Apigenin inhibits proliferation and invasion, and induces apoptosis and cell cycle arrest in human melanoma cells

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Abstract. Malignant melanoma is the most invasive and fatal form of cutaneous cancer. Moreover it is extremely resistant to conventional chemotherapy and radiotherapy. Apigenin, a non-mutagenic flavonoid, has been found to exhibit chemopreventive and/or anticancerogenic properties in many different types of human cancer cells. Therefore, apigenin may have particular relevance for development as a chemotherapeutic agent for cancer treatment. In the present study, we investigated the effects of apigenin on the viability, migration and invasion potential, dendrite morphology, cell cycle distribution, apoptosis, phosphorylation of the extracellular signal-regulated protein kinase (ERK) and the AKT/mTOR signaling pathway in human melanoma A375 and C8161 cell lines in vitro. Apigenin effectively suppressed the proliferation of melanoma cells in vitro. Moreover, it inhibited cell migration and invasion, lengthened the dendrites, and induced G2/M phase arrest and apoptosis. Furthermore, apigenin promoted the activation of cleaved caspase-3 and cleaved PARP proteins and decreased the expression of phosphorylated (p)-ERK1/2 proteins, p-AKT and p-mTOR. Consequently, apigenin is a novel therapeutic candidate for melanoma.

Introduction

Malignant melanoma has recently been reported to have one of the highest incidence rates among all types of cancer, with an increasing number of melanoma-related deaths each year (1). The principal cause of death in melanoma patients is attributed to widespread metastases to the lymphatic system and other organs (2). Following traditional therapy, the average survival time of patients with metastatic melanoma is only 6-12 months and the 5-year survival rate is consistently <10% in most cases (3). However, tremendous progress in both immunotherapy and molecular-targeted therapy has revolutionized the standard of care for terminal melanoma patients. In the meantime, some new challenges for clinicians have also surfaced (4). One such example is molecular-targeted therapy which often leads to fast-acting and significant responses in most patients with the targeted mutation, while the clinical benefit is usually transient due to the rapid emergence of drug resistance. Consequently, it is urgent to develop efficient agents that may be applied for melanoma treatment.

Apigenin, a natural plant flavonoid (4',5,7-trihydroxyflavone), is widespread in common fruits and vegetables. According to the Biopharmaceutics Classification System, apigenin is categorized as a class II drug with poor solubility and high intestinal membrane permeability (5). The oral bioavailability of apigenin is relatively low due to its low solubility in water (~2.16 µg/ml) (5) and in high hydrophilic or nonpolar solvents (0.001-1.63 mg/ml) (6), which has extremely hampered its clinical development. Several formulation strategies have been investigated to improve the bioavailability for application, including liposome (7) and nanocrystals fabricated by high pressure homogenization (8).

Apigenin has been shown to have marked anti-inflammatory, antioxidant and anticarcinogenic properties (9). Recently researchers have demonstrated that apigenin has an anti-proliferative effect on a variety of cancer cells, such as bladder, ovarian, breast and prostate cancer (10-17), including melanoma (16,17). Apoptosis plays a critical role in controlling cell proliferation and thus is pivotal for the prevention of cancer progression and oncogenesis (18). Extracellular signal-regulated kinase (ERK) is a crucial signaling molecule that regulates cell survival and proliferation. The ERK signaling pathway controls various pro- and anti-apoptotic mechanisms that determine cell viability (19). AKT serves as an anti-apoptotic signaling molecule and inhibits apoptosis through mitochondrial pathways (20). Consequently, in the present study, we investigated the effects of apigenin on the viability, migration and invasion potential, dendrite morphology, cell cycle distribution, apoptosis, ERK expression and the AKT/mTOR signaling pathway.

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Materials and methods

Chemicals and reagents. Apigenin (no. A0113, CAS: 520-36-5, purity ≥98%) was purchased from Chengdu Must Bio-Technology Co., Ltd. (Chengdu, China). Dulbecco’s modified Eagle’s medium (DMEM), trypsin and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Triton X-100 and anti-β-actin antibody were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trypsin free of ethylenediaminetetraacetic acid (EDTA) was purchased from Hyclone Co. (Logan, UT, USA). Matrigel was purchased from BD Biosciences (Franklin Lakes, NJ, USA). FITC-Annexin V kit was obtained from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China). Propidium iodide (PI) and RNase were purchased from Takara Bio, Inc. (Otsu, Shiga, Japan). Antibodies for ERK1/2 and phosphorylated (p)-ERK1/2 were purchased from Promega Corp. (Madison, WI, USA). The antibodies for poly(ADP-ribose) polymerase (PARP), caspase-3, AKT, p-AKT (Ser473), mTOR and p-mTOR (Ser2448) were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA).

Cell culture and apigenin treatment. Human malignant melanoma A375 and C8161 cell lines were obtained from Peking Union Cell Resource Center (Beijing, China). The cells were grown at 37˚C in a humidified atmosphere containing 5% CO₂. The cells were cultured and maintained in DMEM supplemented with 1% penicillin-streptomycin and 10% FBS. A375 and C8161 cells were treated with different concentrations of apigenin (dissolved in DMSO) whereas the control cells were treated with an equivalent volume of DMSO.

MTT assays. For cell proliferation assays, the A375 and C8161 cells were seeded in 96-well plates at a concentration of 1x10⁴ cells/well. Cells were allowed to adhere for 24 h and subsequently exposed to different concentrations of apigenin (40, 80, 120, 200, 240 and 280 µM) and incubated at 37˚C for 24, 48, 72 and 96 h. MTT solution was added to each well at the specified time-point and incubated for an additional 4 h. The culture medium in each well was discarded and replaced with DMSO to dissolve the formazan crystals which were formed from the MTT. The absorbance value was evaluated using an automatic microplate reader (T17108U; PerkinElmer, Inc., Waltham, MA, USA) at 490 nm.

Cell migration assays in vitro. Cell migration was performed using the wound healing assay. A375 and C8161 cells were seeded in a 24-well plate at a concentration of 5x10⁴ cells/well and allowed to form a confluent monolayer for 24 h. The monolayer was scratched with a sterile pipette tip (10 µl) then washed with serum-free medium to remove the floating and detached cells. After treatment with 40 and 80 µM of apigenin or DMSO, the cells were observed and photographed (time 0 h and 24 h) using an inverted microscope (Olympus Corporation, Tokyo, Japan). Moreover, the number of cells migrating to the wound was assessed. Data were obtained from three independent experiments.

Cell invasion assay. Cell culture inserts (24-well, pore size 8 µm; BD Biosciences) were seeded with 1x10⁶ cells/ml in 100 µl of serum-free medium with 40 µM apigenin, or DMSO. Inserts were precoated with 10 µl of Matrigel (3 mg/ml; Becton-Dickinson, Mountain View, CA, USA). Medium with 10% FBS (500 µl) was added to the lower chamber and served as a chemotactic agent. After incubation for 72 h, non-invasive cells were wiped from the upper surface of the membrane. Cells on the lower side were fixed with chilled methanol, stained with crystal violet (dissolved in methanol) and counted using an inverted microscope. Each individual experiment had triplicate inserts and five random, non-overlapping fields at a magnification of x200 were counted per insert.

Scanning electron microscopy. A375 and C8161 cells were plated at a concentration of 2x10⁴ cells/well into a 60-mm culture dish. After treatment with 100 µM of apigenin or DMSO for 24 h, the cells were harvested, washed with PBS and fixed with 2.5% glutaraldehyde and 1% osmium tetroxide, followed by an increasing gradient dehydration step using ethanol solutions of 50, 70, 95 and 100%. Samples were sputter-coated with platinum and palladium before being observed under a scanning electron microscope (Quanta 200F; FEI, Hillsboro, OR, USA).

Cell apoptosis. Cells were placed in 6-well culture plates (5x10⁴ cells/ml) and allowed to attach for 8 h. A375 and C8161 cells were treated with apigenin (40 and 100 µM, respectively) or DMSO for 24 h. Following the manufacturer’s instructions, the cells were harvested by trypsinization free of EDTA, washed in cold PBS and resuspended in binding buffer at a concentration of 1x10⁶ cells/ml. FITC-conjugated Annexin V (BioVision, Inc., Milpitas, CA, USA) and PI (5 µl each) (Becton-Dickinson) were added to the cells, gently mixed and then incubated for 15 min at room temperature in the dark. Afterwards binding buffer was added and the cells were analyzed by flow cytometry.

Cell cycle analysis. Cells were seeded in 60-mm culture dishes. After attachment, the cells were treated with 100 µM apigenin or DMSO for 24 h. Then cells were harvested and fixed with ice-cold 75% ethanol. The cell pellets were resuspended in binding buffer consisting of 480 µl PBS, 5 µl PI (5 mg/ml), 5 µl RNase (10 mg/ml) and 10 µl Triton X-100 (10%). After 30 min of incubation at room temperature in the dark, the DNA content of the cells was examined using a flow cytometer (Accuri C6; Becton-Dickinson) for cell cycle phase distribution.

Western blot analysis. Cells were plated in 6-well culture plates at concentrations determined to yield 60-70% confluence within 24 h. Next, the cells were left untreated or treated with 100 µM apigenin for 24 h. After preparing appropriate protein concentrations of 25 µg, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed. Proteins were separated by electrophoresis and transferred onto nitrocellulose membranes and afterwards blocked for 1 h with 5% non-fat dry milk in TBS-T. The membranes were incubated with respective primary antibodies at appropriate concentrations overnight at 4°C. After being washed to remove
unbound primary antibodies, they were incubated with the corresponding secondary antibodies. Proteins were visualized by image scanning and the optical density for each band was assessed using Image Lab software (version 4.0; Bio-Rad, Hercules, CA, USA) after data were normalized to β-actin as an internal reference.

Statistical analysis. All the experiments were carried out in triplicate and the values are expressed as the mean ± standard deviation (SD). SPSS v17.0 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. The repeated experiments used analysis of variance, Dunnett’s test and Student’s t-test for the assessment of differences between groups. A probability value of ≤0.05 was deemed statistically significant.

*P<0.05, **P<0.01 and ***P<0.001 as indicated in the figures, are relative to the controls.

Results

Apigenin inhibits A375 and C8161 cell proliferation. To investigate the growth inhibitory effect of apigenin, A375 and C8161 cells were treated with different concentrations (40, 80, 120, 160, 200, 240 and 280 µM) of apigenin for different periods of time (24, 48, 72 and 96 h). The cell viability was assessed by MTT assay. As shown in Fig. 1, cell growth inhibition caused by apigenin was relatively marked in a dose-dependent, as well as a time-dependent manner (ranging from 40-160 µM within 48 h). The IC50 value at 24 h was estimated to be 100 µM.

Apigenin inhibits A375 and C8161 cell migration potential. To assess whether or not apigenin has an effect on the metastasis of A375 and C8161 cell lines, we examined the number of migratory cells using a wound-healing approach. Migration was significantly inhibited in the A375 and C8161 cell lines after treatment with apigenin (40 and 80 µM) for 24 h (P<0.001) (Fig. 2). When the cells were exposed to 100 µM of apigenin for 24 h, no migrating cells were observed (data not shown).

Apigenin suppresses the invasion of A375 and C8161 cells. We further investigated cell motility by invasion assays. Both the density of the invasive cells on the membrane and the number of invasive cells/field are shown in Fig. 3. Treatment with 40 µM of apigenin for 72 h significantly decreased the invasive ability of the A375 and C8161 melanoma cells compared with the control cells (P<0.001). Following treatment with 80 µM of apigenin, no invading cells were observed (data not shown). These findings demonstrated that apigenin decreased the invasion of melanoma cells in vitro.

Apigenin affects the dendrite morphology of A375 and C8161 cells. Following treatment with 100 µM of apigenin for 24 h, both the A375 and C8161 cells changed their cellular morphology as visualized using scanning electron microscopy. The dendrites became thinner and longer compared to those noted in the untreated control cells (P<0.001) (Fig. 4).

Apigenin promotes the apoptosis of A375 and C8161 cells. To ascertain the underlying mechanism which leads to apigenin-induced inhibition of cell proliferation, we observed the effects of apigenin on the A375 and C8161 cells by detecting their apoptotic rates. Significant apoptosis was found in both the A375 and C8161 cell lines. Treatment with apigenin
Figure 3. Effect of apigenin treatment on the invasive ability of A375 and C8161 cells. The invasive capacity was assessed by employing a Transwell chamber assay. (A and B) Representative photomicrographs of the inhibition of cell invasion following treatment with 40 µM of apigenin (crystal violet staining, magnification, x200; representative image from three independent experiments). (C and D) Histograms of the number of invasive cells after treatment with 40 µM of apigenin. Data are presented as the mean ± SD from at least triplicate wells and of three separate experiments. ***P<0.001 vs. control.

Figure 4. Morphological alteration is induced in A375 and C8161 cells by apigenin. Cells were treated with apigenin (100 µM) for 24 h and then observed by scanning electron microscopy. (A) Dendrites became thinner and longer in the A375 cells compared to the control group. a, DMSO control group; b, A375 cells. (B) Dendrites became thinner and longer in the C8161 cells compared to the control group. a, DMSO control group; b, C8161 cells. (C and D) Quantification of the dendrite length of A375 and C8161 cells after apigenin treatment. Data are presented as the mean ± SD from at least triplicate wells and of three separate experiments. ***P<0.001 vs. control. DMSO, dimethyl sulfoxide.
Apigenin arrests A375 and C8161 cells at the G2/M phase of the cell cycle. Cell cycle analysis was also performed by flow cytometry. A375 and C8161 cells were treated with 100 µM of apigenin for 24 h. As shown in Fig. 6, apigenin treatment resulted in a noticeable accumulation of cells in the G2/M phase with a decrease in the G0/G1 phase during the cell cycle (P<0.05, P<0.01, P<0.001). This blockage of cell progression may be one of the mechanisms by which apigenin exerts its anti-proliferative effect on melanoma cell lines.

Apigenin alters ERK protein expression. Western blot analysis showed that treatment with 100 µM of apigenin significantly increased the expression of cleaved caspase-3 and cleaved PARP in the A375 and C8161 cells, while it decreased the expression of p-ERK1/2 but did not alter the total ERK1/2 level as compared to the DMSO controls (P<0.05, P<0.01, P<0.001) (Fig. 7).

Apigenin inhibits the AKT signaling pathway. Western blot analysis revealed that the expression levels of phosphorylated AKT (p-AKT) and p-mTOR were decreased after treatment with 100 µM of apigenin, whereas no significant difference was observed in total AKT and mTOR, in comparison to the DMSO controls (P<0.001) (Fig. 8).

Discussion

Melanoma is one of the most malignant cancers with a propensity for metastases. The well-established conventional treatments for melanoma, such as cryotherapy, surgery, and chemotherapy (21) and some nonsurgical treatments are usually limited to adjuvant therapies. Therefore, increasing interest has focused on the search for natural dietary phytochemicals both safe and effective against melanoma. It is generally known that many compounds from natural plants possess chemopreventative and chemotherapeutic efficacy in human cancers including melanoma (22). Apigenin, a flavonoid belonging to the flavone subgroup, is present in various vegetables, herbs, fruits and Chinese traditional
Figure 7. Inhibition of ERK phosphorylation following apigenin treatment in A375 and C8161 cells. A375 and C8161 cells were incubated with DMSO or 100 µM of apigenin for 24 h. (A) Investigated proteins were analyzed by western blot analysis. (B) Relative amounts of the investigated proteins expressed in A375 and C8161 cells. The expression of cleaved caspase-3 and cleaved PARP was increased while the content of p-ERK1/2 decreased and the total ERK1/2 level was invariant. Data are presented as the mean ± SD from at least triplicate wells and of three separate experiments. *P<0.05 vs. control. **P<0.01 vs. control. ***P<0.001 vs. control. DMSO, dimethyl sulfoxide.

Figure 8. AKT signaling pathway is inhibited in A375 and C8161 cells treated with 100 µM of apigenin for 24 h. (A) Apigenin significantly decreased the expression of phosphorylated (p)-AKT and p-mTOR, but the expression of total AKT and mTOR was not altered. (B) Relative amounts of the investigated proteins expressed in the A375 and C8161 cells. Data are presented as the mean ± SD from at least triplicate wells and of three separate experiments. ***P<0.001 vs. control.
medications (9,23) and has been shown to suppress tumor growth through inhibition of cell proliferation (24).

In the present study, we investigated the chemotherapeutic capacity of apigenin against human melanoma. We selected the human melanoma A375 and C8161 cell lines which have a different BRAF mutation status. A375 cells harbor the BRAF^{V600E} mutation while C8161 cells contain the BRAF wild-type gene. Apigenin, as shown in this study, significantly suppressed the growth of A375 and C8161 cells. These data suggest that apigenin possesses chemotherapeutic potential against human melanoma.

Dysregulation of the cell cycle is a hallmark of tumorigenesis. The cell cycle is controlled at multiple checkpoints. The G_{2}/M checkpoint inhibits cells from entering mitosis when DNA is impaired, enabling cell repair. Pathways that result in apoptosis may be activated when the damage is irreparable (25). The G_{2}/M checkpoint is controlled by Cdc2/cyclin B, as well as their negative regulators including p21^{Cip1} and p27 (26). Regulating these G_{2}/M checkpoint proteins may enhance the sensitivity of cancer cells to radiotherapy and chemotherapy (27). Therefore, the G_{2}/M checkpoint is a potential target for cancer therapy. It has been reported that apigenin arrested human colon cancer HCT116 cells in the G_{2}/M phase. Moreover, it suppressed the expression of both cyclin B1 and its activating partners, Cdc2 and Cdc25c (28). In addition, apigenin treatment led to a significant accumulation of cells in the G_{2}/M phase via the downregulation of Cdc25c expression in human papillary thyroid carcinoma BCPAP cells (29). We found that apigenin suppressed the growth of human melanoma A375 and C8161 cells by inducing G_{2}/M phase arrest of the cell cycle. Furthermore, apigenin treatment decreased the expression of p-AKT and p-mTOR. Previous studies have indicated that the AKT/mTOR pathway could influence the progression of G_{2} to the mitosis phase through the regulation of the expression of G_{2}/M-related proteins (30). Expression of the active form of AKT increases Cdk1 both at the protein and mRNA level, while its predominant negative mutation suppresses cell proliferation by inducing G_{2}/M arrest (31). Consequently, apigenin may inhibit proliferation of A375 and C8161 cells by arresting G_{2}/M transition in the cell cycle probably via the AKT/mTOR pathway.

Invasion and metastasis are major concerns in the prognosis and progression of cancer. The AKT/mTOR pathway is pivotal in modulating the invasion and migration of tumor cells (32). This pathway promotes resistance to chemotherapy-induced apoptosis in a variety of cancers including melanoma (33). We found that 40 µM of apigenin significantly inhibited cell migration and invasion. Furthermore, western blot analysis demonstrated that the expression levels of p-AKT and p-mTOR were decreased after apigenin treatment, while no significant difference was observed in total AKT and mTOR. These results indicate that the AKT/mTOR pathway plays an important role in the apigenin-induced inhibition of migration and invasion of A375 and C8161 cells. Erdogan et al (34) also showed that apigenin reduced prostate cancer CD44^+ stem cell survival and migration through PI3K/AKT/NF-κB signaling.

Apoptosis, a type of programmed cell death, is a physiological process essential for normal tissue development (35). In mammals, there are two primary apoptotic pathways: the extrinsic pathway (death receptor-mediated pathway) and the intrinsic pathway (mitochondrial-mediated pathway) (36). Caspase-3 is a key executioner caspase and its activation leads to the cleavage of PARP during cell death (37). Cleavage of PARP is regarded as a central indicator of apoptosis. The ERK and AKT signaling pathways are related to cell biological functions and cancer malignancies which could also play an important role in the proliferation and apoptosis of cancers. In our study, we confirmed that apigenin treatment resulted in higher apoptosis rates of the A375 and C8161 cells compared to the control cells in a dose-dependent manner and significantly increased the expression of cleaved caspase-3 and cleaved PARP, while it decreased the expression of p-ERK1/2. The cell death occurring in the A375 and C8161 cells treated with apigenin was probably induced by apoptosis, which was possibly involved with ERK phosphorylation. Pretreatment of A375 and C8161 cells with ERK inhibitors is necessary to reveal whether apigenin-induced apoptosis is dependent on ERK activity and we will investigate the relevant mechanism in future research. Seo et al demonstrated that apigenin induced apoptosis via the extrinsic pathway, increasing p53 and inhibiting STAT3 and NF-κB signaling in HER2-overexpressing breast cancer cells (38). Shukla et al observed that apigenin induced apoptosis by targeting inhibitor of apoptosis proteins and Ku70-Bax interaction in prostate cancer (39). Das et al (40) found that apigenin induced apoptosis in A375 and A549 cells through selective action and dysfunction of mitochondria, suggesting the activation of the intrinsic apoptosis pathway. However, we did not ascertain whether apigenin induced cell apoptosis through the intrinsic or extrinsic pathway and this relevant research will be carried out in the future. In addition, our western blot analysis showed that the expression of p-AKT and p-mTOR was decreased after apigenin treatment, while the expression of total AKT and mTOR was not altered. This indicates that the AKT/mTOR pathway plays a vital role in the apigenin-induced apoptosis observed in A375 and C8161 cells. Zhu et al (30) demonstrated that apigenin induced apoptosis via the PI3K/AKT pathway, the regulation of the Bcl-2 family and activation of caspase-3 and PARP.

Recent studies have implicated glutamate signaling in the development of melanoma (41-43). The antagonists of ionotropic glutamate receptors (iGluRs) have been demonstrated to cause a rapid and reversible change in melanocyte morphology. Metabotropic glutamate 1 (mGlul) receptor has been proposed as a target for metastatic melanoma therapy (44). It is expressed aberrantly in over half of human melanoma cell lines and biopsies (45). In our previous study (46), we found that the antagonists mGlul receptor and N-methyl-D-aspartate (NMDA) receptor increased dendritic branching and inhibited the motility, migration and proliferation of the human metastatic melanoma cell line WM451. We also demonstrated that the invasion and motility effects were significantly inhibited by the combination of increased microtubule-associated protein (MAP)2a (MAP2a) expression and either an mGlul receptor or NMDA receptor antagonist. One plausible explanation for this phenomenon is that the blockade of the glutamate-mediated signaling pathway via the ERK1/2 pathway suppresses cell motility and invasion through a tubulin-dependent mechanism (47). In the present study, we found that the main cytodendrites of human melanoma A375 and C8161 cells following treatment with apigenin became
thinner and longer than those of the controls. Moreover, treatment with apigenin significantly suppressed cell invasion and migration. We deduced that the aforementioned effects of apigenin may be induced by blocking the glutamate-mediated signaling pathway, leading to cytoskeletal protein reorganization and tumor cell differentiation. These results suggest that the blockade of glutamate signaling is a promising novel therapy for the treatment of melanoma.

In conclusion, apigenin is a potent suppressor of cell viability, migration and invasion. Concomitantly, it induces apoptosis in human melanoma A375 and C8161 cells, via activation of caspase-3 and PARP, inhibition of ERK phosphorylation and the AKT/mTOR pathway. Furthermore, it affects the dendrite morphology of the A375 and C8161 cells, which might be involved with the blockade of the glutamate signaling pathway. These findings need to be supported by further experimental evidence. Consequently, apigenin exhibits effective antineoplastic potency and provides a hopeful treatment paradigm for melanoma.

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