The dietary flavonoid apigenin sensitizes malignant tumor cells to tumor necrosis factor–related apoptosis-inducing ligand

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Abstract

Dietary flavonoid apigenin is expected to have preventive and therapeutic potential against malignant tumors. In this report, we show for the first time that apigenin markedly induces the expression of death receptor 5 (DR5) and synergistically acts with exogenous soluble recombinant human tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) to induce apoptosis in malignant tumor cells. TRAIL is a promising candidate for cancer therapeutics due to its ability to selectively induce apoptosis in cancer cells. The combined use of apigenin and TRAIL at suboptimal concentrations induces Bcl-2-interacting domain cleavage and the activation of caspases-8, -10, -9, and -3. Furthermore, human recombinant DR5/Fc chimera protein and caspase inhibitors dramatically inhibit apoptosis induced by the combination of apigenin and TRAIL. On the other hand, apigenin-mediated induction of DR5 expression is not observed in normal human peripheral blood mononuclear cells. Moreover, apigenin does not sensitize normal human peripheral blood mononuclear cells to TRAIL-induced apoptosis. These results suggest that this combined treatment with apigenin and TRAIL might be promising as a new therapy against malignant tumors. [Mol Cancer Ther 2006;5(4):945–51]

Introduction

Apigenin, one of the most common flavonoids, is widely distributed in many fruits and vegetables, including Chinese cabbage, bell pepper, garlic, celery, and guava (1). Apigenin is expected to be a promising cancer-preventive agent contained in foods. The topical application of apigenin in mice decreases the number and size of tumors in the skin induced by chemical carcinogens (2) or by UV exposure *in vivo* (3). Recently, apigenin was also shown to inhibit the motility and invasiveness of carcinoma cells *in vitro* (4, 5). Moreover, apigenin inhibits melanoma growth and metastatic potential *in vivo* by i.p. administration (6). Apigenin induces growth inhibition, cell cycle arrest, and apoptosis in various types of human malignant tumor cells including breast cancer (7), prostate cancer (8), colon cancer (9), and leukemic cells (10). We also previously reported that apigenin induces cell cycle arrest (11) and p21/WAF1 up-regulation in a tumor suppressor p53-independent manner (12). Moreover, although some flavonoids are mutagenic (13), apigenin has no mutagenic activity (14). Thus, apigenin is a promising and safe antitumor agent. However, the functions of apigenin have not been fully elucidated.

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) induces apoptosis in various types of malignant tumor cells through its interaction with the death domain–containing receptor, death receptor 5 (DR5), which is also called TRAIL-R2 (15–18). *In vitro* and *in vivo* data indicate that TRAIL is not cytotoxic toward a variety of normal cells (19–21). Therefore, TRAIL is one of the most promising candidates for cancer therapeutics. However, some tumor cells are resistant to TRAIL-induced apoptosis (22). It is therefore important to overcome this resistance for the clinical use of TRAIL in cancer therapy.

DR5 is a downstream gene of the p53 tumor suppressor gene (23, 24). Inactivation of DR5 significantly increases tumor growth *in vitro* and *in vivo* (25). Thus, the expression of DR5 may contribute to the tumor-selective induction of apoptosis mediated by TRAIL. Accordingly, DR5 is an attractive molecular target for cancer therapy.

In this study, we show for the first time that apigenin up-regulates DR5 expression and synergistically enhances TRAIL-induced apoptosis in various types of malignant tumor cells, but not in normal human peripheral blood mononuclear cells (PBMC). Our results show the novel effects of apigenin and raises the possibility that combined treatment with apigenin and TRAIL can be used as a promising strategy for the treatment of malignant tumors.

Materials and Methods

Reagents

Apigenin (Wako, Osaka, Japan) was dissolved in DMSO to a final concentration of 0.02% in media. Soluble recombinant human TRAIL/Apo2L was purchased from PeproTech (London, United Kingdom), and human recombinant DR5

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(TRAIL-R2)/Fc chimera protein and caspase inhibitors such as zVAD-fmk, zDEVD-fmk, zIETD-fmk, zLEHD-fmk, and zAEVD-fmk were purchased from R&D Systems (Minneapolis, MN). Cycloheximide was purchased from Nacalai Tesque (Kyoto, Japan).

Cell Culture

The human acute lymphoblastic leukemic cell line Jurkat, human prostate cancer cell line DU145, and human colon cancer cell line DLD-1 were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin, and incubated at 37°C in a humidified atmosphere of 5% CO_2. Normal human PBMCs were isolated as previously described (26). PBMCs were acquired from a healthy volunteer after obtaining informed consent.

Detection of Apoptosis

DNA fragmentation was quantified by the percentage of hypodiploid DNA (sub-G_1). In brief, Jurkat cells and PBMC were fixed with 70% ethanol and DLD-1 and DU145 cells were treated with 0.1% Triton X-100. Cells were then treated with 0.1 mg/mL RNase A (Sigma, St. Louis, MO) and the nuclei were stained with 25 μg/mL propidium iodide (Sigma). Measurement and analyses were carried out as previously described (26). For morphologic features, Jurkat cells were washed in PBS, fixed in methanol, incubated with 4′-diamidino-2-phenylindole solution for 30 minutes in the dark, and then analyzed using a fluorescent microscope (Olympus, Tokyo, Japan) at 420 nm.

Northern Blot Analysis and RNase Protection Assay

Total RNA from Jurkat cells was isolated using Sepasol-RNA I (Nacalai Tesque) according to the manufacturer's instructions. Full-length DR5 cDNA was used as a probe for Northern blot analysis. Northern blot analysis and RNase protection assays were carried out as previously described (26).

Luciferase Assay

Jurkat cells were seeded in six-well plates, and 1.0 μg of pDR5PF (pDR5/*Sac*I; ref. 27), or vacant vector plasmid was transfected by the DEAE dextran method using a CellPhect transfection kit (Amersham Pharmacia, Piscataway, NJ). After 24 hours of incubation, the cells were treated with or without apigenin, and 48 hours after the transfection, they were collected for luciferase assay. The luciferase assay was done as previously described (27). All luciferase assays were carried out in triplicate, and each assay was done at least thrice. The data was analyzed using Student's t test. Differences were considered to be statistically significant from the controls ($P < 0.05$).

Western Blot Analysis

Stepwise extraction of cytosolic fraction and organelle/membrane fractionation was done using a subcellular proteome extraction kit (Merck, Darmstadt, Germany) according to the manufacturer's instructions. Western blot analysis was carried out as previously described (26). Rabbit polyclonal anti-DR5 antibody (Cayman Chemical, Ann Arbor, MI), mouse monoclonal anti–Bcl-2-interacting domain (Bid), anti-caspases-8, -9, and -10 antibodies (MBL, Nagoya, Japan), rabbit monoclonal anti-caspase-3 antibody (Cell Signaling Technology, Beverly, MA), and mouse monoclonal anti-β-actin (Sigma) were used as primary antibodies. The relative band intensity was assessed using NIH Image software (NIH, Bethesda, MD).

Results

Apigenin Induces DR5 Expression in Jurkat Cells

To explore the novel functions of apigenin, we first examined the proteins that are regulated by apigenin. As shown in Fig. 1A, we found that apigenin induced DR5 expression in human acute lymphoblastic leukemic Jurkat cells in a dose-dependent manner. Time course studies showed that DR5 protein started to increase 12 hours after treatment with 40 μmol/L of apigenin (Fig. 1B). Furthermore, as shown in Fig. 1C, the up-regulation of DR5

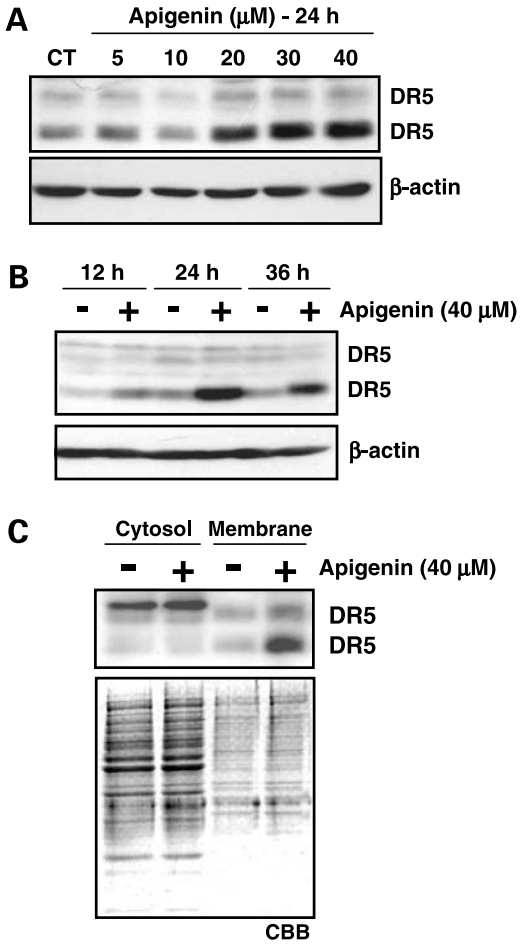

Figure 1. The effects of apigenin on DR5 proteins in Jurkat cells. **A**, Jurkat cells were treated with various concentrations of apigenin for 24 h. **B**, Jurkat cells treated with or without 40 μmol/L apigenin for the indicated times. **C**, Jurkat cells were treated with or without 40 μmol/L apigenin for 24 h and the cytosolic fraction (*left*) and membrane fraction (*right*) were extracted. Western blot analysis was carried out with anti-DR5 antibody. To examine comparable loading, the same blot was probed with anti-β-actin antibody or the gel was stained with Coomassie brilliant blue (*CBB*). Apigenin treatment (CT or −), DMSO only.

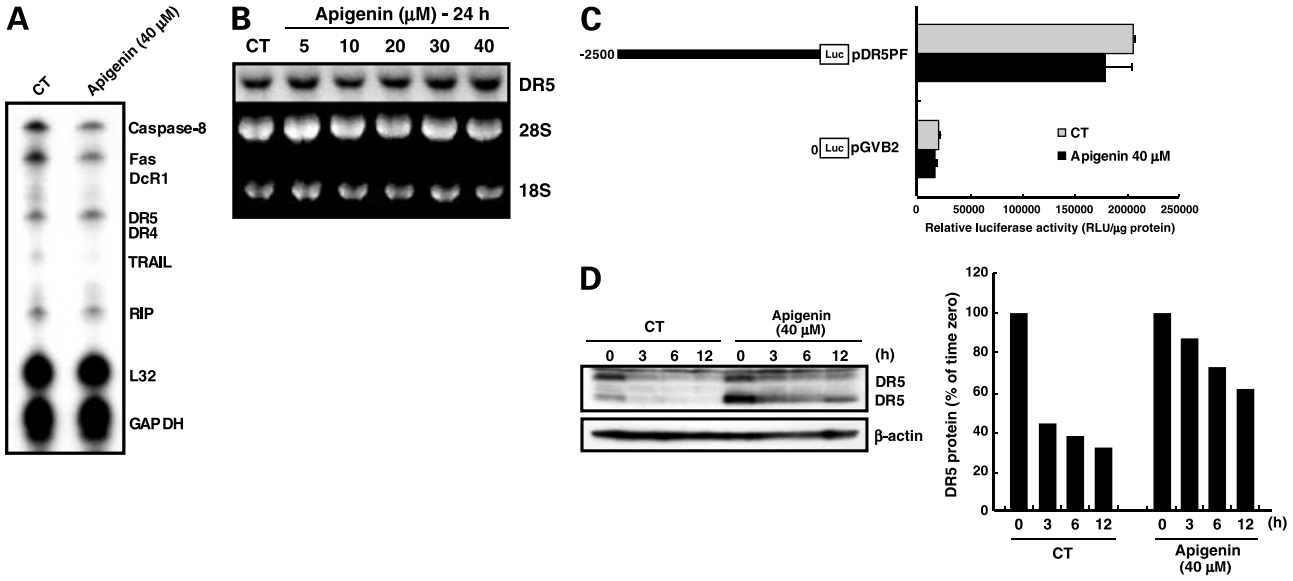

Figure 2. DR5 mRNA expression and DR5 promoter activity are not markedly altered but DR5 protein stability is increased by apigenin in Jurkat cells. **A,** RNase protection assay. Total RNA from Jurkat cells treated with or without 40 μmol/L apigenin for 24 h, was hybridized with probes, and then digested with RNase as described in Materials and Methods. The housekeeping genes GAPDH and ribosomal protein L32 are shown as controls. **B,** Northern blot analysis. Jurkat cells were treated with various concentrations of apigenin for 24 h. Ten micrograms of total RNA was probed with human DR5 cDNA. Ethidium bromide staining of 28S and 18S rRNA are shown as loading controls. **C,** the activation of DR5 promoter activity in Jurkat cells caused by apigenin. Jurkat cells were transiently transfected with a DR5 promoter-luciferase reporter plasmid (*pDR5PF*) or vacant vector (*pGVB2*) for luciferase assay. Relative luciferase activity, raw light units (*RLU*) in cell lysate per microgram of protein. *Columns,* mean; *bars,* ±SD (n = 3). **D,** apigenin treatment prevents degradation of DR5 protein. Jurkat cells were exposed to DMSO or apigenin for 12 h. At this point, 50 μmol/L cycloheximide was added and incubation was continued for an additional 3, 6, or 12 h. Western blot analysis was carried out with anti-DR5 antibody. To examine comparable loading, the same blot was probed with anti-β-actin antibody. The relative band intensity (*DR5/β-actin*) was assessed using NIH Image. *Columns,* results of densitometric analysis of total intensity from two bands of DR5 protein (*right*). CT, DMSO only.

expression by apigenin mainly occurred in the membrane fraction (*right*) and not in the cytosolic fraction (*left*).

DR5 mRNA Expression and DR5 Promoter Activity Are Not Markedly Altered, but DR5 Protein Stability Is Partially Increased by Apigenin in Jurkat Cells

We next investigated the effects of apigenin on the expression of death receptor–related genes by RNase protection assay. As shown in Fig. 2A, apigenin slightly up-regulated DR5 mRNA in Jurkat cells, TRAIL and receptor-interacting protein expressions were not markedly altered, and Fas and caspase-8 expressions decreased. Also, DR4 and DcR1 expression were not detected. To confirm the effect of apigenin on DR5 mRNA, we carried out a Northern blot analysis (Fig. 2B) and found that DR5 mRNA expression was slightly induced by apigenin treatment. We then examined the effect of apigenin on DR5 promoter activity using a DR5 promoter-luciferase reporter plasmid as previously described (27) with a transient luciferase

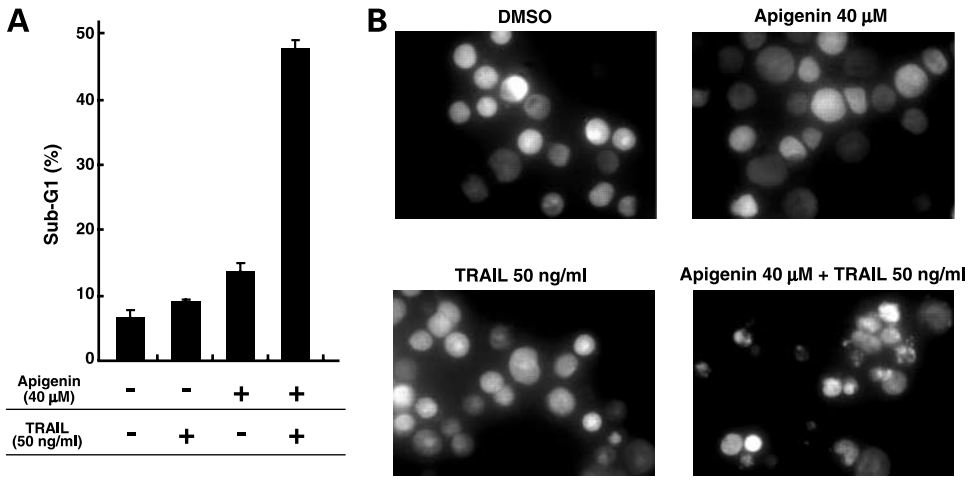

Figure 3. The combination of apigenin and TRAIL drastically induces apoptosis in Jurkat cells. **A,** sub-G_1 populations were detected using flow cytometry as described in Materials and Methods. Jurkat cells were treated with 40 μmol/L of apigenin for 24 h, followed by treatment with or without 50 ng/mL of TRAIL. *Columns,* mean; *bars,* ±SD (n = 3). Apigenin treatment (−), DMSO only. **B,** 4′-diamidino-2-phenylindole staining of Jurkat cells. Nuclear morphology was visualized using 4′-diamidino-2-phenylindole staining under a fluorescence microscope.

assay. As shown in Fig. 2C, apigenin did not increase DR5 promoter activity. These results suggested that apigenin induces DR5 expression at the protein level. To address the possibility that apigenin increased protein stability and thus contributed to the accumulation of DR5, we examined DR5 protein levels following the exposure of Jurkat cells to cycloheximide, which inhibits *de novo* synthesis of protein. As shown in Fig. 2D, in the absence of apigenin, DR5 protein levels decreased to 30% after 12 hours of cycloheximide treatment. On the other hand, in the presence of apigenin, DR5 protein levels decreased to only 60%; suggesting that apigenin treatment partially prevented the degradation of DR5 protein.

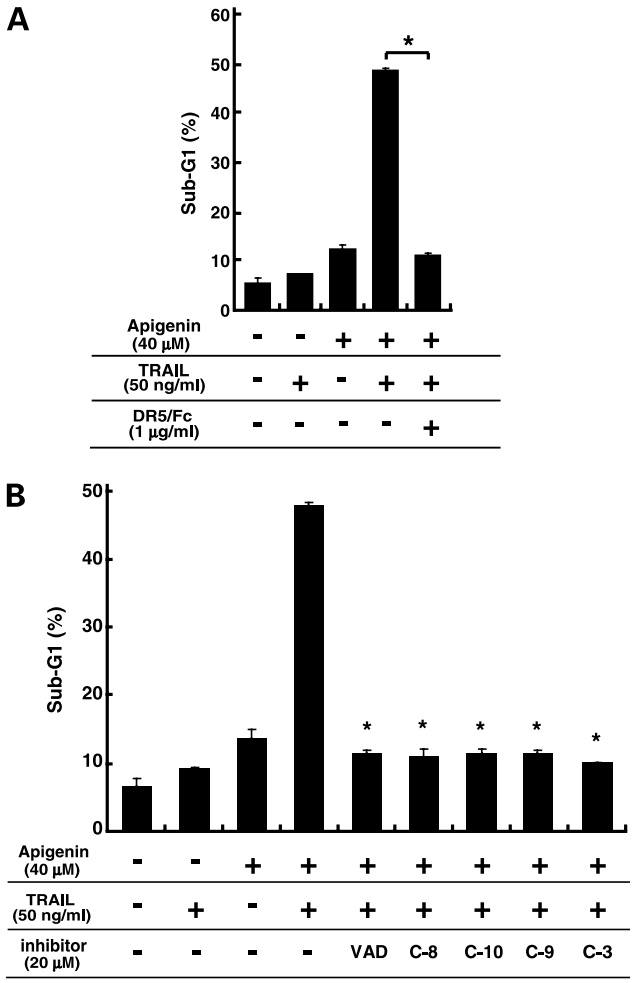

Figure 4. DR5/Fc chimera and caspase inhibitors block apoptosis enhanced by the combination of apigenin and TRAIL. **A,** Jurkat cells were treated with 40 μmol/L of apigenin for 24 h, followed by treatment with or without 50 ng/mL of TRAIL, and/or 1 μg/mL of DR5/Fc for another 12 h. **B,** Jurkat cells were treated with 40 μmol/L of apigenin for 24 h, followed by treatment with or without 50 ng/mL of TRAIL, and/or various inhibitors for another 12 h. *VAD,* treated with zVAD-fmk pan-caspase inhibitor; *C-8,* treated with zIETD-fmk caspase-8 inhibitor; *C-9,* treated with zLEHD-fmk caspase-9 inhibitor; *C-10,* treated with zAEVD-fmk caspase-10 inhibitor; *C-3,* treated with zDEVD-fmk caspase-3 inhibitor. All caspase inhibitors were applied at 20 μmol/L. Sub-G_1 populations were detected using flow cytometry as described in Materials and Methods. *Columns,* mean; *bars,* ±SD (n = 3); *, $P < 0.01$. Apigenin treatment (−), DMSO only.

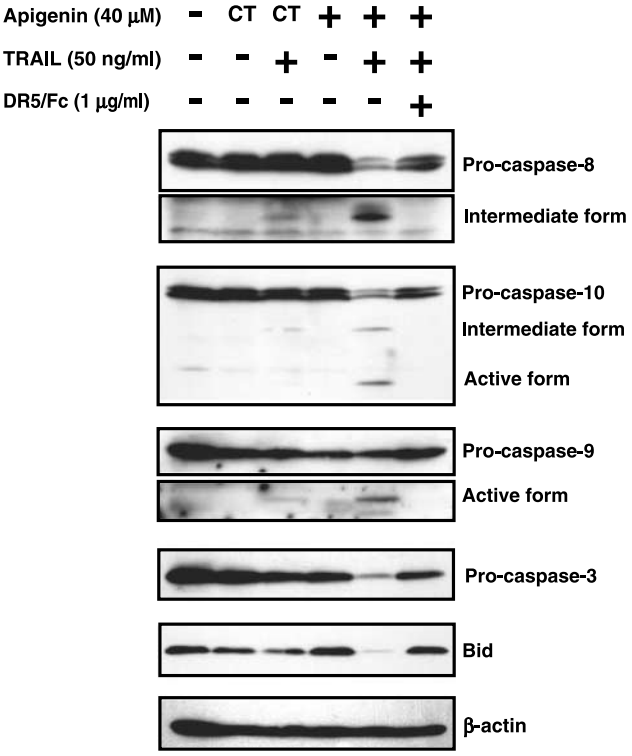

Figure 5. The effects of the combination of apigenin and TRAIL on caspases and Bid in Jurkat cells. Jurkat cells were treated with 40 μmol/L of apigenin for 24 h, followed by treatment with or without 50 ng/mL of TRAIL, and/or 1 μg/mL of DR5/Fc for another 12 h. Western blot analysis was carried out with anti-pro-caspases-3, -8, -9, and -10 antibodies and anti-Bid antibodies. Decreases in the levels of pro-caspases and Bid indicate their activation. To examine comparable loading, the same blot was probed with anti-β-actin antibody. *CT*, DMSO only; (−), no treatment.

Apigenin Sensitizes TRAIL-Induced Apoptosis in Jurkat Cells in a Synergistic Fashion

We hypothesized that apigenin enhances exogenous TRAIL-induced apoptosis due to its ability to increase DR5 expression. TRAIL or apigenin alone weakly induced apoptosis (Fig. 3A). However, interestingly, concurrent treatment with apigenin and TRAIL drastically induced apoptosis. Figure 3B shows the morphologic features of Jurkat cells treated with TRAIL and apigenin. Jurkat cells exhibited the characteristic features of apoptosis including chromatin condensation and nuclear fragmentation by combined treatment of apigenin with TRAIL. These results indicate that apigenin strongly enhanced exogenous recombinant TRAIL-induced apoptosis in a synergistic fashion.

DR5/Fc Chimera Protein and Caspase Inhibitors Block Apoptosis Enhanced by the Combination of Apigenin and TRAIL

To confirm that the induction of apoptosis by the combination of apigenin and TRAIL is mediated through DR5, we used DR5/Fc chimera protein, which has a

dominant-negative function against DR5. The protein efficiently blocked apoptosis caused by the combined treatment of apigenin with TRAIL (Fig. 4A). We also showed that the pan-caspase inhibitor zVAD-fmk and caspases-8, -10, -9, and -3 inhibitors efficiently blocked apoptosis induced by combined treatment of apigenin with TRAIL (Fig. 4B). These results indicate that the apigenin-mediated sensitization to TRAIL occurred in a caspase-dependent manner and are consistent with previous reports on the role of caspases in TRAIL-mediated apoptosis (28). Furthermore, we did Western blot analysis of caspases-8, -10, -9, and -3, and Bid when both apigenin and TRAIL or each alone were given at a suboptimal concentration (Fig. 5). Bid is a substrate of caspase-8 and is cleaved during the activation of TRAIL signaling (29). Combined treatment with apigenin and TRAIL clearly induced the activation of caspases and Bid. Moreover, DR5/Fc chimera protein effectively blocked the activations of Bid and caspases induced by cotreatment with TRAIL and apigenin.

Apigenin Induces DR5 Expression and Sensitizes TRAIL-Induced Apoptosis in Other Human Malignant Tumor Cells in a Synergistic Fashion

We next investigated whether apigenin induces DR5 protein and strongly enhances TRAIL-induced apoptosis in a synergistic fashion in other human malignant tumor cells, using human prostate cancer DU145 cells and colon cancer DLD-1 cells. Apigenin induced DR5 expression in a dose-dependent manner in both cell lines (Fig. 6). Next, we tested this using combined treatment with apigenin and TRAIL, and found that it induced apoptosis more strongly than treatment with TRAIL or apigenin alone. These results indicate that apigenin strongly enhanced exogenous recombinant TRAIL-induced apoptosis in a synergistic fashion not only in Jurkat cells but also in other human malignant tumor cells.

Combined Treatment with Apigenin and TRAIL Does Not Induce Apoptosis in Normal Human PBMCs

Because we found that the combination of apigenin and TRAIL strongly induced apoptosis in malignant tumor cells, we next examined the effect of the treatment on normal human cells. The side effects of chemotherapeutic agents on hematopoietic cells are a major problem during clinical treatment. Thus, we used normal human PBMC as a model. TRAIL or apigenin did not markedly induce apoptosis in Jurkat cells and PBMC (Fig. 7A). Interestingly, the combination of apigenin and TRAIL did not induce apoptosis in normal human PBMC, although it markedly induced apoptosis in Jurkat cells. Furthermore, in normal human PBMC, the level of DR5 expression was significantly lower than in Jurkat cells, and apigenin did not induce DR5 protein expression (Fig. 7B).

Discussion

Apigenin has been suggested as a promising anticancer agent (1–14). In the present study, we found a new function of apigenin, which might be useful for cancer therapy with low side effects. At first, we found that apigenin partially prevented the degradation of DR5 protein (Fig. 2D), and significantly increased DR5 protein in the membrane fraction (Fig. 1), whereas apigenin very slightly increased DR5 mRNA and did not enhance promoter activity (Fig. 2A-C). These results suggest that protein stabilization is at least one of the mechanisms of DR5 induction by apigenin. Recent studies have shown that proteasome inhibitor PS-341 increases DR5 protein levels (30). In addition, it was reported by Chen et al. that apigenin acts as a proteasome inhibitor in Jurkat cells (31). Moreover, in the present study, we also confirmed that apigenin inhibits proteasome activity using Jurkat cell lysate (data not shown). Therefore, apigenin may increase DR5 protein by the inhibition of proteasome. On the other hand, apigenin is also a casein kinase II inhibitor, but casein kinase II inhibitors did not increase DR5 protein expression (32). Thus, induction of DR5 by apigenin might not be related to the function of casein kinase II inhibition.

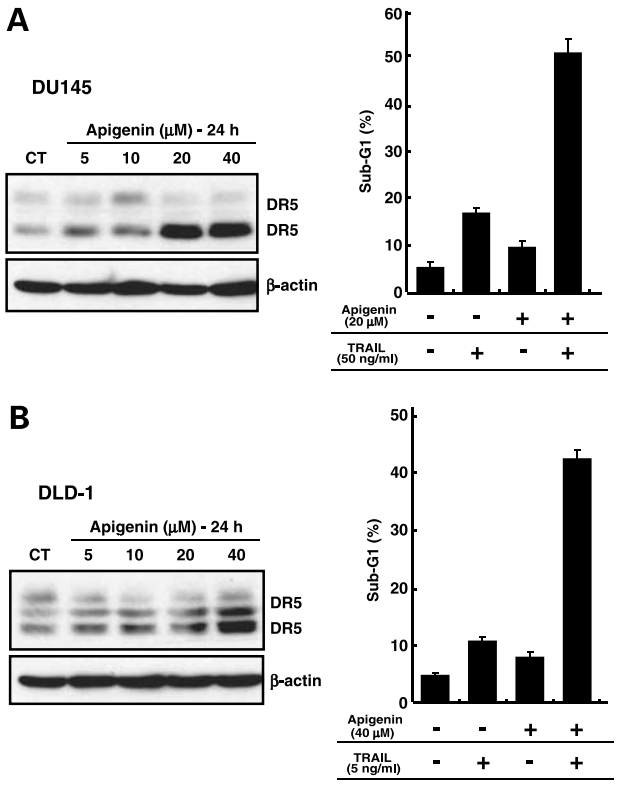

Figure 6. Apigenin also induces DR5 expression and synergistically sensitizes TRAIL-induced apoptosis in other human malignant tumor cells, DU145 and DLD-1. DU145 (**A**) and DLD-1 (**B**) cells were treated with various concentrations of apigenin for 24 h, and then total cell extracts were analyzed by Western blot analysis with anti-DR5 antibody. To examine comparable loading, the same blot was probed with anti-β-actin antibody. Sub-G_1 populations were detected by flow cytometry as described in Materials and Methods. DU145 cells were treated with 20 μmol/L apigenin for 24 h, followed by treatment with or without 50 ng/mL TRAIL and DLD-1 cells were treated with 40 μmol/L apigenin for 24 h, followed by treatment with or without 5 ng/mL TRAIL. *Columns,* mean of triplicate experiments; *bars*, ±SD (n = 3); *, $P < 0.05$. Apigenin treatment (CT or −), DMSO only.

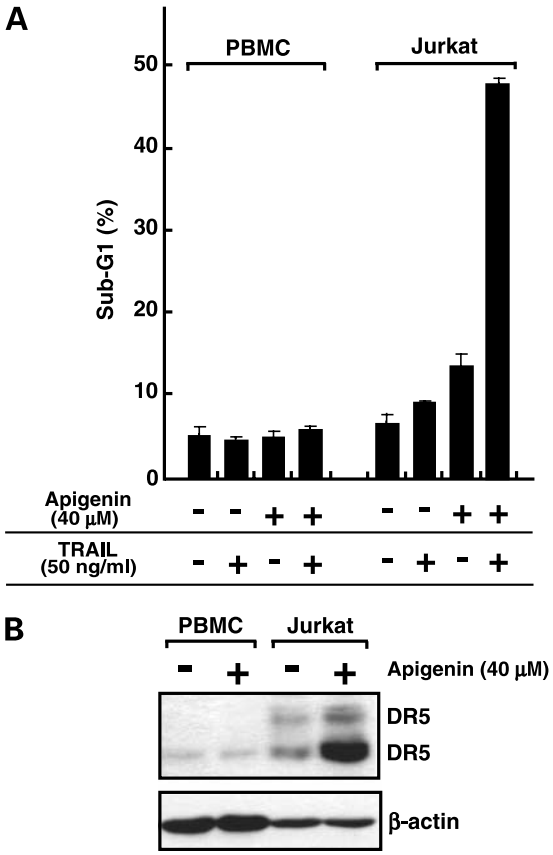

Figure 7. Up-regulation of DR5 expression and apoptosis induced by the combination of apigenin and TRAIL were not observed in normal human PBMC. **A,** apigenin did not sensitize TRAIL-induced apoptosis in normal human PBMC. Jurkat cells and normal human PBMCs were treated with 40 μmol/L of apigenin for 24 h, followed by treatment with or without 50 ng/mL of TRAIL. Sub-G_1 populations were then detected using flow cytometry as described in Materials and Methods. *Columns,* mean; *bars,* ±SD (n = 3). **B,** apigenin did not enhance DR5 protein expression in normal human PBMC. Jurkat cells and normal human PBMCs were treated with 40 μmol/L apigenin for 24 h. Western blot analysis was then carried out with anti-DR5 antibody. To examine comparable loading, the same blot was probed with anti-β-actin antibody. Apigenin treatment (−), DMSO only.

Next, we found that apigenin synergistically sensitizes TRAIL-induced apoptosis associated with the up-regulation of DR5 (Fig. 3). Apigenin lowered the threshold required for TRAIL-induced apoptosis in Jurkat cells. This suggests that apigenin can reduce the minimal effective dose and side effects of TRAIL. We also recently found a similar model of apoptosis induced by the combination of histone deacetylase inhibitors and TRAIL (26).

p53 is an important tumor-suppressor gene and is mutated in more than half of all malignancies (33). Jurkat, DU145, and DLD-1 cells, which we used in the present study, have also inactivated p53 through point mutations. Moreover, we observed the increased expression of DR5 and augmentation of TRAIL-induced apoptosis by apigenin in Jurkat, DU145, and DLD-1 (Figs. 1, 3, and 6). On the other hand, mutations in the *DR5* gene are rare (34–36). Taken together, these observations raise the possibility that the combined treatment of apigenin and TRAIL is effective against various types of malignancies associated with p53 mutations.

In cancer therapies, it is important to specifically induce apoptosis in malignant tumor cells but not in normal cells. In this regard, TRAIL is an attractive candidate for cancer therapy due to its ability to selectively induce apoptosis in cancer cells (19–21). Moreover, apigenin did not induce DR5 protein expression and enhanced TRAIL-induced apoptosis in normal human PBMCs (Fig. 7). These findings suggest that the combined administration of apigenin and TRAIL may be a useful strategy for cancer therapy. These strategies are based on the expression of death receptors on cancer cells. Overexpression of DR5 in TRAIL-resistant cancer cells restored TRAIL sensitivity (37–39). DR5 expression on a number of Jurkat subclones is highly correlated with sensitivity to TRAIL (40). Therefore, apigenin is an attractive agent for inducing DR5 expression and sensitizing tumor cells to TRAIL.

In summary, we showed that apigenin induces DR5 expression through p53-independent regulation and that apigenin synergistically augments TRAIL-induced apoptosis in human malignant tumor cells. Together, these results raise the possibility that the combined treatment of apigenin and TRAIL is a promising new cancer therapy even in tumor cells harboring inactivated p53.

Acknowledgments

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