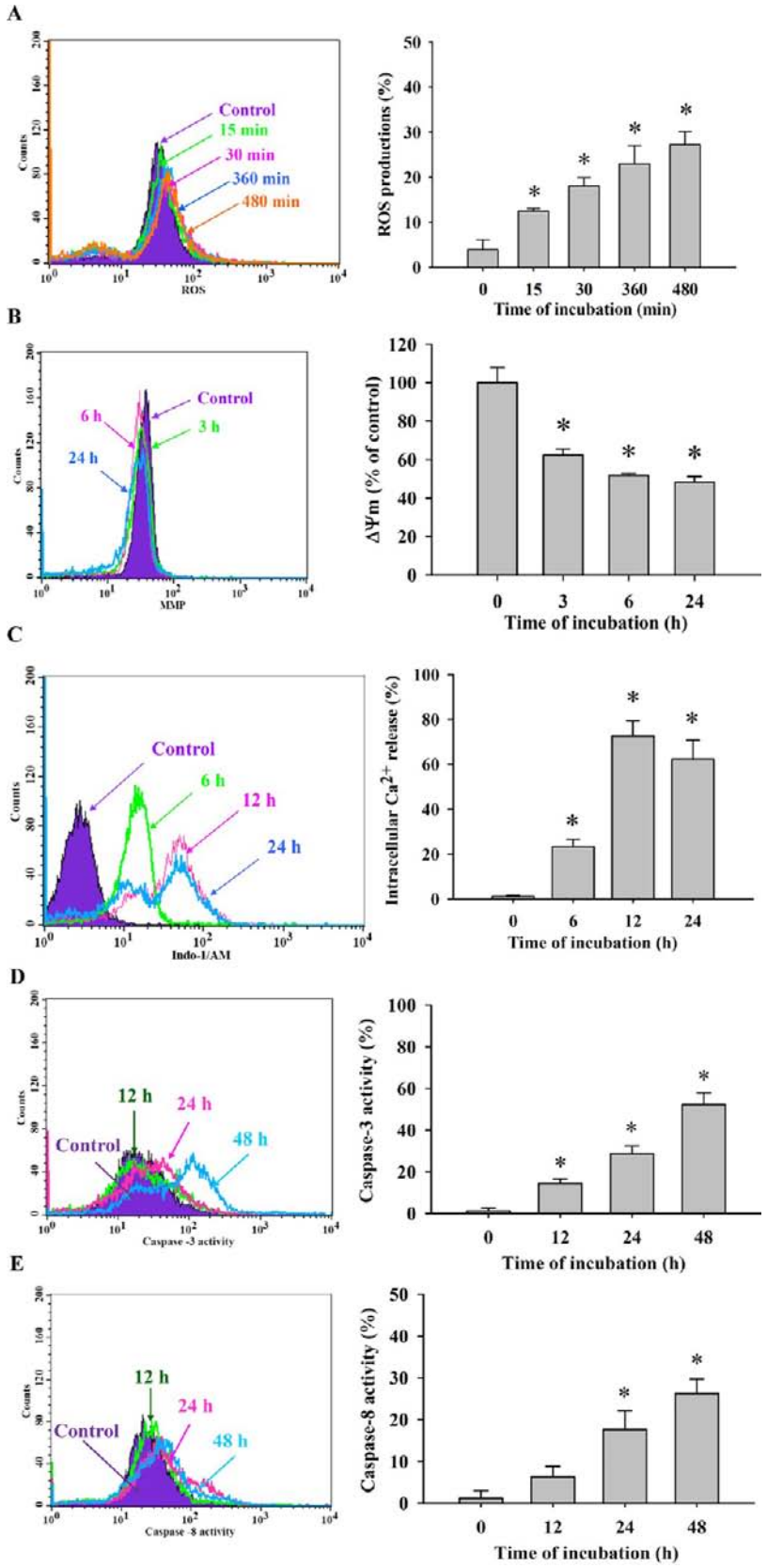

Figure 3. Apigenin affects the levels of reactive oxygen species (ROS) and intracellular Ca^{2+} and mitochondria membrane potential ($\Delta\Psi m$) in U-2 OS cells. Cells were incubated with 75 μM apigenin for various time periods, before being stained by H_2DCFDA for ROS levels determined (A), were stained with $DiOC_6$ for the $\Delta\Psi m$ levels determined (B), stained by Fluo-3/AM for the intracellular Ca^{2+} levels determined (C), were stained with PhiPhiLux-G_1D_2 for the caspase-3 activity (D) and CaspaLux 8-L_1D_2 for caspase-8 activity by flow cytometric analysis as described in Materials and Methods. Each experiment was done with triple sets, *$p < 0.05$ was significantly different from the control.

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DAPI fluorescence intensity. In Figure 2C,D, apigenin resulted in apoptosis of U-2 OS cells in a concentration-dependent manner.

Apigenin Alters the Levels of Reactive Oxygen Species (ROS) and Intracellular Ca^{2+} and Mitochondria Membrane Potential ($\Delta\Psi m$) in U-2 OS Cells. We investigated whether apigenin-induced cell death is involved in the productions of ROS and Ca^{2+} and affecting the levels of $\Delta\Psi m$ in U-2 OS cells. Cells were treated with 75 μM apigenin for various time periods. Cells were then harvested, and the levels of ROS, Ca^{2+} and $\Delta\Psi m$ were measured by flow cytometry. Results are shown in Figure 3A−C. The data indicated that apigenin increased the production of ROS and intracellular Ca^{2+} in a time-dependent manner (Figure 3A,C). However, apigenin decreased the levels of $\Delta\Psi m$ in U-2 OS cells, and this effect also is a time-dependent effect (Figure 3B).

Apigenin Promotes the Activities of Caspase-3 and -8 in U-2 OS Cells. We investigated whether apigenin induces the activities of caspase-3 and -8 in U-2 OS cells. Cells were treated with 75 μM apigenin for various time periods. Then cells were harvested, and the activities of caspase-3 and -8 were measured by flow cytometric assay, and results are shown in Figure 3D,E. The results showed that apigenin promoted the activities of caspase-3 (Figure 3D) and caspase-8 (Figure 3E) in a time-dependent manner. On the basis of those observations, we suggest that apigenin induced apoptosis via the activations of caspase cascade in U-2 OS cells.

Apigenin Affects the Apoptosis-Associated Proteins and Gene Expression in U-2 OS Cells. To confirm if apigenin triggers apoptosis via affecting apoptosis-associated protein expression in U-2 OS cells, cells were treated with 75 μM apigenin for 0, 6, 12, 24, 48, and 72 h and were then measured by Western blotting. The results were presented in Figure 4A,B, which indicated that apigenin stimulated the levels of BAX, caspase-9, AIF, caspase-8, PARP, and p53 (Figure 4A), and it also increased the levels of GRP78, caspase-12, and ATF4 in ER stress (Figure 4B). However, apigenin also decreased the expressions of pro-caspase-9, pro-caspase-3, and pro-caspase-8 (Figure 4A) in U-2 OS cells. Additionally, apigenin promoted the levels of *caspase-3*, *caspase-9*, and *GADD153* gene expression in U-2 OS cells as illustrated in Figure 4C.

Apigenin Stimulates AIF and GADD153 Distribution in U-2 OS Cells. For further confirming if apigenin increases the AIF and GADD153 expression, U-2 OS cells after exposure to apigenin were then harvested and examined for the translocation of AIF and GADD153 by confocal laser microscope. Results are shown in Figure 5A,B, which indicated that apigenin promoted the release of AIF and GADD153 to nuclei followed by apoptosis in U-2 OS cells.

Apigenin Inhibits Tumor Size in a Xenograft Mouse Model. We next examined the in vivo antitumor activities of apigenin in a mouse U-2 OS xenograft model. When xenograft tumor mass reached a volume of about 100 mm^3, mice were intraperitoneally injected with 2 mg/kg of apigenin or a vehicle control every 3 days. Representative tumors in the xenograft mice treated with or without apigenin are shown in Figure 7A, and the treatments of apigenin did not significantly alter body weight (Figure 7E). Figure 7B reveals that apigenin reduced tumor mass compared to control. Apigenin also significantly decreased by 74.5% the tumor weight compared to control (Figure 7B).

DISCUSSION

Numerous studies have demonstrated that apigenin induced cytotoxic effects through causing cell death via cell cycle arrest and induction of apoptosis in many types of cancer cell lines.[12−20] It was reported that apigenin induced apoptosis in human hepatocellular carcinoma (HCC) Huh-7 cells and went through decreased expression of vimentin in apoptosis and it also inhibited through dysfunction of cell migration and angiogenic

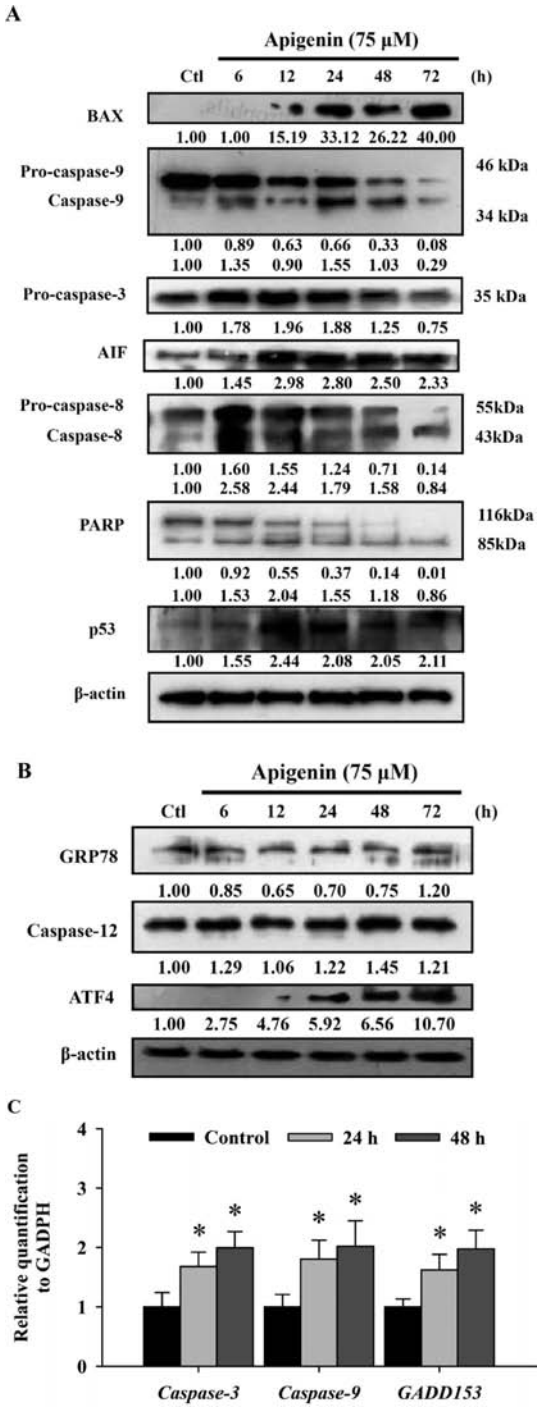

Figure 4. Apigenin alters the apoptotic relative proteins and gene expression in U-2 OS cells. A total of 1×10^6 U-2 OS cells/ml cells were treated with 75 μM apigenin for 0, 6, 12, 24, 48, and 72 h. Cells were harvested and associated proteins were measured by Western blotting. The protein levels of BAX, caspase-9, pro-caspase-3, AIF, and p53 (B) caspase-8 and PARP (A), and GRP78, caspase-12, and ATF4 (C) expressions were examined by using SDS-PAGE gel electrophoresis and Western blotting, and (D) *caspase-3*, *caspase-9*, and *GADD153* mRNA gene expression as described in Materials and Methods.

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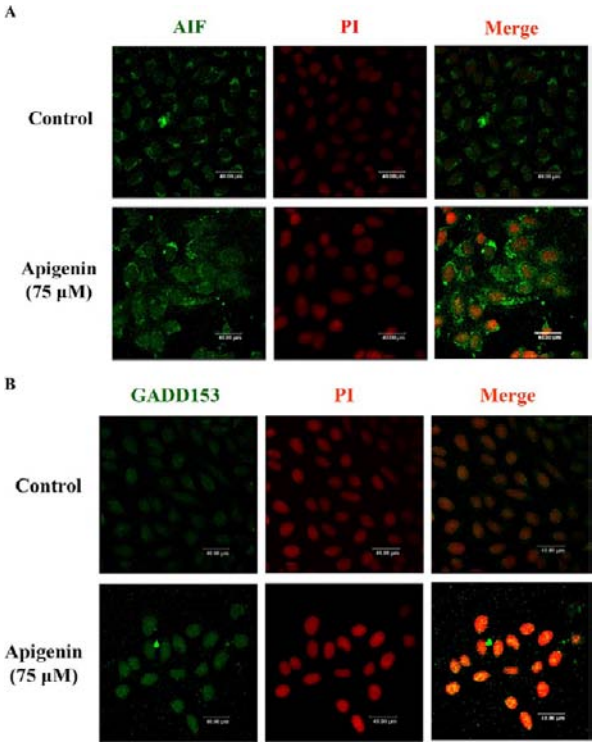

Figure 5. Apigenin stimulates the releases of AIF and GADD153 proteins in U-2 OS cells. A total of 2×10^4 U-2 OS cells/mL cells on the chamber slide were treated with 75 μM apigenin for 24 h. Cells were washed with PBS and then were stained with primary antibodies, including anti-AIF (A) and -GADD153 (B), before being stained with secondary antibody (FITC-conjugated goat antimouse IgG at 1:100 dilution) before all samples were stained with PI (red fluorescence) for nuclei as described in Materials and Methods. Scale bar: 40 μm.

factors.[25] However, there is no report to demonstrate apigenin-induced cytotoxic effects on human osteosarcoma cells. Thus, we investigated the effects of apigenin on human U-2 OS osteosarcoma cells in vitro and results showed that apigenin significantly induced cell morphological changes (cell shrinkage and rounding) (Figure 1A) and decreased percentage of viable cells at 24 and 48 h (Figure 1 B) in U-2 OS cells. Furthermore, DAPI staining showed that apigenin induced apoptosis (DNA condensation and fragmentation) in U-2 OS cells in a concentration-dependent manner (Figure 2A and B) that is nuclear DNA fragmentation which is a biochemical hallmark of apoptosis.[26] Thus, we hope for more studies to show the molecular mechanism of apoptotic induction in U-2 OS cells.

Apoptosis was divided into an extrinsic (death receptor-dependent pathway) and intrinsic pathway (mitochondria-dependent pathway).[27] In the extrinsic pathway, Fas and its receptor Fas ligand (FasL) and caspase-8 are involved in regulating the induction of apoptosis in diverse cell types and tissues.[28] In the intrinsic pathway, BCL-2 family proteins such as pro-apoptotic protein BAX and antiapoptotic protein BCL-2 that play critical roles involved the dysfunction of mitochondria that led to cytochrome *c* or AIF and Endo G release from mitochondria before leading to apoptosis.[22] Recently, the third apoptotic pathway name an ER stress includes the ROS and Ca^{2+} productions that led to activation of caspase-3 before causing apoptosis.[29] In the present study, results showed that apigenin induced ROS (Figure 3A) and Ca^{2+} (Figure 3C) production and decreased the levels of $\Delta\Psi$m (Figure 3B) in U-2 OS cells. We also found that U-2 OS cells after apigenin treatment induced apoptosis and was also involved in ROS through ER stress.

In the present study, results from Western blotting (Figure 4A,B) indicated that apigenin increased the expressions of PARP and active form of caspase-8 (Figure 4A) that demonstrated that apigenin induced apoptosis through the extrinsic pathway (Figure 4A). Furthermore, apigenin increased the expressions of p53, caspase-9, caspase-3, and AIF (Figure 4A), pro-apoptotic protein BAX (Figure 4A), and GRP78, caspase-12, and ATF4 (Figure 4B) in U-2 OS cells. From these results, it showed that apigenin induced apoptosis through the extrinsic pathway in U-2 OS cells. This is also being confirmed due to the results which indicated that apigenin decreased the levels of $\Delta\Psi$m (Figure 3B) in U-2 OS cells. BCL-2 family proteins have been shown to be involved in the mitochondria-dependent pathway and death receptor dependent pathway,[27,29,30] and this family includes BAX, the pro-apoptotic proteins, and antiapoptotic protein BCL-2.[31] It was reported that the changes of the level of $\Delta\Psi$m (Figure 3B) was dependent on the ratio of BAX/BCL-2 in cells after exposed to the agents.[10,31] Flow cytomteric assays also showed that apigenin promoted the activations of caspase-3 and -8 (Figure 3D,E), and these observations indicated that apigenin induced apoptosis through the caspase-dependent pathway. However, our results from Western blotting also demonstrated that apigenin promoted the expression of AIF (Figure 4A), which was also confirmed by confocal laser microscope which indicated that AIF (Figure 5A) and GADD153 (Figure 5B) was released from mitochondria and then translocated to nuclei for causing apoptosis. On the basis of these observations, it also indicated that apigenin induced apoptosis through a caspase-independent pathway. Additionally, our results from the in vivo experiment of U-2 OS tumor xenograft displayed that apigenin suppressed tumor weights (Figure 7C) and volumes (Figure 7D) in the treated group in comparison to untreated control. Also, we found that body weight was not significantly different between untreated control and apigenin-treated mice (Figure 7E).

In summary, we proposed the signaling pathway of apigenin-induced apoptosis in U-2 OS cells that are present in Figure 6.

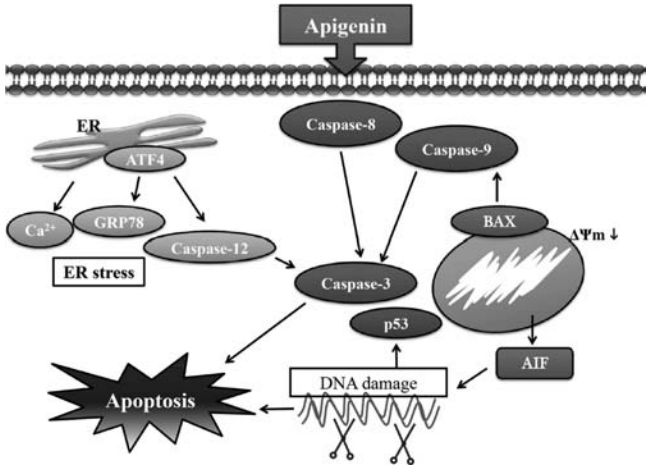

Figure 6. Proposed signaling pathways of apigenin-induced apoptosis in a human osteosarcoma U-2 OS cell line.

The flowchart from Figure 6 indicated that apigenin induced apoptosis via the interaction of caspase-8 (extrinsic pathway),

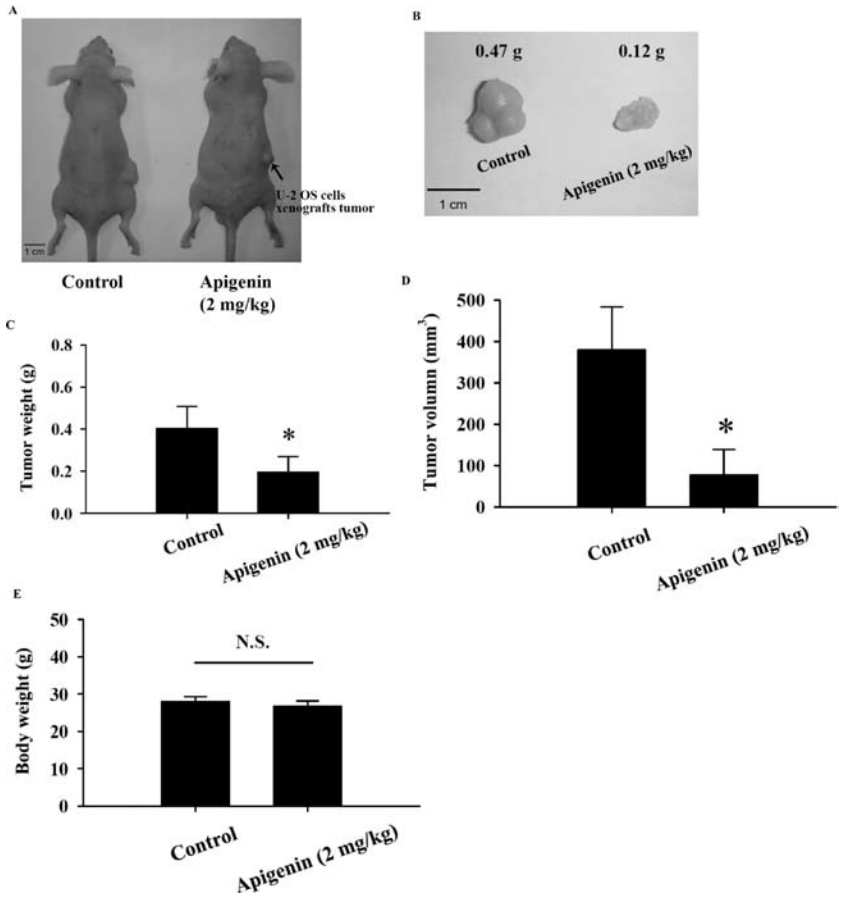

Figure 7. Apigenin inhibits tumor growth on the xenograft animal model. Twenty BALB/c nu/nu nude mice were sc implanted with 1×10^7 U-2 OS cells for 21 days and then randomly divided into two groups. Group 1 was treated with DMSO only and 2 mg/kg of apigenin once every 3 days. At the 30th day, all animals were sacrificed. (A) Representative animals and (B) mice with solid tumor, (C) tumor weight, (D) tumor volume, (E) body weight.

BAX in mitochondria dysfunction (intrinsic pathway), and through activations of the ER stress before leading to caspase-3 activation or through dysfunction of mitochondria before leading to AIF release (caspase-independent pathway) and apoptosis in the present study. Apigenin also decreased the weight and size of U-2 OS cells in xenograft animal models in vivo. The most striking findings are the consistency of effects seen in vitro and in vivo. Based on of these observations, we provide a potential molecular mechanism for apigenin-induced apoptosis, which could be used in antibone cancer in the future.

AUTHOR INFORMATION

Corresponding Author

*For J-G.C.: phone, +886 422053366 ext 2161; fax, +886 422053764, E-mail, jgchung@mail.cmu.edu.tw. For A.-C.H.: phone, +886-03-9897396 ext. 530; e-mail, haj@smc.edu.tw.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

$\Delta\Psi$m, mitochondrial membrane potential; FBS, fetal bovine serum; DAPI, 4′,6,-diamidino-2-phenylindole; $DiOC_6$, 3,3′-dihexyloxacarbocyanine iodide; H_2DCFDA, 2′,7′-dichlorofluorescin diacetate; PI, propidium iodide; ROS, reactive oxygen species

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