Abstract. Background/Aim: The present study aimed to investigate anticancer properties of equol and demonstrate its underlying mechanisms of action in human cervical cancer HeLa cells. Materials and Methods: Inhibition of cell viability was examined by 3-(4,5-dimethylthiazoly-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Apoptosis was evaluated by observation of apoptotic cell morphology, and an increase of annexin-V+ cells. Western blotting was used to examine apoptosis-related proteins. Flow cytometry was used to measure mitochondrial membrane potential (MMP) and reactive oxygen species (ROS). Results: Equol treatment inhibited HeLa cell proliferation in dose- and time-dependent manner. Equol-induced apoptotic cell death was accompanied by the activation of caspases, and alteration of MMP and mitochondrial membrane proteins; equol also rapidly triggered ROS production. Pre-treatment with N-acetylcysteine blocked loss of MMP, caused increase of Bcl-2-associated X protein (Bax)/B-cell lymphoma 2 (Bcl-2) ratio, caspase-8 activation, and apoptosis induced by equol. Conclusion: Equol is a potential anticancer agent against HeLa, with possible mechanisms involved in ROS generation and mitochondrial membrane alteration.

Cervical cancer is the second leading cause of cancer-related death in women (1). Although the incidence of cervical cancer could be prevented by human papilloma virus (HPV) vaccination, it is still the leading cause of cancer-related death, especially in women in developing countries (2, 3). In spite of some therapeutic options for cervical cancer, there are some limitations. Surgical treatment, a preferential choice for patients with early-stage disease, is restricted to patients with appropriate conditions (4). Radiation therapy and chemotherapy can induce systemic cytotoxicity because they influence surrounding normal tissues as well as cancer cells (5). Therefore, new effective and safe therapeutic options for the management of cervical cancer are required.

Previous epidemiological studies have demonstrated an association between intake of soybean or soy isoflavones and a reduced risk of cancer (6-9). In addition, numerous in vivo and in vitro studies have revealed the anticancer activities of soy isoflavones such as daidzein and genistein (10-12). Equol (7-hydroxy-3-(4'-hydroxyphenyl)-chroman), a major metabolite of daidzein, is another soy-derived isoflavone with anticancer activity. The effect of equol on apoptosis and cell-cycle arrest has been reported in prostate and breast cancer cell lines (13-15). Furthermore, recent studies have suggested that equol efficiently enhances the effect of other anticancer agents such as tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (16, 17), tamoxifen (18) and radiation (19) against several cancer cell lines. However, its anticancer effect and precise mechanisms in human cervical cancer cells have not been investigated. In the present study, we demonstrated the antiproliferative effect of equol on HeLa cells and evaluated the underlying mechanisms of action.

Materials and Methods

Materials. Equol was purchased from LC laboratory (Woburn, MA, USA). 3-(4,5-Dimethylthiazoly-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), 3,3'-dihexyloxacarbocyanine iodide (DiOC6), N-acetylcysteine (NAC), and 2',7'-dichlorofluorescein diacetate (DCF-DA) were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). Caspase inhibitors (z-VAD-fmk, z-IETD-fmk, z-LEHD-fmk, and z-DEVD-fmk) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture. HeLa human cervical cancer cells were obtained from the Korean Cell Line Bank (Seoul, Korea) and maintained at 37°C in a humidified atmosphere of 5% CO2. Cells were cultured in RPMI supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin.

Treatment of cells. MTT assay: HeLa cells were treated with different concentrations of equol for 24, 48, 72 h and cell viability was measured by MTT assay. After treatment with equol, MTT reagent was added for 4 h at 37°C. The resulting formazan crystals
were dissolved in dimethyl sulfoxide (DMSO) and the absorbance was analyzed by a microplate reader (EL800, Bio-Tek Instrument Inc., Winooski, VT, USA) at 570 nm.

Flow cytometric analysis for mitochondrial membrane potential (MMP) and reactive oxygen species (ROS) production. HeLa cells were treated with different concentrations of equol in the presence or absence of caspase inhibitors or NAC. For the detection of MMP and ROS generation, cells were harvested and incubated with DiOC6 (40 nM) or DCF-DA (10 μM) at 37°C for 30 min in the dark, respectively. The fluorescent intensity was then measured by flow cytometry (FACSCanto II Flow Cytometer; BD Biosciences, San Jose, CA, USA).

Detection of apoptosis. Cells were treated with different concentrations of equol with or without caspase inhibitors or NAC. At the end of incubation, cells were lysed with lysis buffer (Millipore, Billerica, MA, USA) for 30 min at 4°C and equal amounts of protein were incubated with caspase colorimetric substrate (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and reaction buffer (Millipore, Billerica, MA, USA) at 37°C for 2 h. Caspase activity was examined by measuring absorbance at 405 nm using a microplate reader.

Caspase activity assay. Cells were treated with different concentrations of equol with or without caspase inhibitors or NAC. At the end of incubation, cells were lysed with radio-immunoprecipitation assay (RIPA) buffer (Sigma-Aldrich, St. Louis, USA). Cell lysates containing equal amount of protein were subjected to SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The blots were incubated with primary antibodies against poly (ADP-ribose) polymerase (PARP), caspase-8, cleaved caspase-9, cleaved caspase-3, cleaved caspase-7, BH3-interacting domain death agonist (BID), bcl2-associated X protein (BAX), B-cell lymphoma 2 (BCL2), or β-actin (Cell signaling, Danvers, MA, USA). After washing with tris-buffered saline with Tween 20 (TBS-T), the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and analyzed by using enhanced chemiluminescence (ECL) advance detection kit (Amersham Biosciences, Piscataway, NJ, USA).

Statistical analysis. Values are presented as the mean±SD. Statistical significance was evaluated by using one-way analysis of variance (ANOVA) followed by Turkey’s test. A p-value of less than 0.05 was regarded as statistically significant.

Results

Equol caused HeLa cell growth inhibition in a dose- and time-dependent manner. To evaluate the effect of equol on HeLa cell proliferation, cells were treated with different concentrations of equol and for different times and cell viability was examined by the MTT assay. As shown in Figure 1, equol inhibited cell viability of HeLa cells in a dose- and time-dependent manner.

Equol induced apoptosis of HeLa cells. To investigate whether apoptosis is involved in equol-induced cytotoxicity, morphological changes of HeLa cells were examined after equol treatment for 48 h. As shown in Figure 2A, the control cells were well attached to the dish, with normal morphology. However, equol-treated cells displayed a round and shrunken shape, with an irregularly fragmented morphology. The proportion of floating cells also increased in a dose-dependent manner (Figure 2A). Next, equol-induced apoptosis was further confirmed by flow cytometric analysis after annexin V/PI dual staining. When cells were treated with different concentrations of equol for 48 h, the percentage of apoptotic cells significantly increased in a dose-dependent manner (Figure 2B, and C). We also performed western blot analysis to detect PARP cleavage, another hallmark of apoptosis. As shown in Figure 2D, cleavage of PARP was significantly induced by equol in a dose-dependent manner. Taken together, these results indicate that equol treatment caused HeLa cell death by inducing apoptosis.

Apoptosis of HeLa cells by equol involves the activation of caspase-dependent pathway. Members of the caspase family are well-known for playing a central role in the execution of apoptotic cell death (20). Therefore, we examined whether equol treatment affects caspase activation of HeLa cells. As demonstrated in Figure 3A, results from western blotting showed that exposure to equol for 48 h resulted in the significant activation of initiator caspases (caspase-8 and caspase-9) and their downstream effectors (caspase-3, -6, and -7). The activation of these caspases was confirmed by analyzing their proteolytic cleavage products. As shown in Figure 3B, cleavage of PARP, a key marker of caspase-dependent apoptosis, was observed in equol-treated cells.

These results suggest that equol induces apoptosis in HeLa cells through a caspase-dependent mechanism.
caspase-9) and effector caspases (caspase-3 and caspase-7). In addition, in vitro caspase activity assay also showed the increase of enzymatic activities of caspase-8, -9, and -3 by equol treatment (Figure 3B). Next, to further examine the involvement of caspases, the effect of caspase inhibitors on equol-induced apoptosis was investigated by flow cytometric analysis. As shown in Figure 4A, equol-induced apoptosis was strongly suppressed by z-VAD-fmk (pan-caspase inhibitor), indicating the important role of caspases in equol-induced apoptosis. Moreover, when cells were pre-treated with z-DEVD-fmk (caspase-3 specific inhibitor), z-IETD-fmk (caspase-8 specific inhibitor), and z-LEHD-fmk (caspase-9 specific inhibitor), equol-induced apoptosis was significantly attenuated (Figure 4A). Moreover, caspase-3 activity was markedly suppressed by z-IETD-fmk and z-LEHD-fmk, indicating that both caspase-8 and caspase-9 are involved in the activation of caspase-3, a down-stream caspase (Figure 4B).

Mitochondrial de-polarization was markedly induced by equol treatment. Disruption of MMP (ΔΨm) is a crucial event in the apoptotic pathway (21). To further assess the role of mitochondria in equol-mediated apoptosis, cells were stained with DiOC6 and alteration of MMP was quantified by flow cytometry. When cells were incubated with different concentrations of equol for 48 h, the level of ΔΨm significantly decreased (Figure 5A, and B). Next, to investigate the involvement of mitochondria in equol-induced caspase activation, cells were pre-treated with z-VAD-fmk or z-IETD-fmk before equol treatment. The broad caspase inhibitor z-VAD-fmk almost completely inhibited equol-induced mitochondrial de-polarization. The caspase-8 specific inhibitor z-IETD-fmk also significantly blocked equol-triggered disruption of ΔΨm (Figure 5C). Collectively, these results indicate that equol mediates mitochondrial dysfunction in HeLa cells through a caspase-dependent pathway.
BCL2 family regulation by equol-treatment. BCL2 family members are closely related to mitochondria-mediated apoptosis signaling. Because depletion of ΔΨm by equol was dependent on caspase-8 activation, we further examined the expression levels of BID, a specific substrate for caspase-8, which serves as a link between caspase-8 and mitochondria-mediated apoptotic pathway. As shown in Figure 5D, the level of full-length BID was significantly reduced by equol treatment. Equol also caused the reduction of anti-apoptotic BCL2 protein, with concomitant increase of pro-apoptotic BAX protein, leading to the substantial increase of the BAX/BCL2 ratio (Figure 5D, and E). Consistent with previous results, these data indicate that equol induces mitochondrial alteration in HeLa cells.

ROS generation was partially involved in equol-induced apoptosis. Many previous reports have demonstrated the important role of ROS on mitochondria-mediated apoptosis, which is regulated by BCL2 family members (22-24). To evaluate equol-induced ROS generation, cells were examined after DCF-DA staining. As shown in Figure 6A, ROS levels were significantly increased within 0.5 h after equol treatment and consistently maintained until 4 h. Next, to examine whether ROS production is involved in equol-induced apoptosis, cells were pre-treated with NAC before equol treatment and apoptosis was determined by PARP cleavage and annexin V/PI staining assay. As shown in Figure 6B and C, scavenging of ROS by NAC resulted in the significant attenuation of PARP cleavage and apoptosis.
Moreover, equol-induced loss of MMP, activation of caspase-8, and alteration of BAX/BCL2 ratio were also blocked by NAC (Figure 6D-G). These results indicate that equol-induced apoptosis and associated mitochondrial changes are, at least in part, involved in ROS production.

**Discussion**

In the present study, we demonstrated that equol inhibits HeLa cell proliferation through inducing apoptosis. Equol-induced apoptosis was evaluated by morphological
observation, dual annexin V/PI staining assay, PARP cleavage, and activation of caspases.

There are two well-known pathways leading to apoptotic cell death: the mitochondrial pathway and the death receptor pathway. In both pathways, the caspase family of proteins plays a critical role in activating apoptosis. Mitochondria-mediated apoptosis is triggered by intrinsic factors and induces the disruption of mitochondrial membrane, leading to the release of cytochrome c from mitochondria. Released cytochrome c causes the activation of caspase-9 and
subsequent activation of executioner caspases such as caspase-3 and caspase-7 (25, 26). According to our results, equol substantially increased caspase-9 activation. Furthermore, equol-induced apoptosis and caspase-3 activation were significantly blocked by z-LEHD-fmk, a specific inhibitor of caspase-9, suggesting the involvement of mitochondria-mediated apoptosis. Concomitantly, the MMP was markedly reduced by equol treatment in a concentration-dependent manner. In addition, we examined the effect of equol on BAX/BCL2 ratio because BAX and BCL2 play an important role in regulation of mitochondria-mediated apoptosis (27). We found that equol treatment induced the significant elevation of the BAX/BCL2 ratio in a dose-dependent manner at 48 h. Taken together, these results demonstrated the crucial role of mitochondria in equol-induced apoptosis.

The death receptor-mediated or extrinsic apoptotic pathway is induced by external signals and mediates activation of caspase-8. Active caspase-8 can induce mitochondrial damage by triggering BID cleavage or directly inducing effector caspases (28). In the present study, we found that equol mediates caspase-8 activation in inducing apoptosis of HeLa cells. Inhibition of caspase-8 significantly attenuated equol-induced mitochondrial membrane dysfunction and apoptosis, suggesting that equol-induced caspase-8 activation could be involved in intrinsic apoptosis. Consistently, BID, a molecular linker to the mitochondrial pathway, was markedly activated by equol treatment. All together, these results demonstrate that equol-induced apoptosis of HeLa cells is related to both extrinsic and intrinsic apoptotic pathways.

Oxidative stress has been reported to contribute to regulation of apoptotic cell death (29-30). Accumulating reports have demonstrated that various anticancer agents, including bortezomib, cisplatin, paclitaxel, and radiation, cause excessive ROS production, leading to mitochondrial damage and caspase activation (31-34). In the present study, equol treatment resulted in a rapid increase of ROS generation and pre-treatment with NAC significantly blocked equol-induced MMP disruption, caspase-8 activation, increase of BAX/BCL2 ratio, and subsequent apoptosis. However, scavenging of ROS by NAC was not enough to completely rescue the cells from equol-mediated damage, suggesting that multiple mechanisms could be involved in equol-induced HeLa cell death. A detailed investigation of the exact mechanisms by which equol initiates ROS-dependent and independent cell death in HeLa cells will be performed in the near future.

In summary, the present study demonstrated that equol has an anti-proliferative effect against human cervical cancer HeLa cells by inducing apoptosis. Our findings provided a basic mechanism for the anticancer effect of equol in human cervical cancer. Further study should be performed to confirm the antigen tumor effect of equol on cervical cancer in vivo.

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References


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