Apigenin induces caspase-dependent apoptosis by inhibiting signal transducer and activator of transcription 3 signaling in HER2-overexpressing SKBR3 breast cancer cells

HYE-SOOK SEO1, JIN MO KU1, HAN-SEOK CHOI1, JONG-KYU WOO2, BO-HYOUNG JANG1, HOYEON GO3, YONG CHEOL SHIN1 and SEONG-GYU KO1

1Laboratory of Clinical Biology and Pharmacogenomics and Center for Clinical Research and Genomics, College of Korean Medicine, Kyung Hee University, Dongdaemun-gu, Seoul 130-701; 2College of Pharmacy, Gachon University of Medicine and Science, Yeonsu-gu, Incheon 406-840; 3Department of Oriental Medicine, Semyung University, College of Korean Medicine, Checheon, Chungbuk 390-711, Republic of Korea

Received May 2, 2014; Accepted January 21, 2015

DOI: 10.3892/mmr.2015.3698

Abstract. Phytoestrogens have been demonstrated to inhibit tumor induction; however, their molecular mechanisms of action have remained elusive. The present study aimed to investigate the effects of a phytoestrogen, apigenin, on proliferation and apoptosis of the human epidermal growth factor receptor 2 (HER2)-expressing breast cancer cell line SKBR3. Proliferation assay, MTT assay, fluorescence-activated cell sorting analysis, western blot analysis, immunocytochemistry, reverse transcription-polymerase chain reaction and ELISA assay were used in the present study. The results of the present study indicated that apigenin inhibited the proliferation of SKBR3 cells in a dose- and time-dependent manner. This inhibition of growth was accompanied by an increase in the sub-G0/G1 apoptotic population. Furthermore, apigenin enhanced the expression levels of cleaved caspase-8 and -3, and induced the cleavage of poly(adenosine diphosphate ribose) polymerase in SKBR3 cells, confirming that apigenin promotes apoptosis via a caspase-dependent pathway. Apigenin additionally reduced the expression of phosphorylated (p)-janus kinase 2 and p-signal transducer and activator of transcription 3 (STAT3), inhibited CoCl2-induced vascular endothelial growth factor (VEGF) secretion and decreased the nuclear localization of STAT3. The STAT3 inhibitor S31-201 decreased the cellular proliferation rate and reduced the expression of p-STAT3 and VEGF. Therefore, these results suggested that apigenin induced apoptosis via the inhibition of STAT3 signaling in SKBR3 cells. In conclusion, the results of the present study indicated that apigenin may be a potentially useful compound for the prevention or treatment of HER2-overexpressing breast cancer.

Introduction

Apigenin (4',5,7-trihydroxyflavone) is a non-toxic dietary flavonoid present in numerous herbs, including parsley, thyme, peppermint, chamomile, horsetail herb, lemon balm, perilla, vervain and yarrow (1). Apigenin has been demonstrated to exert anti-oxidant (1), anti-inflammatory (2), anti-telomerase (3) and anti-depressant activities (4). Of note, apigenin also possesses anti-tumor properties and is therefore of particular interest in the development of novel drugs for the treatment and/or prevention of cancer (5,6). It has previously been demonstrated that apigenin is able to reduce the volume and mass of implanted androgen-sensitive 22Rv1 and androgen-insensitive PC-3 tumor cells (7). Furthermore, apigenin suppresses inducible cyclooxygenase and nitric oxide synthase in mouse macrophages (8), and inhibits ultraviolet light-induced skin carcinogenesis in SKH-1 mice (9). Apigenin has also been shown to inhibit growth and induce apoptosis in numerous cancer cell lines, including those of breast (10), lung (11), colon (12,13), prostate (14), leukemia (15) and pancreatic cancer (16).

Apoptosis, also known as programmed cell death, is a fundamental physiological process, required for normal development and tissue homeostasis (17,18). Apoptotic progression is associated with various caspases, which comprise a group of aspartate-specific cysteine proteases, which are members of the interleukin-1-converting enzyme family (17,18). The caspase cascade signaling pathway has crucial roles in the induction, transduction and amplification of intracellular apoptotic signals (17,18). In the majority of tumor cells, apoptosis is induced via two distinct signaling pathways: The extrinsic and intrinsic apoptotic pathways. The extrinsic pathway is associated with the activation of death receptors, including Fas and the tumor necrosis factor receptors, and the cleavage of caspase-8.
and caspase-3 (19-21). The intrinsic pathway is associated with the cleavage of caspase-9 and -3, as well as alterations in the mitochondrial membrane potential and the mitochondrial permeability transition (22). Caspase-3 is responsible for the cleavage of poly(adenosine diphosphate-ribose) polymerase (PARP) during cell death in each of these pathways (23).

Overexpression of the human epidermal growth factor receptor 2 (HER2) tyrosine kinase is associated with the pathogenesis and aggressive characteristics underlying ~25% of invasive human breast cancers (24). Clinical and experimental evidence has suggested that aberrant HER2 signaling may contribute to the initiation of tumor development and disease progression (24). HER2-positive tumors are associated with more aggressive phenotypes, characterized by more frequent recurrence and shorter overall survival, compared with that of HER2-negative tumor subtypes (25). The recombinant humanized anti-HER2 monoclonal antibody trastuzumab (herceptin) is frequently used in the treatment of patients with HER2-overexpressing subtypes of cancer (26). Trastuzumab induces downregulation of HER2/Neu, resulting in the disruption of receptor dimerization and signaling (27). Trastuzumab also induces cell cycle arrest during G_{1} phase and inhibits the phosphorylation of p27_{kip1}, suppressing cdk2 activity and reducing proliferation (28). Trastuzumab suppresses angiogenesis via the induction of anti-angiogenic factors and the repression of pro-angiogenic factors. However, numerous patients with breast cancer are unresponsive to trastuzumab treatment or develop a resistance to this drug (29). There have therefore been numerous studies devoted to the identification of alternative compounds with the ability to effectively treat HER2-overexpressing subtypes of breast cancer.

Previously, our group reported that apigenin promoted apoptosis via the extrinsic pathway, inducing p53 and inhibiting STAT3 and NfκB signaling in HER2-transfected MCF-7 cells (30). The present study aimed to investigate whether apigenin exerted growth-suppressive activity in natural HER2-overexpressing breast cancer cells, using the SKBR3 cell line. The effects of apigenin on the proliferation and apoptosis of SKBR3 cells were therefore evaluated. Thyroid cancer cells were therefore evaluated. Thyroid cancer cells were used for comparison, in order to demonstrate that the effect of apigenin was not cell-specific. The mechanism underlying the regulation of SKBR3 cell growth by apigenin was also investigated, by analysis of the cell cycle and determination of the expression levels of apoptotic and intracellular signaling molecules. In addition, whether apigenin was able to inhibit the STAT3 signaling pathway, resulting in growth suppression of HER2-overexpressing breast cancer cells was examined.

Materials and methods

Compounds. Apigenin (4',5,7-trihydroxyflavone) genistein and quercetin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Apigenin was dissolved in dimethyl sulfoxide (DMSO), and the final concentration of DMSO in the controls and each sample did not exceed 0.1%. It was found that 0.1% DMSO did not influence the cell growth rate compared with 0% DMSO (no treatment) in the breast cancer cells (data not shown). The caspase-8 inhibitor Z-IETD-fmk and the caspase-9 inhibitor Z-LEHD-fmk were obtained from R&D Systems, Inc. (Minneapolis, MN, USA). The STAT3 inhibitor S31-201 was obtained from Calbiochem (Billerica, MA, USA) and an EZ-western chemiluminescent detection kit was purchased from Daeilab Service Co. (Seoul, Korea).

Cell culture. The human breast cancer cell line SKBR3 (American Type Culture Collection, Manassas, VA, USA) was cultured in Dulbecco's modified Eagle's medium (Life Technologies Korea LLC, Seoul, Korea) containing 50 U/ml penicillin, 50 mg/ml streptomycin and 10% fetal bovine serum (FBS; Welgene, Daegu, Korea) at 37°C in an atmosphere of 5% CO_{2}. The human thyroid cancer cell lines SNU790 and SNU80 were obtained from the Korean Cell Line Bank (Seoul, Korea) and cultured in RPMI (Life Technologies Korea LLC) containing 50 U/ml penicillin, 50 mg/ml streptomycin (Life Technologies Korea LLC) and 10% FBS at 37°C in an atmosphere of 5% CO_{2}.

Antibodies. Primary antibodies against cleaved caspase-8 (9496) and PARP (9542), were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Primary antibodies against B cell lymphoma 2 (BCL2; sc-7382), BCL2-associated X (BAX; sc-7480), p53 (sc-126) and hypoxia-inducible factor (Hif)-1α (sc-13515) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Primary antibodies against STAT3 (06-596), p-STAT3 (Tyr705; 05-485), vascular endothelial growth factor (VEGF; 05-1116) and p-janus kinase 2 (JAK2, Tyr1022/Tyr1023; 06-255) were obtained from EMD-Millipore (Billerica, MA, USA). The anti-tubulin antibody (T3526) was from Sigma-Aldrich. Horseradish peroxidase (HRP)-conjugated secondary antibodies (mouse and rabbit) were purchased from Calbiochem (San Diego, CA, USA) and the anti-goat secondary antibody was from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA).

Cell proliferation assay. Cells were seeded in 12-well culture plates at a density of 5x10^{4} cells/well. After the cells were exposed to various concentrations of apigenin genistein or quercetin (0, 20, 40, 60, 80 or 100 µM) and incubated at 37°C for three days, the cells were harvested by trypsinization (Sigma-Aldrich), resuspended in 1-2 ml medium, and counted using a hemocytometer (Sigma-Aldrich).

MTT assay. Cells were seeded in 96-multiwell culture plates at a density of 2x10^{3}-3x10^{3} cells/well and incubated for 24 h at 37°C. Subsequently, the cells were treated with different concentrations of apigenin (0-80 µM) for 24, 48 or 72 h. Following incubation, MTT reagent (0.5 mg/ml) was added to each well, and the plates were incubated in the dark at 37°C for 2 h. At the end of the incubation, the medium was removed, the resulting formazan was dissolved in DMSO, and the optical density was measured at 570 nm using an ELISA plate reader (Gemini EM Microplate reader, Versa Max; Molecular Devices, Sunnyvale, CA, USA).

Cell cycle analysis by flow cytometry. Cells were harvested with 0.25% trypsin and washed once with phosphate-buffered saline (PBS). Following centrifugation, the cells were fixed in cold 95% ethanol with 0.5% Tween-20 and stored at -20°C for at least 30 min. The cells were incubated in 50 µg/ml propidium iodide (PI, Sigma-Aldrich), including 1% sodium citrate.
(Sigma-Aldrich) and 50 µg/ml RNase A (Sigma-Aldrich) at room temperature in the dark for 30 min. Analysis of the apoptotic cells was performed with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA), and the data were analyzed using CellQuest software (BD Biosciences, San Jose, CA, USA).

**Immunocytochemistry.** Cells (4x10^4 cells/well) were seeded in eight-well chamber slides, incubated for 24 h at 37°C and treated with apigenin (40 µM) in the presence or absence of CoCl_2 (Sigma-Aldrich) for a further 24 h. The cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) for 30 min and treated with 3% hydrogen peroxide (H_2O_2; Sigma-Aldrich) in methanol for 20 min to quench the endogenous peroxidase activity. The cells were washed with PBS, blocked with 5% bovine serum albumin in PBS for 1 h and incubated with the anti-STAT3 primary antibody (1:200 dilution) overnight at 4°C. After washing with PBS, the cells were incubated with the anti-rabbit biotin-conjugated secondary antibody for 1 h at room temperature. The cells were treated with Vectastain ABC reagent (Vector Laboratories, Inc. Burlingame, CA, USA) for 30 min at 4°C and stained with diaminobenzidine tetrachloride (DAB; Thermo Fisher Scientific, Waltham, MA, USA) and hematoxylin (Sigma-Aldrich). The cells were mounted with mounting medium (Vector Laboratories, Inc., Burlingame, CA, USA) and subsequently analyzed by microscopy (CKX41; Olympus America Inc., Center Valley, PA, USA).

**Measurement of VEGF secreted from SKBR3 cells by ELISA.** To assess the level of VEGF in the SKBR3 cell supernatants, the cells were treated with apigenin (0-80 µM) in the presence or absence of CoCl_2 (100 µM) to mimic hypoxia. After 24 h, the media were collected, centrifuged at 15,000 x g at room temperature to remove the cellular debris, and stored at -70°C until assayed for VEGF. The amount of VEGF secreted into the culture medium was measured by ELISA according to the manufacturer's instructions (Human VEGF Quantikine ELISA kit; R&D Systems, Minneapolis, MN, USA). Briefly, 96-well plates were coated with capture antibody in ELISA coating buffer and incubated overnight at 4°C. The plates were then washed with PBS with 0.05% Tween 20 (PBS-T) and subsequently blocked with 10% FBS in PBS for 1 h at 20°C. Serial dilutions of standard antigen or sample in dilution buffer (10% FBS in PBS) were added to the plates, and the plates were incubated overnight at 4°C. The plates were then washed with PBS with 0.05% Tween 20 (PBS-T) and subsequently blocked with 10% FBS in PBS for 1 h at 20°C. Following washing, biotin-conjugated anti-mouse immunoglobulin E and streptavidin-conjugated horseradish peroxidase (SAv-HRP) (R&D Systems, Inc.) were added to the plates, and the plates were incubated for 1 h at 20°C. Finally, the tetramethylbenzidine (TMB) substrate was added to the plates, and after 15 min of incubation in the dark, 2 N H_2SO_4 was added for stoppage of the reaction. The optical density was measured at 450 nm. The amount of VEGF secreted into the culture medium was calculated by interpolation from the standard curve and was expressed in pg/ml.
added to stop the reaction. The optical density was measured at 450 nm on the automated ELISA reader (Molecular Devices).

**Western blot analysis.** Cells were lysed in modified radiolabeling immunoprecipitation assay buffer [150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris (pH 8.0), 1 mM EDTA, 1 mM phenylmethylsulfon fluoride, 1 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub> and a protease inhibitor mixture (Life Technologies Korea LLC). The lysates were cleared by centrifugation at 10,000 x g for 15 min, and the supernatants were collected.
The protein concentration was quantified using a Bradford Protein Assay (Bio-Rad, Hercules, CA, USA). Equal amounts of protein lysates were used for western blot analysis with the indicated antibodies (primary antibody, 1:1,000 dilution, 4˚C; secondary antibody, 1:3,000 dilution, room temperature). The immunoreactive protein bands were detected using an EZ-Western Detection kit (Daeilab Service Co., Seoul, Korea).

Statistical analysis. All experiments were performed in triplicate. Results of the cell proliferation assay and MTT assay are expressed as the mean ± standard deviation. The standard deviations for all of the measured biological parameters are displayed in the appropriate figures. A Student's t-test (Microsoft Excel, Albuquerque, NM, USA) was used for single variable comparisons, and P<0.05 was considered to indicate a statistically significant difference.

Results

Apigenin suppresses the growth of SKBR3 cells. The growth suppressive activity of three phytoestrogens (apigenin, genistein and quercetin) was evaluated in SKBR3 cells using a cell proliferation assay. As indicated in Fig. 1A, apigenin, genistein and quercetin significantly inhibited SKBR3 cell proliferation in a dose-dependent manner (0-100 µM) following 72 h of treatment. Among the three phytoestrogens, apigenin exerted the greatest growth suppressive activity in the SKBR3 cells. Therefore, apigenin was selected for use in the present study. In addition, the time-dependent growth suppressive activity of apigenin was measured by MTT assay, as shown in Fig. 1B. It was demonstrated that the proliferation assay was more sensitive than the MTT assay with respect to measuring the extent of cell growth inhibition, as indicated by the comparison between Fig. 1A and B. Furthermore, the growth inhibition exerted by apigenin was confirmed by microscopic cellular evaluation. The results in Fig. 1C demonstrated that apigenin was able to effectively attenuate the growth of SKBR3 monolayer cells following 72 h of treatment. Of note, apigenin also induced morphological changes in these cells (Fig. 1C). Furthermore, the growth suppressive activity of apigenin was not limited to breast cancer cells. The results presented in Fig. 1D demonstrated that apigenin...
also inhibited the growth of papillary and anaplastic thyroid cancer cells (SNU790 and SNU80).

**Growth-suppressive activity of apigenin is accompanied by an increase in the sub G_0/G_1 apoptotic population in SKBR3 cells.** To investigate whether apigenin inhibited cell proliferation via the induction of alterations in cell cycle progression, the effects of apigenin on the cell cycle were evaluated in SKBR3 cells. Cells were treated with apigenin (0-80 µM) for 72 h and cell cycle distribution was determined by flow cytometric analysis. The results demonstrated that apigenin induced an increase in the sub G_0/G_1 apoptotic population in SKBR3 cells (Fig. 2).

**Apigenin induces apoptosis via caspase-dependent pathways in SKBR3 cells.** Whether apigenin activated caspase-dependent apoptosis was assessed via evaluation of the expression levels of caspase-8, caspase-3 and PARP. It was revealed that apigenin upregulated the expression levels of cleaved caspase-8 and -3, and induced PARP cleavage in SKBR3 cells (Fig. 3A). It was also demonstrated that the cleavage of caspase-8, caspase-3 and PARP was inhibited by the caspase-8 inhibitor Z-IETD-fmk and the caspase-9 inhibitor Z-LEHD-fmk (Fig. 3B). However, apigenin abrogated this inhibition and induced the cleavage of caspase-8, caspase-3 and PARP in the presence of Z-IETD-fmk and Z-LEHD-fmk (Fig. 3B). Furthermore, the caspase-8 and caspase-9 inhibitors did not suppress cell growth, but apigenin was still able to induce apoptosis in their presence (Fig. 3C). These results confirmed that apigenin promoted apoptosis via caspase-dependent mechanisms.

**Apigenin decreases cell growth rate of SKBR3 cells via the JAK2-STAT3-VEGF signaling pathway.** Whether apigenin regulated the levels of p53, BCL2 and BAX was subsequently evaluated. As indicated in Fig. 4A, apigenin did not influence the expression of p53, BCL2 and BAX. However, apigenin downregulated the expression levels of p-STAT3, p-JAK2 (an upstream kinase of STAT3) and VEGF in SKBR3 cells (Fig. 4B). In addition, apigenin suppressed the expression of p-STAT3 and Hif-1α, which were upregulated by hypoxia mimic CoCl2 (Fig. 4C). Immunocytochemical staining indicated that apigenin decreased the nuclear localization of STAT3 in the presence and absence of CoCl2 (Fig. 5A). Of
note, apigenin significantly attenuated the CoCl₂-induced upregulation of VEGF (Fig. 5B). These results suggested that apigenin decreased the cell growth rate via inhibition of the JAK2-STAT3-VEGF signaling pathway.

S31-201 inhibits cell growth and expression of oncogenic molecules in SKBR3 cells. Whether the STAT3 inhibitor S31-201 inhibited cell proliferation and STAT3 activation in SKBR3 cells was also investigated. As indicated in Fig. 6A and B, S31-201 decreased cell growth in a dose- and time-dependent manner. Furthermore, S31-201 reduced the expression levels of p-STAT3 and VEGF (Fig. 6C). These results demonstrated that STAT3 inhibition induced cell growth inhibition and suppressed the expression of oncogenic molecules.

Discussion

The present study aimed to evaluate the potential anti-proliferative activity of apigenin in SKBR3 HER2-overexpressing breast cancer cells, and elucidate its mechanism of action (Fig. 7). Apigenin suppressed the growth of SKBR3 cells in a dose- and time-dependent manner. Apigenin also inhibited the growth of papillary and anaplastic thyroid cancer cells (SNU790 and SNU80) in a dose-dependent manner, which suggested that the anti-proliferative effect of apigenin was not limited to breast cancer cells.

The results of the present study indicated that the growth inhibition induced by apigenin was associated with an increase in the sub-G₀/G₁ apoptotic population of SKBR3 cells. Apigenin increased the number of apoptotic cells in a dose-dependent manner, as revealed by fluorescence-assisted cell sorting analysis. Of note, apigenin induced apoptosis via caspase-dependent pathways by enhancing the cleavage of caspase-8, caspase-3 and PARP. In order to determine whether apigenin-induced apoptosis occurred via a caspase-8 and caspase-3-dependent pathway, SKBR3 cells were treated with caspase-8 inhibitor Z-IETD-fmk and the caspase-9 inhibitor Z-LEHD-fmk and the expression of caspase-pathway associated factors was evaluated by western blot analysis. It was revealed that the cleavage of caspase-8, caspase-3 and PARP was inhibited by the caspase-8 and caspase-9 inhibitors. However, this inhibition was abrogated by apigenin, suggesting that it is a potent inducer of apoptosis.

Caspases are members of a cysteine-dependent aspartate-regulated protease family, frequently associated with cell death (31). Caspases are initially synthesized as relatively inactive zymogens, which are subsequently activated by scaffold-mediated transactivation or cleavage by upstream proteases in the relevant intracellular cascade (31). Following activation, the caspases cleave various intracellular polypeptides, including major cytoplasmic and nucleic structural elements, components of the DNA repair machinery and numerous protein kinases (31).

The results of the western blot analyses in the present study revealed that apigenin did not influence the expression levels of apoptotic molecules, including p53, BCL2 and BAX, which suggested that apigenin may not be able to regulate the levels of p53 (32). However, apigenin reduced the expression of p-STAT3 and p-JAK2 in SKBR3 cells. The VEGF promoter contains various transcription factor binding sites, including sites for STAT3 (33) and Hif-1 (34). The physical interaction between STAT3 and Hif-1 regulates the transcriptional activation of VEGF via their binding to the VEGF promoter (35). In the present study, it was demonstrated that apigenin inhibited VEGF expression and production, as well as p-STAT3 expression and nuclear localization in the presence or absence of CoCl₂. The STAT3 inhibitor S31-201 decreased the expression of p-STAT3 and VEGF. Culturing of SKBR3 cells under conditions that mimicked hypoxia or normoxia did not induce the expression or activation of MMP-2 or MMP-9 (data not shown), as indicated in a previous study (36). Conversely, the co-culture of SKBR3 with another cell line or tumor-associated macrophages induces the expression and activation of MMP-2 and MMP-9 (36). The results of the present study demonstrated that apigenin suppressed cell growth via inhibition of the STAT3-VEGF signaling pathway in SKBR3 cells (Fig. 7).

STAT3 is a transcription factor which modulates gene expression in response to certain cellular stimuli and has significant roles in the mediation of cell growth and apoptosis. STAT3 frequently functions as a tumor promoter; however, a tumor-suppressor role for STAT3 has also been reported (37,38). STAT3 enhances cellular proliferation and angiogenesis, inhibits apoptosis and promotes invasion and metastasis (39-41). The expression of STAT3 in melanoma tumors is associated with poor prognosis (39-41). Constitutive STAT3 phosphorylation is regulated by upstream kinases (Jak and Src) and has been suggested to be a crucial stage in oncogenesis (42,43). Resveratrol (a phytoestrogen) has been shown to inhibit STAT3 signaling and induce apoptosis in malignant cells expressing activated STAT3 (44).

HER2 overexpression is associated with ~20-25% of invasive breast carcinomas (45). A normal, healthy breast cell has 20,000 HER2 receptors, compared with up to 1.5 million in a breast cancer cell. HER2 is a member of the HER/ErbB2/Neu protein family, which comprises multiple receptors, including HER1/EGFR, HER3 and HER4. Crosstalk between the HER2 and estrogen receptor (ER) signal transduction pathways has been identified (46), and ER is able to modulate HER2 expression levels. In the present study, it was demonstrated that apigenin was able to significantly inhibit the growth of HER2-overexpressing breast cancer cells. This result revealed that apigenin may represent a potential natural therapeutic for the treatment and prevention of HER2-overexpressing breast cancer.

Acknowledgements

The present study was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (NRF-2012R1A1A0304797). This study was also supported by a grant from the Korean Medicine R&D project of the Ministry of Health and Welfare (B120014).

References


