Sub-Toxic Dose of Apigenin Sensitizes HepG2 Cells to TRAIL through ERK-Dependent Up-Regulation of TRAIL Receptor DR5

Eun Young Kim, Ji Sun Yu, Mihi Yang, and An Keun Kim*

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is regarded as a promising candidate for anticancer therapy due to its selective toxicity to cancer cells. Nevertheless, because of TRAIL resistance in some cancer cells, combined treatment with sensitizing agents is required to enhance the anticancer potential of TRAIL. In this study, we investigated the underlying mechanism of apigenin-induced sensitization of HepG2 cells to TRAIL-induced cell death. Synergistic induction of apoptosis by combination was confirmed by examining the typical morphology changes of apoptosis, PARP-cleavage, and activation of effector caspases. Z-VAD-fmk, a pan-caspase inhibitor, inhibited the enhanced cell death by combined treatment of apigenin and TRAIL, demonstrating that a caspase-dependent pathway is involved in apigenin/TRAIL-mediated apoptosis. In addition, we found that apigenin/TRAIL co-treatment up-regulates DR5 cell surface expression. The synergistic induction of cell death by the apigenin/TRAIL combination was significantly attenuated by DR5 blocking chimera antibody. Next, using pharmacological inhibitors, we found that ERK activation is involved in the induction of DR5 expression. Inhibition of ERK1/2 by U0126 significantly decreased the apigenin/TRAIL-induced DR5 expression and apoptosis. Taken together, our results indicate that apigenin can enhance the apoptotic effect of TRAIL *via* ERK-induced up-regulation of DR5.

INTRODUCTION

Apoptosis is a tightly regulated process of the self-destruction of cells *via* a complex signaling cascade. Failure of apoptosis regulation is considered as a major feature of many cancers (Kasibhatla and Tseng, 2003). So, cancer therapy such as radiation and chemotherapy are mainly intended to induce apoptosis (Rupnow and Knox, 1999; Russo et al., 2006). However, these methods kill normal cells as well as cancer cells, which is the cause of many of the severe side effects associated with these treatments (Cuzick et al., 1994; Redding, 2005). Therefore, the development of a more effective and selective

strategy for cancer management is required.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a member of the TNF family, is a type II transmembrane protein that shows homology to other TNF family members. TRAIL binds to the death receptors DR4 and DR5, and triggers the apoptosis signaling pathway by recruiting Fas-associated death domain (FADD), and subsequently activating caspase-8. Caspase-8 activates executioner caspases such as caspase-3, -6, and -7, leading to cleavage of numerous intracellular proteins. Unlike FasL and TNF- α , TRAIL selectively induces cell death in malignant cells but not in normal cells (Kim and Seol, 2003; Walczak and Krammer, 2000). Accordingly, TRAIL has been considered as a safe and effective anti-cancer agent. However, recent studies have demonstrated that some cancer cells, including hepatoma cells, are resistant to TRAIL (He et al., 2005; Malhi and Gores, 2006). It has been reported that resistance to TRAIL can occur at different levels in the TRAIL-mediated signaling pathway. For example, defects of death receptors, overexpression of survival proteins, and a reduction in the levels of proapoptotic proteins can lead to TRAIL resistance (Zhang and Fang, 2004). These data suggest that potential strategies to overcoming this resistance are required for treating TRAIL-resistant cancer cells. Current trials are focusing on overcoming TRAIL-resistance by combining TRAIL with other agents such as chemotherapeutic drugs or natural products. Combined therapy should prove to be an inherently effective strategy, because any given resistance mechanisms can be affected by combination (Jalving et al., 2005; Kruyt, 2008).

In this present study, we evaluated the sensitizing effect of apigenin on TRAIL-resistant HepG2 cells, and demonstrated the underlying molecular mechanisms of sensitization.

MATERIALS AND METHODS

Materials

Apigenin was purchased from Sigma-Aldrich (USA) and dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 0.01%. Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate buffered saline (DPBS), fetal bovine serum (FBS), trypsin-EDTA and penicillin/streptomycin were purchased

College of Pharmacy, Sookmyung Women's University, Seoul 140-742, Korea
*Correspondence: akkim@sookmyung.ac.kr

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sed from Welgene (Korea). Soluble recombinant human TRAIL Apo2L was purchased from Peprotech (USA). Pan-caspase inhibitor z-VAD-fmk, human recombinant DR4/Fc and DR5/Fc chimera protein were obtained from R&D Systems (USA). N-acetylcysteine (NAC), caspase-3/7 substrate, and DMSO were purchased from Sigma-Aldrich (USA). Caspase-6 substrate was purchased from Santa Cruz Biotechnology, Inc. (USA). All the antibodies for Western blotting and MAPK inhibitors were purchased from Cell Signaling (USA).

Cell culture

Human hepatocellular carcinoma HepG2 cell line was obtained from the Korean Cell Line Bank (Korea) and maintained in DMEM with 10% FBS and penicillin/streptomycin. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂.

Cell viability analysis

Cell viability was assessed by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Briefly, cells were seeded in 96-well plate and then were allowed to attach overnight. Then, cells were treated with various concentrations of chemicals for 24 h in humidified 5% CO₂ atmosphere. Following incubation, MTT solution was added to each well and cells were incubated for 4 h at 37°C. Then, MTT solution was removed and the resulting formazan crystals were dissolved in DMSO. The absorbance of each well was measured at 570 nm by a microplate reader (EL800, Bio-Tek Instrument Inc., USA) and the cell viability (%) was calculated.

Observation of apoptotic morphological change

Cells were seeded in a 6-well plate and incubated for 24 h, then treated with apigenin and/or TRAIL for 24 h. HepG2 cell morphology was observed and photographed with convert light microscopy (Olympus BH Series, Japan).

Analysis of apoptosis

Apoptotic cell death was quantified by flow cytometry using annexin V and PI double staining kit from BD biosciences (USA) according to the manufacturer's protocol. Cells were incubated with apigenin and/or TRAIL for 24 h and harvested and resuspended in 1× binding buffer and stained with annexin V and PI for 15 min at room temperature. Apoptotic cell distribution was evaluated by FACSCanto II Flow Cytometer (BD Biosciences, USA). Cells that were annexin V⁺/PI⁻ were considered as apoptotic cells.

In vitro caspase activity assay

Enzymatic activities of caspase-3/7 and caspase-6 were determined by colorimetric assay. Cells were lysed in the lysis buffer (Millipore, USA) and the supernatants were collected. Reaction buffer (Millipore, USA) and colorimetric caspase-specific substrates (Ac-DEVD-pNA for caspase-3/7 and Ac-VEID-pNA for caspase-6) were added to each sample and incubated at 37°C for 2 h in the dark. Caspase activity was determined by measuring the relative absorbance at 405 nm using the microplate reader.

Analysis of cell surface expression of DR4 and DR5

Cells were analyzed for the surface expression of DR4 and DR5 by indirect staining with primary mouse anti-DR4 and DR5, followed by phycoerythrin (PE)-conjugated goat anti-mouse IgG1 (Santa Cruz Biotechnology, Inc., USA). As a negative control, a normal mouse IgG1 antibody (Santa Cruz Biotechnology, Inc., USA) was used as isotype nonbinding antibody. After

staining with primary antibodies, cells were washed twice and reacted with PE-conjugated secondary antibody at 4°C in the dark. Cells were washed twice with PBS and the cell surface expression of DR4 and DR5 was analyzed by FACSCanto II Flow Cytometer.

Western blotting

After treatment, cell were lysed with RIPA buffer (50 mM Tris-HCL, pH 8.0, with 150 mM NaCl, 0.1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing protease inhibitor (Roche). Lysates were centrifuged at 17,000 rpm for 20 min. Protein contents was measured with Bio-Rad protein assay kit. The equal amount of protein was loaded on 10% SDS-PAGE gels and transferred onto poly-vinylidene difluoride (PVDF) membranes. After blocking with 5% nonfat dry milk in TBS-T (25 mM Tris, 137 mM NaCl and 0.1% Tween 20) for 2 h at room temperature, the blots were incubated overnight at 4°C with primary antibodies. After washing with TBS-T, the membrane was incubated with secondary horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies. Finally, the protein signals were visualized by enhanced chemiluminescence (ECL) advance detection kit. Multi Gauge software (Fuji Photo Film, Japan) was used to quantify the protein signals.

Statistical analysis

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

RESULTS

Apigenin sensitizes HepG2 cells to TRAIL-induced apoptosis

We first evaluated the effect of apigenin on HepG2 cell viability, whereupon treatment of HepG2 cells with apigenin was found to reduce cell viability in a dose-dependent manner (Fig. 1A). In order to examine the sensitizing effect of apigenin on TRAIL-induced cytotoxicity, cells were treated with apigenin in the range of 1 to 12 µg/ml, as this range of apigenin, when used as the sole treatment, had induced only limited cell growth inhibition (< 30%) over 24 h. As shown in Fig. 1B, TRAIL (5 ng/ml) alone did not show significant cytotoxic effect. However, co-treatment of TRAIL with apigenin resulted in a marked reduction of cell viability in an apigenin dose-dependent manner (Fig. 1B). We then investigated whether the enhanced cytotoxicity of the apigenin/TRAIL combination is mediated through the induction of apoptosis by using flow cytometry, which detects the percentage of early apoptotic cells (annexin V⁺/PI⁻ cells). Since the externalization of phosphatidylserine (PS) on the cell surface is a typical feature of early apoptosis, increased binding of annexin V-FITC to PS indicates the cells which are undergoing apoptosis (van Engeland et al., 1998). Combined treatment with both TRAIL (5 ng/ml) and apigenin (6 µg/ml) for 24 h resulted in a dramatic increase of annexin V⁺/PI⁻ cells, while treatment with TRAIL or apigenin alone did not, indicating that combined treatment with apigenin and TRAIL effectively induces apoptosis in HepG2 cells (Fig. 1C). Next, in order to confirm the enhanced apoptosis by the combination of apigenin with TRAIL, we monitored for morphological changes under light microscope. As shown in Fig. 1D, treatment of HepG2 cells with TRAIL or apigenin alone did not induce any changes of cell morphology when compared with the control group. In contrast, the combined treatment markedly induced apoptosis-associ-

Fig. 1. Apigenin sensitizes HepG2 cells to TRAIL. (A) Effect of apigenin on cell viability. Cells were treated with various concentrations of apigenin (2–30 µg/ml) for 24 h and cell viability was measured by MTT assay. Statistical significance: * $p < 0.001$. (B) Effects of apigenin and/or TRAIL on HepG2 cell viability. Cells were treated with various concentrations of apigenin (1–12 µg/ml) for 24 h in the presence or absence of 5 ng/ml TRAIL. Cell viability was evaluated with MTT assay. Statistical significance: * $p < 0.05$, ** $p < 0.001$ versus control TRAIL-untreated cells. (C) Effect of apigenin on TRAIL-induced apoptosis. Cells were treated with apigenin (6 µg/ml) and/or TRAIL (5 ng/ml) for 24 h and apoptosis was measured by flow cytometric analysis after double-staining with annexin V-FITC/PI. Representative result was shown in upper panel. Lower panel is the bar graph of early apoptotic cells (%). The value represents mean $\pm$ SD ($n = 3$). Statistical significance: * $p < 0.001$ versus apigenin/TRAIL co-treated group. (D) Morphological change by apigenin/TRAIL combination. Cells were treated with apigenin (6 µg/ml) and/or TRAIL (5 ng/ml) for 24 h and cell morphology was investigated by inverted microscope.

Fig. 2. Caspase is involved in the combination treatment-induced apoptosis in HepG2 cells. (A) Cells were pretreated with 20 μ M pan-caspase inhibitor (z-VAD-fmk) for 30 min prior to the combined treatment of apigenin (6 μ g/ml) and TRAIL (5 ng/ml) for 24 h. Cell viability was evaluated by MTT assay. Data are presented as mean $\pm$ SD (n = 3). Statistical significance: * p < 0.001. (B) The effects of combined treatment on activity of executioner caspases were determined by colorimetric assay. Cells were treated with apigenin (6 μ g/ml) and/or TRAIL (5 ng/ml) for 24 h before measuring caspase activities. Results are presented as bar graphs from three independent experiments. Statistical significance: * p < 0.001. (C) The effects of combined treatment on effector caspases and PARP cleavage were examined by Western blot analysis. Cells were treated with apigenin (6 μ g/ml) and/or TRAIL (5 ng/ml) for 24 h and cell lysates were separated by SDS-PAGE. The β -actin was used as loading control.

ted morphological changes such as cellular shrinkage, nucleus condensation, and the formation of apoptotic bodies, further suggesting that apigenin sensitizes HepG2 cells to TRAIL-induced apoptosis (Fig. 1D).

Apigenin/TRAIL-induced apoptosis is involved in activation of effector caspases

Caspases have been reported to play a central role in the apoptotic signaling pathway (Cohen, 1997; Fan et al., 2005). Therefore, to estimate whether apigenin promotes TRAIL-mediated cell death by activating caspases, we examined the effect of z-VAD-fmk, a broad spectrum caspase inhibitor, on apigenin plus

TRAIL-induced cell death. As shown in Fig. 2A, z-VAD-fmk significantly blocked the cell death induced by the combined treatment, indicating that apigenin enhances TRAIL-induced cell death via a caspase-dependent pathway. Members of the apoptotic caspases are generally divided into initiator and effector caspases, according to their structural and functional differences (Kumar, 2007). It has been reported that morphological changes during apoptosis are mainly occurred due to the activation of effector caspases such as caspase-3, caspase-7 and caspase-6 (Häcker, 2000). As typical apoptotic morphological alterations were observed in combined apigenin/TRAIL treated HepG2 cells, we further investigated whether effector caspases

Fig. 3. Up-regulation of DR5 by apigenin is essential for apigenin/TRAIL-induced cytotoxicity. (A, B) Cells were incubated with apigenin (6 µg/ml) and/or TRAIL (5 ng/ml) for 24 h and the surface expression of (A) DR4 and (B) DR5 was analyzed by flow cytometry. X axis, fluorescence intensity; Y axis, relative number of cells. Representative data is shown. (C) DR5/Fc chimera protein blocked the enhanced cell death by apigenin/TRAIL combination. Cells were treated with 6 µg/ml apigenin, 5 ng/ml TRAIL and/or DR4/Fc or DR5/Fc chimera for 24 h. Cell viability was measured by MTT assay. The bar represents the mean $\pm$ SD (n = 3). Statistical significance: * p < 0.001.

Fig. 4. Up-regulation of DR5 is mediated through ERK activation. (A) Cells were pretreated with indicated concentrations of NAC for 1 h and treated with apigenin (6 μg/ml) plus TRAIL (5 ng/ml) for 24 h, and whole cell extracts were analyzed by Western blotting. Protein band in-intensity was measured by image analyzer and presented as a bar graph. The bar represents the mean ± SD (n = 3). Statistical significance: **p* < 0.001 compared to control group. (B, C) Time-course effects of apigenin/TRAIL on DR5 and ERK. Cells were treated with apigenin (6 μg/ml) and TRAIL (5 ng/ml) for 0.5 h, 1 h, 4 h, 8 h, 16 h, and 24 h and cell extracts were prepared for Western blotting for (B) DR5/β-actin and (C) p-ERK/ERK. (D, E) Cells were pretreated with the indicated concentrations of U0126 for 30 min and treated with apigenin (6 μg/ml) and TRAIL (5 ng/ml) for another 24 h. Then cell lysates were evaluated by Western blot analysis using (D) anti-DR5 antibody and (E) anti-PARP antibody. Protein band intensity was measured by image analyzer and presented as bar graphs. The bar represents the mean ± SD (n = 3). Statistical significance: **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

are activated by the combined treatment of apigenin and TRAIL. As shown in Fig. 2B, treatment with apigenin or TRAIL alone did not induce any significant activation of effector caspases. However, combined treatment with apigenin and TRAIL led to a marked increase of caspase enzymatic activity. Also, enhanced activation of effector caspases in apigenin/TRAIL-treated cells was further confirmed by Western blotting. Consistent with the results of a colorimetric caspase activity assay, combined treatment with apigenin and TRAIL significantly induced the cleavage of effector caspases (Fig. 2C). We also investigated the effect of apigenin and TRAIL on PARP cleavage, since poly (ADP-ribose) polymerase (PARP) is one of the major target substrate of effector caspases and PARP cleavage is considered as a hallmark of apoptosis (Yang et al., 2004). As shown in Fig. 2C, apigenin and TRAIL combined treatment induced a significant increase of PARP cleavage, while TRAIL or apigenin alone did not.

Combined treatment of apigenin with TRAIL enhances HepG2 cell death by DR5 up-regulation

It is well known that TRAIL triggers apoptosis on binding to two types of death receptors, DR4 (TRAIL-R1) and DR5 (TRAIL-R2), on the cell surface (Ashkenazi, 2002). Therefore, we examined whether the combined treatment modulates cell surface expression of DR4 and/or DR5. Flow cytometric analysis showed that the surface expression of both DR4 and DR5 were increased in apigenin/TRAIL-treated cells (Figs. 3A and 3B). To elucidate the functional role of DR4 or DR5 in the effects of apigenin/TRAIL combination, we next examined the effect of DR4 or DR5-specific chimera antibody on apigenin/TRAIL-induced cell death. We found that DR5-specific blocking antibody significantly attenuates cell death by co-treatment with apigenin and TRAIL. However, DR4-specific chimera antibody did not affect the apigenin/TRAIL-induced cell death (Fig. 3C). These results indicate that enhanced cell death by combination of apigenin and TRAIL is mediated through DR5.

Apigenin/TRAIL-induced up-regulation of DR5 is independent of ROS generation

Next, we attempted to determine the upstream regulator of apigenin/TRAIL increased DR5 expression. Reactive oxygen species (ROS) mediate various signal transduction pathways leading to cell growth, differentiation, or cell death (Simon et al., 2000). In the previous study by Choi et al. (2007) ROS generation has been proposed to play an essential role in apigenin-induced apoptosis in HepG2 cells. Therefore, we hypothesized that ROS could contribute to DR5 up-regulation in apigenin-treated HepG2 cells. Accordingly, we examined the role of ROS in DR5 regulation by using NAC, the ROS scavenger. Cells were pretreated with different concentrations of NAC before apigenin/TRAIL treatment, and activation of DR5 was detected by Western blot analysis. Through this method we determined that pretreatment with NAC failed to block apigenin/TRAIL-induced DR5 up-regulation, indicating that ROS is not involved in the combination-mediated DR5 regulation in HepG2 cells (Fig. 4A).

ERK activation is the upstream signal in apigenin/TRAIL-induced up-regulation of DR5

Since it has been reported that members of the mitogen-activated protein kinases (MAPK) family can mediate the TRAIL-induced apoptotic pathway *via* DR5-regulation, we examined whether these signaling pathways are involved in apigenin/TRAIL-induced DR5 up-regulation. To do so, we monitored the

expression of DR5 and MAPKs at various time points by Western blot analysis. As shown in Fig. 4B, DR5 was increased within 4 h of apigenin/TRAIL treatment. Also, ERK1/2, JNK1/2, and p38 were markedly activated by combined treatment (Fig. 4C, Supplementary Figs. S1A and S2A). Next, to further investigate the role of MAPKs in apigenin/TRAIL-induced DR5 expression, cells were pre-incubated with various concentrations of respective inhibitors for 30 min before co-treatment of apigenin/TRAIL and DR5 expression was analyzed by Western blotting. We found that inhibition of MEK, the upstream kinase of ERK1/2, by U0126 significantly attenuated apigenin/TRAIL-induced DR5 up-regulation in a dose-dependent manner (Fig. 4D). Also, pre-treatment with U0126 significantly abolished apigenin/TRAIL-induced PARP cleavage in a dose-dependent manner (Fig. 4E). On the other hand, neither the presence of JNK inhibitor SP600125 nor p38 inhibitor SB203580 had a significant effect on apigenin/TRAIL-induced DR5 up-regulation (Supplementary Figs. S1B and S2B, respectively).

Overall, these results indicate that ERK (but not JNK and p38) plays an important role in the sensitization of HepG2 cells to TRAIL-induced apoptosis through DR5 up-regulation.

DISCUSSION

Apigenin is a flavonoid widely distributed in various fruits and vegetables. It has been well documented that apigenin induces cell growth inhibition, apoptosis, and cell cycle arrest in multiple malignant cells without mutagenic activity (Czeczot et al., 1990; Shukla and Gupta, 2006; Xu et al., 2008). Moreover, the anticancer property of apigenin is selective, as it has been previously reported that apigenin does not induce cytotoxic effects toward normal cells (Gupta et al., 2001). Thus, apigenin could be developed as a safe and promising anticancer agent. A recent study has demonstrated that apigenin sensitizes human malignant cells to paclitaxel-induced apoptosis, suggesting that the combination of apigenin with other anticancer agents would be an effective strategy for cancer therapy (Xu et al., 2011). In our present study, we examined the synergistic anticancer effect of apigenin in combination with TRAIL in human hepatocellular carcinoma (HCC) HepG2 cell line. HCC cells have been reported to be resistant to TRAIL, but several anticancer agents have been shown to enhance their susceptibility to TRAIL-induced apoptosis (Lu et al., 2010; Shigeno et al., 2003).

In this study, we found that apigenin effectively sensitizes HepG2 cells to TRAIL-mediated cytotoxicity by inducing apoptosis. During the apoptotic process, caspases play a critical role in the induction of cell death in response to various apoptotic signals (Fan et al., 2005). Caspases are present in the cells in zymogen, the inactive pro-caspase form, and are activated sequentially through proteolytic cleavage. Activation of executioner caspases and the following cleavage of target proteins such as PARP, are considered to be the final step in apoptosis. In our results, combined treatment of apigenin with TRAIL caused the synergistic induction of effector caspase activation and subsequent PARP cleavage. Furthermore, we have demonstrated that the enhanced cytotoxicity achieved through the combination of apigenin and TRAIL is mediated through DR5. There are five TRAIL receptors that have been reported to interact with TRAIL, including DR4 (TRAIL-R1), DR5 (TRAIL-R2), DcR1 (TRAIL-R3), DcR2 (TRAIL-R4) and osteoprotegerin (OPG) (Degli-Esposti et al., 1997; Pan et al., 1997a; 1997b; Walczak et al., 1997). Among them, DR4 and DR5 are considered as death-inducing receptors because they contain death domain (DD) motif, which is required for triggering the extrinsic apop-

totic signaling pathway. On the other hand, DcR1 and DcR2 act as TRAIL antagonistic receptors that prevent apoptosis triggered by TRAIL. Since there is a relatively higher expression of DR4 and DR5 death receptors in tumor cells than in normal cells, TRAIL selectively kills the malignant cells by inducing apoptosis (van Noesel et al., 2002). In line with this, many reports have demonstrated that TRAIL-resistant cancer cells could be sensitized to TRAIL through the up-regulation of DR4 or DR5 (Mahalingam et al., 2009). It has previously been noted that apigenin sensitizes malignant tumor cells to TRAIL-induced apoptosis by DR5 up-regulation and caspase activation, while this effect was not observed in normal cells (Horinaka et al., 2006). However, the potential effect and the underlying mechanisms of apigenin and TRAIL combinations in HCC cells have not been fully investigated. In our result, the combined use of apigenin with TRAIL induced up-regulation of both DR4 and DR5. A human recombinant DR5-specific chimera antibody significantly blocked the apigenin/TRAIL-induced cytotoxicity, while DR4 chimera antibody did not, suggesting that DR5 plays a key role in the sensitizing effect of apigenin on TRAIL-induced cytotoxicity. This finding is similar to a previous report that apigenin enhances TRAIL-mediated apoptosis in several cancer cell lines by DR5 up-regulation (Horinaka et al., 2006).

Mitogen-activated protein kinase (MAPK) pathway regulation is one of the mechanisms that can potentially sensitize TRAIL-resistant cells through death receptor up-regulation (Nakshatri et al., 2004; Yodkeeree et al., 2009). In mammals, three major kinases of MAPK family have been identified: the extracellular-receptor kinases (ERK), the c-Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPK) and p38 MAPK. They mediate a wide range of cellular processes, including proliferation, differentiation, inflammation and apoptosis. Accumulating data indicate that the fundamental role of each MAPKs in the regulation of cell survival or apoptosis is complex and controversial (Wada and Penninger, 2004). In our results, we found that ERK activation is required for DR5 activation, leading to the sensitization of HepG2 cells to TRAIL. Although all 3 MAPK family members were activated by combination treatment, only the ERK inhibitor U0126 abolished apigenin/TRAIL-induced DR5 up-regulation. Interestingly, densitometry analysis of the kinetics of DR5 up-regulation and MAPK activation upon apigenin/TRAIL treatment showed that the activation of ERK peaked much earlier (0.5-8 h; Fig. 4C) than DR5 up-regulation (4-24 h; Fig. 4B), whereas JNK and p38 activation peaked later (both 8 h; Supplementary Figs. S1A and S2A) than DR5 activation. These results demonstrate that ERK activation is an upstream event of DR5 up-regulation. Previous studies have reported the upstream role of ERK in death receptor-mediated signaling in sensitizing TRAIL-resistant cancer cells. In human colon cancer cells, ERK activation sensitized cells to TRAIL by up-regulating DR4 and DR5 (Drosopoulos et al., 2005). In another study, butein induced ERK-mediated DR5 expression to sensitize human hepatoma cells to TRAIL-induced apoptosis (Moon et al., 2010). Furthermore, piceatannol, a phenolic compound of grapes, enhanced TRAIL-mediated apoptosis via DR5 up-regulation through activation of ERK and Sp1 (Kang et al., 2011). In accordance with these studies, our results suggest the critical role of ERK in sensitizing HepG2 cells to TRAIL-induced cell death via DR5 up-regulation.

Many reports have demonstrated the role of ROS in the up-regulation of DR5 and in the triggering of apoptosis in cancer cells (Chen et al., 2010). Additionally, several agents have been demonstrated to enhance TRAIL-induced apoptosis through ROS-dependent activation of MAPK family members and the

subsequent up-regulation of death receptors, suggesting that ROS generation could be an upstream event of MAPK pathway activation to induce death receptor regulation (Gupta et al., 2011; Prasad et al., 2011; Sung et al., 2010). However, pretreatment of NAC did not alter apigenin/TRAIL-induced DR5 expression, indicating that apigenin/TRAIL-mediated DR5 up-regulation is ROS-independent.

In summary, we showed that a combined treatment with apigenin and TRAIL enhances apoptosis in HepG2 hepatocellular carcinoma cells. Our results are the first to reveal the molecular mechanisms of apigenin sensitization of HepG2 cells to TRAIL-induced cell death. However, additional *in vivo* studies are, of course, required prior to the therapeutic application of apigenin with TRAIL in clinical trials.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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