

Combination Effect of Equol and TRAIL against Human Cervical Cancer Cells

EUN YOUNG KIM and AN KEUN KIM

College of Pharmacy, Sookmyung Women's University, Seoul, Korea

Abstract. *Background/Aim:* Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promising candidate for cancer therapy due to its selective ability to induce apoptosis of cancer cells. However, some cancer cells are resistant to TRAIL. Here, we demonstrated that treatment with TRAIL, in combination with equol, sensitizes TRAIL-mediated apoptosis of HeLa cells. *Materials and Methods:* Cell viability was evaluated by the colorimetric cell viability assay (MTT). Apoptotic cell death was analyzed by flow cytometry and microscopy. Western blotting was performed to examine protein expression and cell surface expression was evaluated by flow cytometry. Enzymatic activity of caspases was measured by the colorimetric assay. *Results:* Equol enhanced TRAIL-induced apoptosis through activation of caspase-3, -8, -9, and cleavage of BID. Furthermore, DR4/Fc chimera protein and DR5/Fc chimera protein efficiently reduced the activation of caspases and BID cleavage, as well as apoptotic cell death induced by co-treatment with equol and TRAIL. *Conclusion:* Equol enhances TRAIL-induced apoptosis of HeLa cells through a death receptor-mediated caspase pathway.

Cervical cancer is the second most common type of cancer in women, and is the most frequent female malignancy in developing countries (1). Although the worldwide death rates from cervical cancer have decreased, it remains the leading cause of mortality for women in developing countries (2). Although conventional treatments, such as surgery, radiation, and chemotherapy are used effectively, the survival benefit is limited, and relapse can occur after treatment (3). Therefore, new therapeutic options are needed in order to improve the treatment of cervical cancer.

Correspondence to: An Keun Kim, College of Pharmacy, Sookmyung Women's University, Hyochanwon-Gil 52, Yongsan-Ku, Seoul, 140-742, Republic of Korea. Tel: +82 27109566, Fax: +82 27109871, e-mail: akkim@sookmyung.ac.kr

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Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a member of the TNF family, has been considered as an attractive anticancer agent, as it preferentially induces apoptosis of malignant or transformed cells without toxicity to most normal cells (4). To date, five different cell surface receptors have been identified for TRAIL. Two of them, death receptor-4 (DR4) and death receptor-5 (DR5), contain the cytoplasmic death domain that triggers apoptosis upon TRAIL binding through the formation of a death-inducing signaling complex (DISC). The other three serve as decoy receptors that compete with DRs for TRAIL binding, without transducing apoptotic signals (5). Although TRAIL is a potent anticancer agent in susceptible cells, recent studies have shown that many cancer cells, including human cervical cancer cells, are resistant to TRAIL (6, 7). Therefore, identification of the sensitizing agents which enable TRAIL resistance to be overcome would represent a valuable therapeutic approach for the clinical use of TRAIL in cancer therapy. Numerous studies have demonstrated that the combination of TRAIL with traditional chemotherapy, radiation, or natural chemopreventive compounds could sensitize TRAIL-resistant cancer cells to TRAIL (8-10).

Previous epidemiological studies have revealed the cancer chemopreventive effect of phytochemicals (11). Soybean, an edible plant with such chemopreventive phytochemicals, has been used for centuries as a dietary staple in Asia. Several epidemiological and pre-clinical studies have suggested that soybean and its isoflavones reduces the risk of cancer incidence in different cell lines (12-14). Genistein and daidzein, the major isoflavones in soybean, have been reported to possess potent antitumor effects on several cancer cell lines through multiple signaling pathways (15, 16). Equol, an isoflavone metabolite of daidzein, has also been demonstrated to inhibit cell viability *in vitro* and tumor formation *in vivo*, through the induction of apoptosis (17, 18). Here, we investigated whether soy isoflavones can enhance TRAIL-induced cell death. Our results showed, to our knowledge for the first time, that equol sensitizes HeLa cells to TRAIL-induced cell death *via* apoptosis.

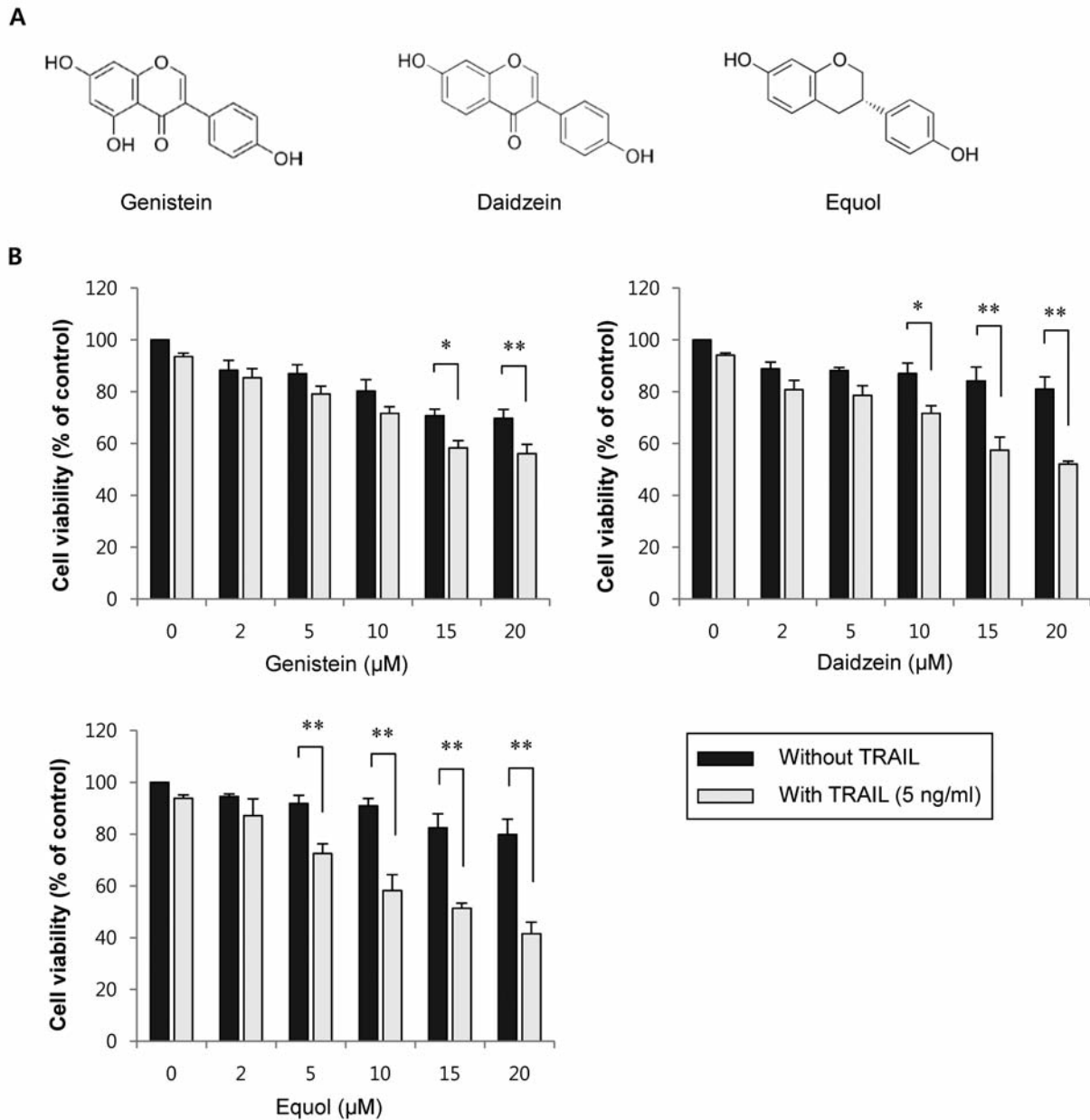


Figure 1. Soy isoflavones potentiate tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated cytotoxicity in HeLa cells. A: Chemical structures of soy isoflavones used in combination with TRAIL. B: Effect of isoflavones on TRAIL-induced cell death. Cells were treated with isoflavones (2-20 μM) alone, or combination with TRAIL (5 ng/ml) for 24 h. Cell viability was measured by 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyl-tetrazolium bromide assay. Statistical significance: * $p < 0.01$, ** $p < 0.001$ vs. control TRAIL-untreated cells.

Materials and Methods

Materials. Equol was purchased from LC lab (Woburn, MA, USA). Genistein and daidzein were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). Soluble recombinant human TRAIL Apo2L was purchased from Peprotech (Rocky Hill, NJ, USA). Human recombinant DR4/Fc and DR5/Fc chimera protein, and caspase inhibitors were obtained from R&D Systems (Minneapolis, MN,

USA). Antibodies for western blotting were purchased from Cell Signaling (Beverly, MA, USA).

Cell culture. Human cervical cancer HeLa cells were obtained from the Korean Cell Line Bank (Seoul, South Korea) and maintained in RPMI with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 $\mu\text{g/ml}$ streptomycin. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 .

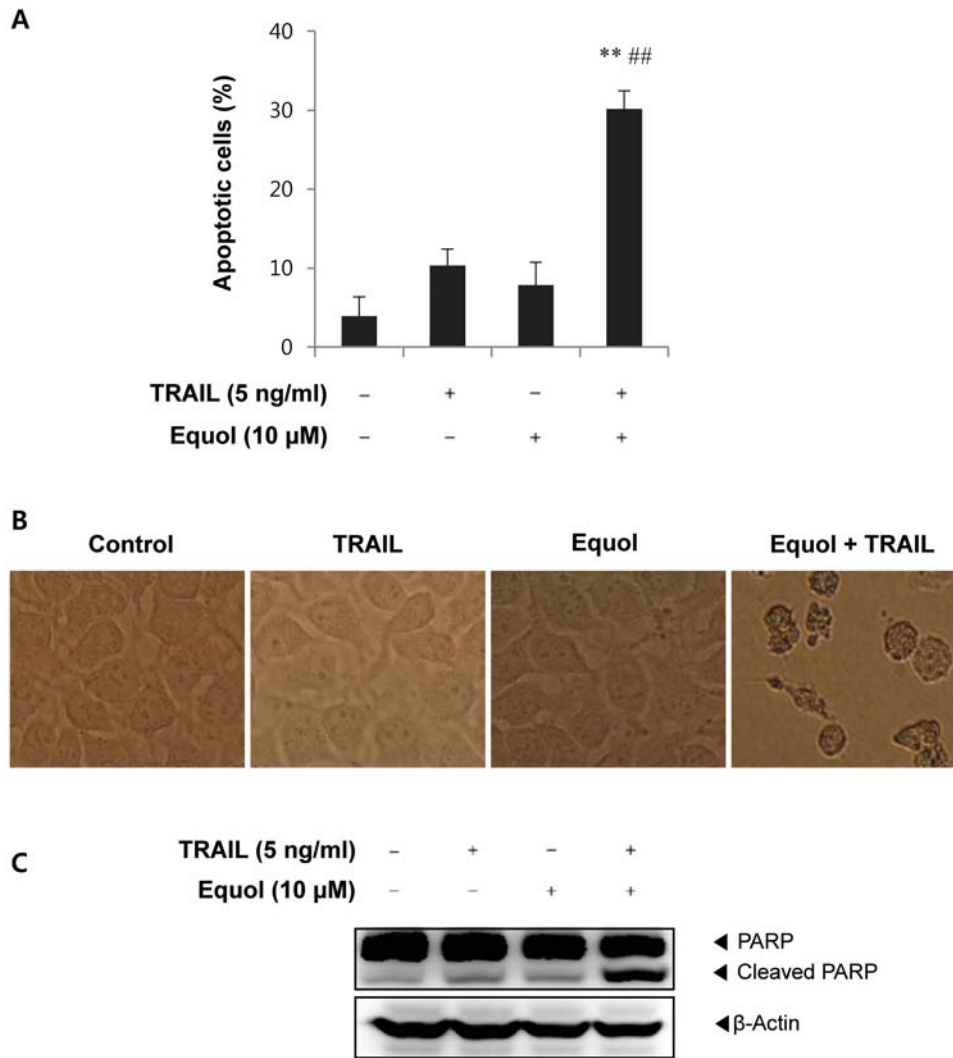


Figure 2. Apoptotic effect of equol and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in HeLa cells. Cells were treated with equol (10 μ M) and/or TRAIL (5 ng/ml) for 24 h. A: Apoptosis was quantified by flow cytometric analysis after Annexin V/propidium iodide (PI) double staining assay. Data in the bar graph represent the mean \pm SD of three independent experiments. ** p <0.001 vs. untreated control; ## p <0.001 vs. cells treated with equol or TRAIL alone. B: Morphological changes in cultures were examined by light microscopy. Magnification, \times 400. C: Whole-cell extracts were prepared and analyzed by western blot for detecting poly (ADP-ribose) polymerase (PARP) cleavage. β -Actin was used as a loading control.

Cell viability analysis. Cells were treated with various concentrations of isoflavones such as genistein, daidzein and equol with or without TRAIL (5 ng/ml) for 24 h and cell viability was determined by 3-(4,5-dimethylthiazoly-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. The optical density was measured at 570 nm by a microplate reader (EL800; Bio-Tek Instrument Inc., Winooski, VT, USA) and cell viability (%) was calculated.

Apoptosis detection. Cells were treated with equol (10 μ M) and/or TRAIL (5 ng/ml) for 24 h and the induction of apoptosis was evaluated with flow cytometry and light microscopy. For quantifying apoptosis, cells were double-stained with Annexin V-

FITC/propidium iodide (PI) and fluorescence was detected by FACSCanto II Flow Cytometer (BD Biosciences, San Jose, California, USA). Annexin V⁺/PI⁻ cells were considered as early apoptotic cells. For light microscopy, cells were seeded in a 6-well plate. After treatment, morphological changes of cells were observed and photographed with Olympus BH series microscope (Shinjuku-ku, Tokyo, Japan).

Caspase activity assay. Cells were treated with equol (10 μ M) and/or TRAIL (5 ng/ml) for 24 h. To measure enzymatic activity of caspases, a caspase colorimetric protease assay kit (Millipore, Billerica, MA, USA) was used. In brief, harvested cell pellets were lysed in the lysis buffer and supernatants were collected. Equal

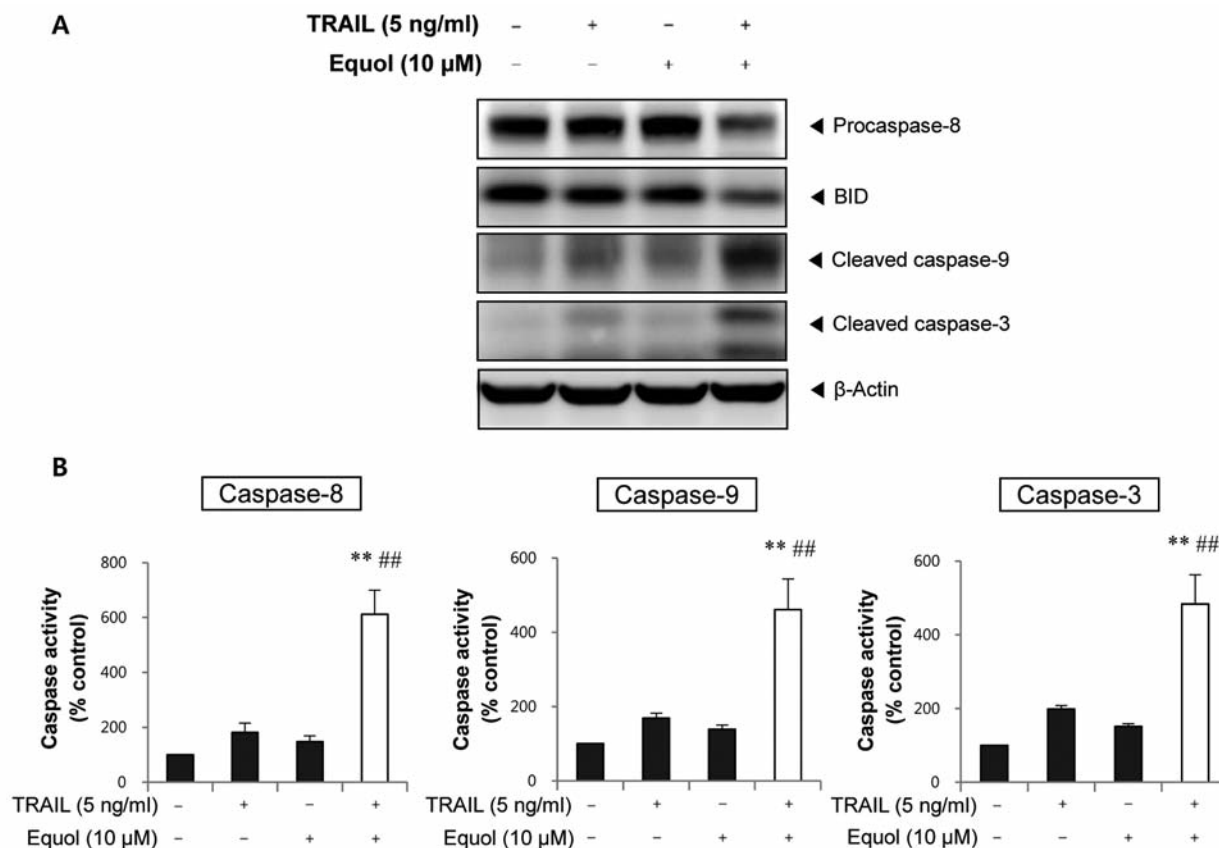


Figure 3. Continued

amounts of protein were incubated with reaction buffer and colorimetric substrate, acetyl-Ile-Glu-Thr-Asp p-nitroaniline (Ac-IETD-pNA) for caspase-8, acetyl-Leu-Glu-His-Asp- p-nitroaniline (Ac-LEHD-pNA) for caspase-9, and acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) for caspase-3, at 37°C for 2 h in the dark. Caspase activity was calculated following the manufacturer’s instructions.

Flow cytometry of DR4 and DR5. Cells were incubated with or without equol (10 μM) for 24 h. Then, surface expression of DRs was analyzed by indirect staining with primary mouse antibodies against DR4 and DR5 from Santa Cruz Biotechnology (Santa Cruz, CA, USA), followed by incubation with phycoerythrin (PE)-conjugated goat anti-mouse IgG1. Normal mouse IgG1 antibody was used as an isotype non-binding antibody. The expression of DR4 and DR5 was analyzed by FACSCanto II Flow Cytometer.

Western blotting. Cells were harvested and re-suspended in a protein lysis buffer (Sigma Aldrich), and the lysates were centrifuged at 2,000 ×g for 20 min at 4°C. Equivalent amounts of proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and followed by transferring to a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% skimmed milk, the blot was incubated with protein-specific antibodies against DR5, poly (ADP-ribose) polymerase

(PARP), cleaved caspase-3, caspase-8, cleaved caspase-9, BH3 interacting domain death agonist (BID) or β-actin, followed by horseradish peroxidase-conjugated secondary antibody. Bands were visualized by enhanced chemiluminescence advance detection kit.

Statistical analysis. All data are presented as the mean±SD from three independent experiments. Statistical significance was evaluated by 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

The cytotoxic effects of soybean isoflavones in combination with TRAIL in HeLa cells. In order to investigate whether soy isoflavones (Figure 1A) potentiate TRAIL-mediated cell death of HeLa cells, cells were treated with isoflavones (2 to 20 μM) with or without TRAIL (5 ng/ml), and the cytotoxicity was evaluated by the MTT assay. As a result, all the tested compounds augmented TRAIL-mediated cytotoxicity in a dose-dependent manner, while TRAIL (5 ng/ml)-alone had a small effect. As shown in Figure 1B,

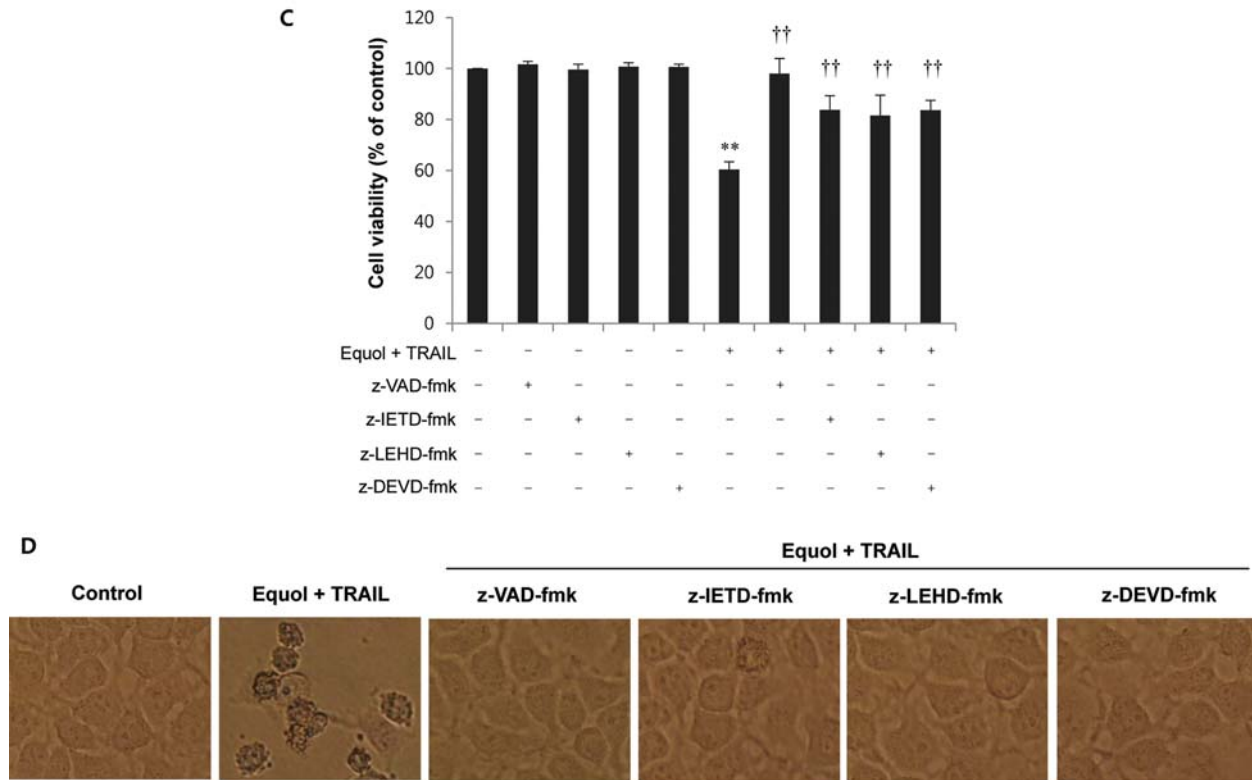


Figure 3. Combined treatment with equol and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces cell death through caspase activation. A,B: Cells were treated with equol (10 μ M) and/or TRAIL (5 ng/ml) for 24 h. Caspase activation and BH3 interacting domain death agonist (BID) cleavage was examined by western blotting (A) and caspase enzymatic activity was evaluated by *in vitro* colorimetric caspase activity assay (B). In (B), data in bar graphs represent the mean \pm SD of three independent experiments. ** p <0.001 vs. untreated control; ## p <0.001 vs. cells treated with equol- or TRAIL-alone. C,D: Cells were pre-treated with 20 μ M pan-caspase inhibitor (z-VAD-fmk), caspase-8 inhibitor (z-IETD-fmk), caspase-9 inhibitor (z-LEHD-fmk), or caspase-3 inhibitor (z-DEVD-fmk) for 30 min and then treated with the combination of 10 μ M equol plus 5 ng/ml TRAIL for 24 h. The effect of caspase inhibitors on the combination treatment was evaluated by 3-(4,5-dimethylthiazoly-2-yl)-2,5-diphenyl-tetrazolium bromide assay (C) and examination of cell morphology (D). In (C), ** p <0.001 vs. untreated control; †† p <0.001 vs. cells treated with equol plus TRAIL.

genistein significantly enhanced TRAIL-induced cell death at a dose of 15 and 20 μ M. Meanwhile, daidzein potentiated TRAIL-mediated cytotoxicity at the concentrations that daidzein-alone induced limited cytotoxicity (<20%) towards HeLa cells (Figure 1B). Importantly, equol displayed greater potency than the other agents in the sensitization of HeLa cells to TRAIL-induced cytotoxicity, even at concentrations for which genistein or daidzein had no similar effect. For example, treatment with isoflavones at 5 μ M resulted in a similarly slight decrease of the viability of HeLa cells; 13.1 \pm 3.5% for genistein, 11.9 \pm 1.3% for daidzein, and 8.1 \pm 3.1% for equol. However, when cells were co-treated with these agents plus TRAIL, only the equol and TRAIL combination induced a significant increase in cell death compared to the single-agent-alone. Accordingly, equol was selected for further experiments to elucidate the underlying mechanisms of enhanced TRAIL-mediated cell death.

Equol enhances TRAIL-induced apoptosis in HeLa cells. In order to examine whether enhanced cytotoxicity of the equol and TRAIL combination is involved in apoptosis, cells were analyzed by annexin V-FITC/PI double-staining assay, which detects apoptotic cell death. As shown in Figure 2A, the combination of equol (10 μ M) plus TRAIL (5 ng/ml) for 24 h significantly increased the percentage of early apoptotic cells (annexin V⁺/PI⁻ cells). Furthermore, the cells treated with equol and TRAIL displayed typical morphological features of apoptosis, such as condensed and fragmented nuclei with cellular shrinkage, compared to control or single-agent-treated cells (Figure 2B). In addition, western blot analysis revealed that cleaved PARP, another hallmark of apoptosis, was significantly increased by the combination of equol and TRAIL compared to equol- or TRAIL-alone. Overall, these findings indicate that equol potentiates TRAIL-mediated cell death by inducing apoptosis of HeLa cells.

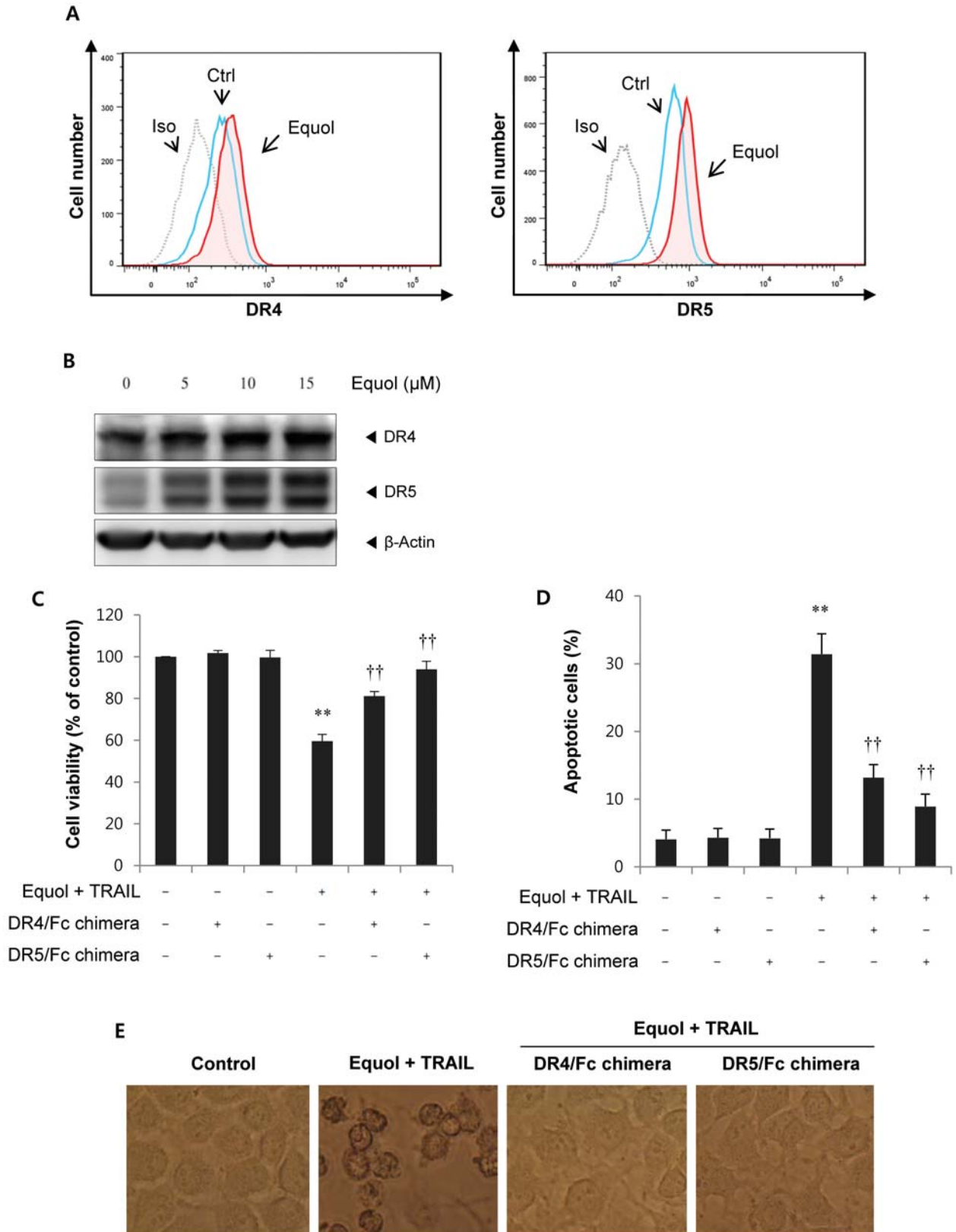


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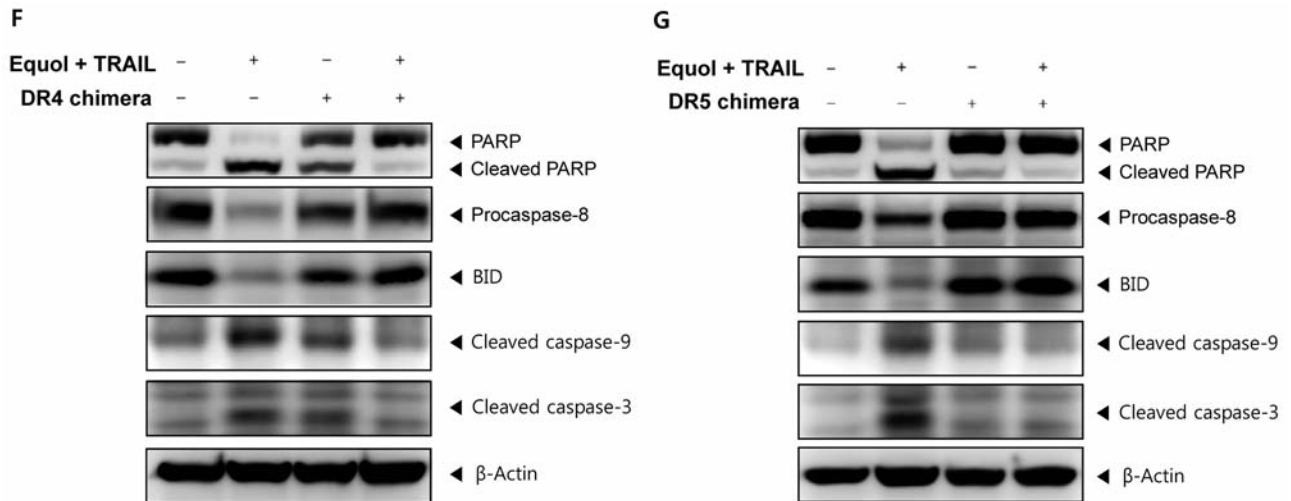


Figure 4. Equol and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis is involved in up-regulation of DRs, leading to activation of caspases. A: Cells were treated with or without equol (10 μ M) for 24 h and surface expression of DR4 and DR5 were analyzed by flow cytometry using antibodies against DR4 and DR5, as described in the Materials and Methods. Isotype-matched non-binding antibodies (Iso) were used for control for unspecific binding. X axis, fluorescence intensity; Y axis, relative number of cells. Representative data are shown. B: Cells were incubated with the indicated doses of equol for 24 h and the protein levels of DR4 and DR5 were evaluated by western blotting. C-E: Cells were treated with 10 μ M equol, 5 ng/ml TRAIL and/or DR4/Fc (50 ng/ml) or DR5/Fc chimera protein (50 ng/ml) for 24 h prior to 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyl-tetrazolium bromide assay (C), annexin V/propidium iodide (PI) double staining assay (D), and examination of cell morphology (E). In (C) and (D), ** p <0.001 vs. untreated control; †† p <0.001 vs. cells treated with equol plus TRAIL. F,G: Cells were treated with equol and TRAIL combination with or without 50 ng/ml DR4 chimera protein (F) or 50 ng/ml DR5 chimera protein (G) for 24 h, and then whole-cell extracts were analyzed by western blotting. β -Actin was used as a loading control.

Critical role of caspases in the induction of cell death by co-treatment with equol and TRAIL. To determine whether enhanced apoptosis *via* the combination of equol and TRAIL involves in caspase activation, western blot analysis was performed. As shown in Figure 3A, combination treatment markedly induced the activation of caspase-3, -8, and -9. In addition, treatment with equol and TRAIL resulted in a significant disappearance of full-length BID compared with control or single agent-treated groups (Figure 3A). The level of caspase activity was measured by colorimetric *in vitro* caspase activity assay. In accordance with the results from western blotting, caspase-3, -8, and -9 activity was significantly increased through the combination of equol and TRAIL (Figure 3B). Next, to further evaluate the involvement of caspase-dependent signaling pathway in equol and TRAIL-induced cell death, we used general and potent inhibitors of caspase-3, -8, and -9. As shown in Figure 3C, combined treatment of equol with TRAIL induced significant cytotoxic effect on HeLa cells and the pan-caspase inhibitor z-VAD-fmk completely blocked the induction of cell death. We also demonstrated that pre-treatment with the caspase-8 inhibitor: z-IETD-fmk, the caspase-3 inhibitor: z-DEVD-fmk, and the caspase-9 inhibitor: z-LEHD-fmk, significantly blocked the enhanced cytotoxicity induced by the combination of equol and TRAIL

(Figure 3C). In addition, similar results were determined through the observation of morphological changes under microscopy. As shown in Figure 3D, the apoptotic cell death induced by treatment with equol and TRAIL was markedly blocked by caspase inhibitors. Collectively, these results indicate that caspases play an essential role in cell death mediated by combination treatment of equol and TRAIL.

Up-regulation of DRs is important for equol and TRAIL-induced apoptosis. It is well-known that TRAIL interacts with the two pro-apoptotic DRs, DR4 and DR5, which lead to the oligomerization of the receptors, and the subsequent activation of caspases (19). As equol sensitized TRAIL-mediated apoptosis through a caspase-dependent pathway, we further examined whether equol affects the cell surface expression of DRs. As shown in Figure 4A, treatment with equol (10 μ M) for 24 h, up-regulated cell surface expression of DR4 and DR5. Western blot analysis also revealed that protein levels of both DR4 and DR5 were markedly increased in response to treatment with equol in a dose-dependent manner (Figure 4B).

To clarify the functional role of DRs in equol and TRAIL-induced apoptosis, we used human recombinant DR4/Fc and DR5/Fc chimera proteins to block TRAIL from binding to DR4 and DR5 receptors, respectively. As shown in Figure

4C and D, equol and TRAIL-induced apoptotic cell death was significantly attenuated by addition of DR4- and DR5-specific blocking antibodies. However, DR5-specific blocking had a more dramatic effect on equol and TRAIL-induced apoptosis than DR4 blocking. These results were further confirmed by light microscopy and western blotting. As shown in Figure 4E, combined treatment with equol plus TRAIL resulted in a markedly increased number of apoptotic cells, and the addition of DR4/Fc- and DR5/Fc-chimera proteins significantly reduced apoptosis. Moreover, both DR4- and DR5-specific antibodies significantly blocked the combination treatment-induced cleavage of PARP, activation of caspases, and decrease of full-length BID (Figure 4F and G). In most cases, DR5 blocking had a more dramatic effect on combination-induced apoptosis than did DR4 blocking. Taken together, these results indicate that the up-regulation of DR4 and DR5 by equol is critical for facilitating caspase activation and the subsequent induction of apoptosis in TRAIL-treated cells.

Discussion

It has been reported that natural dietary compounds in foods and herbs are effective in preventing development of cancer and other chronic diseases (20). These naturally-occurring products have advantages in that they are generally relatively safe and non-toxic (21). Various natural compounds have been reported to possess strong chemopreventive effects on different cell lines and animal models of cancer (22). Therefore, the investigation of natural products which effectively sensitize TRAIL-induced apoptosis may provide safe and efficient anticancer strategies for cancer therapy, with minimal toxicity. Indeed, numerous recent studies have shown that the combined therapy of TRAIL with natural dietary agents such as silibinin, quercetin, curcumin, and resveratrol, sensitize malignant cells to TRAIL (23-26).

In the present study, we compared the sensitizing effect of the soybean isoflavones genistein, daidzein, and equol in combination with TRAIL on human cervical cancer cells. Several previous reports have demonstrated the synergistic effect of genistein plus TRAIL in TRAIL-resistant cancer cells: pancreatic cancer (27), gastric cancer (28), malignant glioma (29), and hepatocellular carcinoma cells (30). A similar study with daidzein also showed augmentation of TRAIL-induced apoptosis of malignant glioma cells (31). However, information on the effect of soy isoflavones on TRAIL-mediated apoptosis of cervical cancer cells is limited. Here, we tested the combination effect of three soy isoflavones with TRAIL of HeLa cells. The results showed that all three agents enhanced cell death induced by TRAIL. Among the three agents, equol exhibited the most potent sensitizing effect for TRAIL-mediated cell death. Therefore, we chose equol in order to explore the possible mechanisms of combination

effect. We found that the combined treatment of equol and TRAIL induced cell death by triggering apoptosis, as indicated by typical morphological features and cleavage of PARP.

Apoptotic cell death can be induced by two major pathways, the extrinsic (or death receptor) and the intrinsic (or mitochondrial) pathway. Both pathways are involved in the sequential activation of caspases, which in turn mediates morphological and biochemical changes associated with apoptosis (32). TRAIL is known to trigger the extrinsic apoptotic pathway by binding to its DRs (DR4 and DR5), which recruit the adaptor protein Fas-associated protein with death domain (FADD) and caspase-8, forming the DISC (33). Formation of the DISC activates caspase-8, the essential mediator of the death receptor pathway, leading to the direct activation of effector caspase-3. In some cells, caspase-8 also triggers the mitochondrial pathway *via* activating or cleaving the BID protein, which then induces permeabilization of the mitochondria, followed by activation of caspase-9 (34). In this study, the combined use of equol and TRAIL resulted in the significant activation of BID and caspase-9, as well as to activation of caspase-3 and caspase-8, suggesting the involvement of both intrinsic and extrinsic pathways in combination-treated cells. Indeed, the cell death which occurred in response to the equol and TRAIL combination, was significantly blocked by caspase-3, -8, and -9 specific inhibitors, and pan-caspase inhibitor. These results are consistent with previous reports for other TRAIL-sensitizing agents (35, 36).

Recently, Szliszka and Krol reported that soy isoflavones, including genistein, daidzein, and equol, sensitized TRAIL-resistant LNCaP prostate cancer cells to TRAIL-mediated apoptosis through disruption of the mitochondrial membrane potential (37). They have demonstrated that combined treatment with TRAIL and soy isoflavones did not alter the expression of DRs in LNCaP cells. Chimeric proteins also failed to block apoptosis induced by isoflavones/TRAIL, indicating that the synergistic apoptotic effects of isoflavones with TRAIL on LNCaP cells does not involve DRs. However, in our study, equol clearly induced a dose-dependent increase of DR4 and DR5 expressions at the protein level. Cell surface expression of DR4 and DR5 was also induced by equol treatment. Next, we found that the combination of equol and TRAIL induced activation of caspases and subsequent apoptosis through the up-regulation of DRs, as shown by our results that chimeric proteins efficiently abolished caspase activation, leading to the reverse of the synergistic induction of apoptosis by equol and TRAIL. In our results, the blocking of DR5 was more effective than that of DR4, as shown by the DR5-specific blocking chimera abrogating the sensitizing effects of equol on TRAIL-induced apoptosis, whereas the DR4-selective chimeric protein had a relatively weak influence on equol

and TRAIL combination-induced apoptosis. These results are consistent with those of previous reports for other chemopreventive natural products, such as zerumbone, gossypol, and β -phenylethyl isothiocyanate, which have also revealed the critical role of DRs in sensitizing malignant cells to TRAIL-mediated apoptosis (38-40).

Overall, our results showed that equol effectively potentiates TRAIL-mediated cell death through the up-regulation of DRs, leading to the subsequent activation of caspase-dependent apoptosis. The findings of this study suggest that the combined use of equol and TRAIL might be a useful strategy against cancer that is less sensitive to TRAIL treatment.

Acknowledgements

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