Apigenin Induces Apoptosis in Human Leukemia Cells and Exhibits Anti-Leukemic Activity In Vivo

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Abstract

In this study, we investigated the functional role of Akt and c-jun-NH2-kinase (JNK) signaling cascades in apigenin-induced apoptosis in U937 human leukemia cells and anti-leukemic activity of apigenin in vivo. Apigenin induced apoptosis by inactivation of Akt with a concomitant activation of JNK, Mcl-1 and Bcl-2 downregulation, cytochrome c release from mitochondria, and activation of caspases. Constitutively active myristilated Akt prevented apigenin-induced JNK, caspase activation, and apoptosis. Conversely, LY294002 and a dominant-negative construct of Akt potentiated apigenin-induced apoptosis in leukemia cells. Interruption of the JNK pathway showed marked reduction in apigenin-induced caspase activation and apoptosis in leukemia cells. Furthermore, in vivo administration of apigenin resulted in attenuation of tumor growth in U937 xenografts accompanied by inactivation of Akt and activation of JNK. Attenuation of tumor growth in U937 xenografts by apigenin raises the possibility that apigenin may have clinical implications and can be further tested for incorporating in leukemia treatment regimens. Mol Cancer Ther; 11(1); 132–42. ©2011 AACR.

Introduction

Apigenin is a flavonoid belonging to the flavones structural class and is chemically known as 4’,5,7-trihydroxyflavone (1). It is present in abundance in fruits and vegetables such as oranges, grapefruit, celery, parsley, onions, chamomile, and wheat sprouts (1, 2). It has been reported that apigenin is a potent inhibitor of cell growth and inducer of apoptosis in various cancer cells including breast (3), prostate (4, 5), lung (6), and hematologic malignancies (7, 8). Studies have revealed that apigenin induces apoptosis through different cellular signaling transduction pathways such as NFκB (9), p53 (10), MAPK (11), and PI3K/Akt (12, 13).

The PI3K/Akt signaling pathway plays an important role in cell survival and apoptosis. Activation of phosphoinositide 3-kinase (PI3K) through growth receptor kinases leads to phosphorylation of PIP2 at 3’-position at its inositol ring and converts PIP2 to PIP3 at plasma membrane. Successively, PIP3 recruits Akt and PDK1 through their Pleckstrin homology domain. Full activation of Akt occurs when it is phosphorylated by PDK1 at Thr308 and by mTORC2 at Ser473 (14). Activated Akt inactivates several proapoptotic factors including BAD, procaspase-9, and forkhead transcription factors (14). Constitutively active Akt has been reported in various types of leukemia (15, 16) and is responsible for uncontrolled proliferation and resistance to apoptosis in leukemia cells, providing a potential therapeutic target in leukemia.

The c-jun-NH2-kinase (JNK) belongs to the superfamily of mitogen-activated protein kinases that are involved in various cellular processes such as proliferation, differentiation, and apoptosis. JNKs can promote apoptosis by different mechanisms. First, activated JNK translocates to the nucleus where it phosphorylates and transactivates c-Jun, which leads to the increased expression of proapoptotic genes such as TNF-α, Fas-L, and Bak. Second, activated JNK can be translocated to mitochondria where it can phosphorylate Mcl-1 and Bcl-2 to antagonize their antiapoptotic activity. JNK can also stimulate the release of cytochrome c from mitochondria through Bid-Bax-dependent mechanism, which leads to apoptosis (17).

Apigenin exposure to different leukemia cells resulted in selective apoptosis in monocytic and lymphocytic leukemias (18). Exposure of human promyelocytic leukemia HL60 cells to apigenin resulted in induction of apoptosis (18). Apigenin induced apoptosis by inactivation of Akt with a concomitant activation of JNK, Mcl-1 and Bcl-2 downregulation, cytochrome c release from mitochondria, and activation of caspases. Constitutively active myristilated Akt prevented apigenin-induced JNK, caspase activation, and apoptosis. Conversely, LY294002 and a dominant-negative construct of Akt potentiated apigenin-induced apoptosis in leukemia cells. Interruption of the JNK pathway showed marked reduction in apigenin-induced caspase activation and apoptosis in leukemia cells. Furthermore, in vivo administration of apigenin resulted in attenuation of tumor growth in U937 xenografts accompanied by inactivation of Akt and activation of JNK. Attenuation of tumor growth in U937 xenografts by apigenin raises the possibility that apigenin may have clinical implications and can be further tested for incorporating in leukemia treatment regimens.

Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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cell-cycle arrest, caspase-3 and PARP cleavage (7, 19). Another study showed that apigenin induces apoptosis in primary effusion lymphoma cells via suppression of Akt pathway (20). The relationships between apigenin-induced apoptosis and cell signaling cascades have not yet been examined in-depth in human leukemia cells. In this study, we have elucidated the functional role of Akt and JNK pathways in apigenin-induced lethality in leukemia cells. Our results suggest a hierarchical model of apigenin-induced apoptosis in human leukemia cells. In this model, apigenin-induced Akt inactivation represents a primary event resulting in JNK activation, downregulation of Mcl-1 and Bcl-2, and culminating in caspase activation, and apoptosis. In addition, we have shown that apigenin attenuated tumor formation in U937 xenograft in athymic nude mice, suggesting that apigenin is not only effective in vitro but also in vivo.

**Materials and Methods**

**Chemicals**

Apigenin was purchased from Sigma. LY294002 and SP600125 were from EMD Biosciences. Antibodies against Akt1, phospho-JNK, JNK1, and β-actin were from Santa Cruz Biotechnology. Antibodies against Bad, Bax, cytochrome c, and Mcl-1 were from BD Pharmingen. Cleaved caspase-3, cleaved caspase-7, cleaved caspase-9, Bcl-XL, phospho-Akt (Ser473), phospho-Bad (Ser136) and Akt kinase assay kit were from Cell Signaling Technology. Antibodies against PARP and Bcl-2 were from Biomol and DAKO, respectively.

**Cell culture and plasmid transfection**

U937, Jurkat, and HL60 human leukemia cells were obtained from American Type Culture Collection and grown in RPMI-1640 medium with 10% FBS. Normal peripheral blood mononuclear cells (PBMC) were obtained from AllCells and maintained in RPMI-1640 with 10% FBS. Dr. Ruth Craig (Dartmouth Medical School, Hanover, NH) kindly provided U937 cells stably overexpressing Mcl-1 and their empty vector counterpart (pCEP). Authors carried out no further cell line authentication in the last 6 months. Dr. Bing-Hua Jiang (West Virginia University, Morgantown, WV) kindly provided U937 xenograft of human U937 cells were used. Athymic nude mice (nu/nu, 4 weeks old; The Jackson Laboratory) were housed in a specific pathogen-free room within the animal facilities at the University of Kentucky, Lexington, KY. Animals were allowed to acclimatize to their new environment for 2 weeks prior to use. All animals were handled according to the Institutional Animal Care and Use, University of Kentucky. U937 cells (2 × 10⁶) were resuspended in serum-free RPMI-1640 medium with Matrigel basement membrane matrix (BD Biosciences) at a 1:1 ratio (total volume: 100 µL) and then were subcutaneously injected into the flanks of nude mice. Four days after tumor inoculation, mice were randomly divided into 3 groups (n = 6 in each group), and apigenin (0, 20, and 40 mg/kg body weight) was administered intraperitoneally in 150 µL of dimethyl sulfoxide/0.9% physiologic saline (1:0.5) daily for 5 days a week for 4 weeks. Body weight and tumor mass were measured every 5 days throughout the study. Tumor volumes was determined by a caliper and calculated according to the formula (width² × length)/2. The dose of the apigenin for in vivo study was selected as described previously (22).

**Statistical analysis**

Statistical analysis was conducted using GraphPad Prism version 3. For analysis of apoptosis, values were presented as mean ± SD. Statistical differences between control and treated groups were determined by the Student t test for unpaired observations. Differences were considered statistically significant for values of P < 0.05 or P < 0.01.

**Analysis of apoptosis**

For Annexin V/propidium iodide (PI) assay, cells were stained with Annexin V-fluorescein isothiocyanate and PI and apoptosis was evaluated by flow cytometry according to manufacturer’s protocol (BD Pharmingen) and described previously (21).

**Measurement of Akt kinase activity**

U937 cells were seeded in 6-well plate and then treated with apigenin (40 µmol/L) for 24 hours. In vitro Akt kinase assay was then used to measure Akt kinase activity as per manufacturer’s instruction.

**Western blotting**

Western blotting was carried out using NuPAGE Bis-Tris electrophoresis system (Invitrogen). For tissue sections, radioimmunoprecipitation assay (RIPA) buffer was added to the sections and homogenized with electric homogenizer. After incubation for 20 minutes on ice, samples were centrifuged for 30 minutes at 12,000 rpm at 4°C and supernatant was collected as total cell lysate. SDS-PAGE was carried out as described previously (21). Blots shown were representative for 3 separate experiments.

**U937 xenograft assay**

To evaluate the therapeutic effect of apigenin in vivo, xenograft of human U937 cells were used. Athymic nude mice (nu/nu, 4 weeks old; The Jackson Laboratory) were housed in a specific pathogen-free room within the animal facilities at the University of Kentucky, Lexington, KY. Animals were allowed to acclimatize to their new environment for 2 weeks prior to use. All animals were handled according to the Institutional Animal Care and Use, University of Kentucky. U937 cells (2 × 10⁶) were resuspended in serum-free RPMI-1640 medium with Matrigel basement membrane matrix (BD Biosciences) at a 1:1 ratio (total volume: 100 µL) and then were subcutaneously injected into the flanks of nude mice. Four days after tumor inoculation, mice were randomly divided into 3 groups (n = 6 in each group), and apigenin (0, 20, and 40 mg/kg body weight) was administered intraperitoneally in 150 µL of dimethyl sulfoxide/0.9% physiologic saline (1:0.5) daily for 5 days a week for 4 weeks. Body weight and tumor mass were measured every 5 days throughout the study. Tumor volumes was determined by a caliper and calculated according to the formula (width² × length)/2. The dose of the apigenin for in vivo study was selected as described previously (22).

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Apigenin induced apoptosis, activated caspases and cleaved PARP in dose- and time-dependent manners in U937 cells

Apigenin induced a dose-dependent apoptosis in U937 cells. Moderate increase in apoptosis was observed after 12 and 24 hours of exposure to apigenin at concentration of 20 μmol/L and marked increase in apoptosis was observed at concentrations of 30 μmol/L or higher (Fig. 1A). Apigenin exposure of U937 cells at concentration of 40 μmol/L also caused apoptosis in a time-dependent manner and a significant increase in apoptosis was observed as early as 6 hours after apigenin exposure (Fig. 1B).

Western blotting revealed that apigenin induced apoptosis in a caspase-dependent manner. Exposure of U937 cells at indicated concentrations of apigenin for 12 and 24 hours activates caspases-3, 7 and 9 and cleaved PARP (Fig. 1C). In addition, a time course study of U937 cells exposed to 40 μmol/L apigenin showed marked increase in activation of caspases-3, 7 and 9 and PARP cleavage (Fig. 1D). Figure 1E shows structure of apigenin.

Together, these results indicate that apigenin induces apoptosis in dose- and time-dependent manners in U937 cells.

Exposure of U937 cells to apigenin resulted in downregulation of Bcl-2 and Mcl-1

Furthermore, we evaluated the expression of various members of Bcl-2 family of proteins. A dose- and time-dependent exposure of U937 cells to apigenin showed cleavage of Bcl-2 and downregulation of Mcl-1 (Fig. 2A and B). No changes in Bcl-XL, Bax, and XIAP expression were observed in U937 cells in dose- as well as time-dependent manners (Fig. 2A and B). These results suggested that exposure of leukemia cells to apigenin resulted in cleavage or downregulation of antiapoptotic members of Bcl-2 family members such as Bcl-2 and Mcl-1.

Exposure of U937 cells to apigenin resulted in inactivation of Akt and pronounced increase in JNK activation

Next, we examined the effects of apigenin on cell survival and stress-induced signaling pathways. A dose-dependent study showed that apigenin dephosphorylates Akt at Ser473 and its downstream targets mTOR (Ser2448) and Bad (Ser136) at concentrations of 30 μmol/L or higher. Total Akt1 and mTOR levels were also decreased (Fig. 2C). In addition, we observed that JNK phosphorylation levels increased concomitantly with a decrease in Akt phosphorylation in dose-dependent
manner whereas JNK1 levels remained unchanged (Fig. 2C).

A time course study showed that exposure of U937 cells to 40 μmol/L apigenin resulted in dephosphorylation of Akt as early as 6 hours after drug exposure and a concomitant increase in JNK phosphorylation, which reached maximum level at 24 hours (Fig. 2D).

Exposure of U937 cells to apigenin for 24 hours reduced kinase activity of Akt as shown by decreased phosphorylation of GSK-3α/β (Ser21/9; Fig. 2E). Collectively, these results suggest that inactivation of Akt with a concomitant activation of JNK may play an important role in apigenin-induced apoptosis.

Apigenin induced apoptosis in leukemia cells via caspase-independent inactivation of Akt and activation of JNK

To determine whether inactivation of Akt and activation of JNK were secondary to caspase activation, we treated U937 cells with 40 μmol/L apigenin in the presence or absence of the broad-spectrum caspase inhibitor Z-VAD-FMK at 20 μmol/L. Exposure of Z-VAD-FMK to U937 cells attenuated apigenin-induced apoptosis (Supplementary Fig. S1A), PARP cleavage, and caspase activation (Supplementary Fig. S1B). In addition, Z-VAD-FMK failed to inactivate Akt, activate JNK, and downregulate the expression of Mcl-1 (Supplementary Fig. S1C).
and S1D). Interestingly, Z-VAD-FMK inhibited apigenin-induced cleavage of Bcl-2 (Supplementary Fig. S1D), suggesting that the cleavage of Bcl-2 was caspase dependent. Together these findings suggest that apigenin-induced inactivation of Akt, activation of JNK, and Mcl-1 downregulation were caspase independent.

**Apigenin induced apoptosis in leukemia cells via mitochondrial dependent mechanism**

It has been reported that exposure of leukemia cells to apigenin resulted in mitochondrial injury, release of cytochrome c into cytosol, and caspase activation (7). We investigated apigenin-induced mitochondrial alterations in U937 cells by DiOC₆ staining. As shown in Supplementary Fig. S2A and S2B, exposure of cells to apigenin in dose- and time-dependent manners increased the number of cells with low mitochondrial transmembrane potential as compared with control. In addition, apigenin induced release of cytochrome c from mitochondria to cytosol in dose- and time-dependent manners (Supplementary Fig. S2C and S2D). Such findings indicate that apigenin induced mitochondrial injury in leukemia cells that leads to cytochrome c release, caspase activation, and apoptosis.

**Apigenin induced similar effects in other leukemia cells but not in NPBMNCs**

To assess whether apigenin-induced effects are restricted to monocytic (U937) leukemia cells, we conducted similar studies in T-cell lymphoblastic leukemia cells (Jurkat) and acute promyelocytic leukemia (HL60). The cells also showed apoptotic effects on apigenin exposure but were less sensitive than U937 cells (Supplementary Fig. S3A). Moreover, apigenin showed no significant apoptotic effect on NPBMNCs at concentration of 40 μmol/L (Supplementary Fig. S3B). Supplementary Figure S3C revealed that in HL60 cells there was very little or no PARP cleavage or caspase-3 activation or expression of phospho-Akt (Ser473). These observations suggest that HL60 cells were less sensitive to apigenin-induced apoptosis at the same experimental condition. In addition, Mcl-1 expression was downregulated in all 3 cell lines. To elucidate the mechanism by which the NPBMNCs did not undergo apoptosis, we carried out Western blotting and the results show that treatment of NPBMNCs with apigenin at various concentrations neither cause cleavage of PARP nor dephosphorylation of Akt, downregulation of Mcl-1, and activation of JNK (Supplementary Fig. S3D). Collectively, these results show that apigenin induced apoptosis in several leukemia cells but not in NPBMNCs.

**Inactivation of Akt is responsible for apigenin-induced JNK and caspases activation and apoptosis**

Results in Fig. 2 show that inactivation of Akt may play an important role in apigenin-induced apoptosis. To test this hypothesis, we pretreated U937 cells with PD3 inhibitor LY294002 (10 μmol/L) for 1 hour, followed by exposure to apigenin (20 μmol/L) for 12 and 24 hours. As shown in Fig. 3A, pretreatment of cells with LY294002 and then to apigenin resulted in a sharp increase in apoptosis as compared with apigenin alone. Figure 3B revealed that pretreatment with LY294002 and then apigenin exposure resulted in a pronounced increase in activation of caspases-3, 7, 9, and PARP cleavage. In addition, LY294002 potentiates apigenin-induced Akt inactivation, phosphorylation of JNK, Bcl-2 cleavage, and Mcl-1 downregulation. However, LY294002 showed no effect on total Akt1 and JNK1 levels (Fig. 3C and D). Figure 3E shows structure of LY294002.

To assess the functional significance of Akt inactivation in apigenin-induced apoptosis, we used Akt-DN construct. As shown in Fig. 4A, Akt-DN–expressing cells were more sensitive to apigenin-induced apoptosis than pcDNA3.1 vector control cells. Western blotting revealed that Akt-DN construct potentiates apigenin-induced activation of caspases-3, 7, 9, and PARP cleavage as compared with vector control (Fig. 4B). Consistent with these findings, Akt-DN construct potentiates apigenin-induced Akt inactivation and JNK activation with no change in the levels of total JNK1 (Fig. 4C). In addition, apigenin-induced Bcl-2 cleavage and Mcl-1 downregulation were enhanced in Akt-DN–expressing cells (Fig. 4D). Overexpression of Akt by a constitutively active m-Akt prevented apigenin-induced apoptosis in U937 cells as compared with vector control (Fig. 4E). In addition, there was a marked increase in levels of total Akt1 and ability of apigenin to dephosphorylate Akt was inhibited in cells expressing m-Akt (Fig. 4E, Western). Apigenin-induced activation or caspases-3, 7, 9, and PARP cleavage were drastically reduced in U937 cells expressing m-Akt (Fig. 4F). Interestingly, the ability of apigenin to induce JNK activation was abrogated in cells expressing m-Akt (Fig. 4G). Furthermore, overexpression of Akt essentially abolished apigenin-induced Bcl-2 cleavage and Mcl-1 downregulation (Fig. 4G). Together, these findings indicate that inactivation of Akt plays a critical role in apigenin-induced apoptosis and that this event lies upstream of Mcl-1 and Bcl-2 downregulation and JNK activation.

**Activation of JNK played an important role in apigenin-induced caspase activation and apoptosis**

To dissect the possible functional significance of JNK activation in apigenin-induced apoptosis, we used both pharmacologic and genetic approaches. Pretreatment of U937 cells with JNK inhibitor SP600125 (15 μmol/L) for 1 hour diminished apigenin-induced apoptosis (Fig. 5A). Figure 5B revealed that pretreatment with SP600125 blocked apigenin-induced caspases-3, 7, and 9 activation and PARP cleavage. In addition, SP600125 reduced apigenin-induced phosphorylation of JNK and showed no effect on phosphorylation of Akt, total Akt1 or JNK1 levels (Fig. 5C). Furthermore, interruption of JNK by SP600125 inhibited apigenin-induced Bcl-2 cleavage and Mcl-1 downregulation (Fig. 5D).

To confirm the role of JNK in apigenin-induced apoptosis, short interfering RNA (siRNA) of JNK1 was used. Apigenin-induced apoptosis was sharply reduced in
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JNK1 siRNA–transfected cells and that JNK1 levels were reduced to one third (approximately) as compared with control siRNA (Fig. 5E). As shown in Fig. 5F, JNK1 siRNA–transfected cells diminished apigenin-induced caspases-3, 7, and 9 activation and PARP cleavage. Furthermore, JNK1 siRNA cells showed no change in phosphorylation of Akt and total Akt levels (Fig. 5G). Apigenin-induced Bcl-2 cleavage and Mcl-1 downregulation were inhibited in JNK1 siRNA–transfected cells (Fig. 5G). Collectively, these results indicate that apigenin-induced JNK activation played an important functional role in apoptosis and that the activation of JNK occurred downstream of Akt inactivation.

Apigenin-induced Mcl-1 downregulation proceeds via transcripitional and proteasome-dependent mechanisms

Results from Fig. 2 indicate that downregulation of Mcl-1 upon apigenin treatment is tightly connected to Akt dephosphorylation and JNK activation. Therefore, to elucidate the mechanism underlying Mcl-1 downregulation by apigenin, we used quantitative reverse transcriptase PCR analysis. As shown in Supplementary Fig. S4A and S4B, exposure of U937 cells to apigenin resulted in significant decrease in Mcl-1 mRNA levels in dose- and time-dependent manners. Inhibition of transcription by exposing cells to actinomycin D (5 μg/mL) in the presence or absence of apigenin failed to reduce apigenin-mediated Mcl-1 downregulation (data not shown).

To further delineate the mechanism by which apigenin diminishes Mcl-1 expression in U937 cells, we exposed the cells to apigenin (40 μmol/L) for various intervals in the presence or absence of the proteasome inhibitor MG132 (10 μmol/L). As shown in Supplementary Fig. S4C, MG132 essentially blocked the downregulation of Mcl-1. In addition, co-administration of protein synthesis inhibitor cycloheximide (20 μmol/L) hastened the rate of Mcl-1 downregulation (Supplementary Fig. S4D). Together, these findings suggest that apigenin not only blocks Mcl-1 transcription but also degrades Mcl-1 via proteasome-dependent mechanism.

Overexpression of Mcl-1 substantially diminished apigenin-induced apoptosis, caspases activation, and PARP cleavage in U937 cells

To assess the functional significance of Mcl-1 in apigenin-induced apoptosis, we used U937 cells overexpressing Mcl-1. As shown in Supplementary Fig. S5A, overexpression of Mcl-1 substantially diminished the apigenin-induced apoptosis, whereas empty vector control (pCEP) cells were as sensitive as parental cells. Treatment with apigenin diminished Mcl-1 expression in pCEP cells but failed to downregulate Mcl-1 in overexpressing cells (Supplementary Fig. S5B). Supplementary Fig. S5C revealed that apigenin failed...
to activate caspases-3, -9 and cleave PARP in Mcl-1–overexpressing cells. In addition, apigenin-induced cytochrome c release from mitochondria was diminished in Mcl-1–overexpressing cells (Supplementary Fig. S5D). These findings show that apigenin-induced downregulation of Mcl-1 is an essential event in apigenin-induced apoptosis in U937 cells.

Apigenin inhibited tumor formation in xenografts of U937 human leukemia cells

On the basis of the in vitro studies described in above sections, we extend our studies to test the anti-leukemic activity of apigenin in vivo in U937 human leukemia xenografts. Athymic nude mice were inoculated with U937 cells subcutaneously, after which mice were injected with vehicle or apigenin (20 and 40 mg/kg intraperitoneally) daily for 5 days a week for 4 weeks as described in Materials and Methods. As shown in Fig. 6A and B, treatment of mice with 20 and 40 mg/kg apigenin resulted in 58.4% and 71% inhibition of tumor growth as compared with control group on day 20. In addition, no statistically significant change in body weight was observed in control and apigenin-treated animals (Fig. 6C), indicating that apigenin was not toxic. Figure 6D and E revealed that apigenin (40 mg/kg) dephosphorylated Akt and activated JNK as compared with vehicle in tissue sections. PARP cleavage, an indicator of apoptosis, was also increased in treatment group (Fig. 6D). Downregulation in Mcl-1 levels in apigenin-treated mice was consistent with
in vitro finding (Fig. 6F). Collectively, these findings suggest that inactivation of Akt and activation of JNK signaling contributes to apigenin-induced apoptosis not only in vitro but also in vivo.

Discussion

Previous studies have shown that apigenin-induced apoptosis in several types of cancer cells such as breast, prostate, lung, and hematologic (3–8). Presently, no information is available about the functional importance of Akt and JNK pathways in apigenin-induced lethality in leukemia cells. The results in the present study indicate that exposure of apigenin to leukemia cells leads to mitochondrial injury, caspase activation, and apoptosis. In addition, our results provide the mechanistic information of how apigenin exerts its proapoptotic effects in leukemia cells, that is, by inactivation of Akt and activation of JNK.

Because phosphorylation by upstream kinases is required for complete activation of Akt (14), it is not surprising that various protein phosphatases dephosphorylate Akt. Activation of Akt generally involves PTEN inactivation and results in attenuation of apoptosis (23). In our study, apigenin exposure resulted in dephosphorylation of Akt. Thus, it would be enticing to relate this observation to PTEN activation. However, the absence of
wild-type PTEN in U937 cells does not support this possibility (24). In addition, Akt can be dephosphorylated by phosphatases such as PP2A and PHLPP (25, 26). It is likely that apigenin increases the activity or expression of these phosphatases that in turn dephosphorylate Akt. In fact, our unpublished results show that apigenin enhanced the expression of PP2A in U937 cells. Additional mechanistic studies are required to show the role of these phosphatases in apigenin-induced Akt dephosphorylation in leukemia cells. It is possible that apigenin, through a mechanism not known yet, blocks the actions of PI3K. The findings that LY29004 augmented apigenin-induced inactivation of Akt, caspase activation, and PARP cleavage were in agreement with this hypothesis.

Akt is a well-known substrate of caspases. For instance, cleavage of Akt1 was caspase dependent in U937 and Jurkat cells exposed to UV light, etoposide, and Fas ligation (27). The results shown in the present study showed that pretreatment of leukemia cells with caspase inhibitor Z-VAD-FMK failed to prevent inactivation of Akt, suggesting that inactivation of Akt by apigenin was caspase independent.

Results from the present study suggest that Akt inactivation by apigenin plays a functional role in apigenin-induced lethality in human leukemia cells. First, pretreatment with the LY29004 followed by treatment with apigenin enhanced apigenin-induced caspases activation and apoptosis. Second, U937 cells expressing Akt-DN enhanced apigenin-induced lethality as compared with vector control. Last, overexpression of Akt by a constitutively active m-Akt prevented apigenin-induced caspase activation and apoptosis. In addition, we have shown that apigenin downregulated Akt kinase activity, suggesting its reduced ability to phosphorylate downstream targets.

Furthermore, we elucidated the functional role of JNK pathway in apigenin-induced apoptosis in leukemia cells. We have shown a dose- and time-dependent correlation between Akt inactivation and JNK activation. Inhibition of JNK by SP600125 and JNK1 siRNA had no effect on Akt, suggesting that Akt lies upstream of JNK. Constitutively active m-Akt prevented apigenin-induced JNK activation, indicating that one of the mechanisms by which Akt pathway protects leukemia cells from apigenin-induced lethality is due to the inhibition of JNK. Conversely, inhibition of Akt pathway by LY29004 and Akt-DN potentiated apigenin-induced JNK activation and apoptosis. The following evidences can explain the correlation between Akt inactivation and JNK activation. It has been shown that Akt suppresses the JNK pathway by phosphorylating and negatively regulating ASK-1 (apoptosis signal-regulating kinase-1), MLK3 (mixed-lineage protein kinase), and MKK4/SEK1 (mitogen-activated protein/ERK kinase; refs. 28–30). In addition, Akt suppresses JNK activation by directly interacting with JNK-interacting protein (JIP) and thus preventing the recruitment of upstream kinases to JNK (31).
JNK can induce apoptosis by phosphorylating and inhibiting antiapoptotic function of Bcl-2 and Mcl-1 (32, 33). In this study, we found that apigenin downregulated Mcl-1 and induced Bcl-2 cleavage. We have also shown that inhibition of JNK decreased apigenin-induced Mcl-1 downregulation and Bcl-2 cleavage, suggesting that these antiapoptotic proteins lie downstream of JNK. Collectively, these findings showed an important functional role of JNK pathway in apigenin-induced apoptosis.

Notably, apigenin exposure resulted in downregulation of Mcl-1, an antiapoptotic protein that may play an important role in apoptosis in malignant hematopoietic cells (34). Overexpression of Akt inhibited apigenin-induced Mcl-1 downregulation, suggesting that Mcl-1 downregulation is significant for apigenin-induced apoptosis. This finding was supported by the evidence that Mcl-1 is upregulated by PI3K/Akt pathway through a transcription factor complex CREB (35). In addition, we have shown that apigenin inhibited Mcl-1 transcription, and overexpression of Mcl-1 substantially diminished apigenin-induced apoptosis, caspases activation, PARP cleavage, and mitochondrial injury (cytochrome c release). Together, these findings suggest that apigenin-induced downregulation of Mcl-1 plays an essential role in apigenin-induced apoptosis in leukemia cells.

Apigenin has been shown to inhibit tumor growth of prostate, ovarian, and lung xenografts by inducing apoptosis (36, 37). The present study shows that apigenin exhibits significant inhibitory effects on the growth of U937 leukemia tumor xenograft. We found an increase in phospho-JNK expression, PARP cleavage, dephosphorylation of Akt (Ser473), and downregulation of Mcl-1 in the apigenin-treated group compared with the vehicle group, providing the apoptotic evidence in apigenin-treated U937 xenograft mice. The expression levels of phospho-Akt, phospho-JNK, PARP cleavage, and Mcl-1 in tissue sections of U937 xenograft tumor were closely correlated with the reduction of U937 tumor xenografts.

In summary, the results obtained from the present study provide an evidence that Akt and JNK signaling pathways are potential targets for apigenin-induced apoptosis in leukemia cells and in vivo. Further efforts are required to understand the mechanism by which apigenin inactivates Akt and induces apoptosis in human leukemia cells and U937 tumor xenografts, which could provide a reasonable evidence to incorporate apigenin in leukemia treatment regimens.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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