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Trichostatin A sensitizes human ovarian cancer cells to TRAIL-induced apoptosis by down-regulation of c-FLIP$_L$ via inhibition of EGFR pathway

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ABSTRACT

TRAIL-resistant cancer cells can be sensitized to TRAIL by combination therapy. In this study, we investigated the effect of trichostatin A (TSA), a histone deacetylase inhibitor, to overcome the TRAIL resistance in human ovarian cancer cells. Co-treatment of human ovarian cancer cells with TSA and TRAIL synergistically inhibited cell proliferation and induced apoptosis. The combined treatment of ovarian cancer SKOV3 cells with TSA and TRAIL significantly activated caspase-8 and truncated Bid, resulting in the cytosolic accumulation of cytochrome *c* as well as the activation of caspase-9 and -3. Moreover, we found that down-regulation of c-FLIP$_L$ might contribute to TSA-mediated sensitization to TRAIL-induced apoptosis in SKOV3 cells, and this result was supported by showing that down- or up-regulation of c-FLIP$_L$ with transfection of siRNA or plasmid sensitized or made SKOV3 cells resistant to TRAIL-induced apoptosis, respectively. TSA or co-treatment with TSA alone and TRAIL also resulted in down-regulation of EGFR1/2 and dephosphorylation of its downstream targets, AKT and ERK. Treatment of SKOV3 cells with PKI-166 (EGFR1/2 inhibitor), LY294002 (AKT inhibitor), and PD98059 (ERK inhibitor) decreased c-FLIP$_L$ expression and co-treatment with TRAIL further reduced the level of c-FLIP$_L$, respectively, as did TSA. Collectively, our data suggest that TSA-mediated sensitization of ovarian cancer cells to TRAIL is closely correlated with down-regulation of c-FLIP$_L$ via inhibition of EGFR pathway, involving caspase-dependent mitochondrial apoptosis, and combination of TSA and TRAIL may be an effective strategy for treating TRAIL-resistant human ovarian cancer cells.

1. Introduction

TNF-related apoptosis-inducing ligand (TRAIL), a member of the TNF family, is being developed as an anticancer agent, since it induces apoptosis in a wide range of cancer cells but not in most normal cells [1,2]. TRAIL can interact with two specific cell surface death receptors TRAIL-R1 (DR4/KILLER) and TRAIL-R2 (DR5/TRICK2) [3] and initiate the extrinsic apoptotic cascade via activation of pro-caspase-8 [1]. Activated caspase-8 can either directly cleave downstream effector caspases such as caspase-3, -6, and -7 without involving mitochondria activation (type I cells) or can require the engagement of the mitochondria-mediated pathway (intrinsic pathway) through cleavage of the pro-apoptotic Bcl-2 family protein BID (type II cells) [4,5]. However, recent reports have demonstrated that many tumor cells including ovarian cancer cells acquire resistance to the apoptotic effects of TRAIL and this could limit the efficiency of TRAIL in cancer therapy [4,6]. One of the most important regulators of resistance to TRAIL, c-FLIP (FLICE-like inhibitory protein), is an apoptosis inhibitory protein with homologous to caspase-8 that lacks catalytic activity [7] and is highly expressed in ovarian cancer cell lines [8]. To overcome the resistance of cancer cells to TRAIL, the enhanced effects using combination of TRAIL and chemotherapy have been reported [9,10]. However, the sensitivity of human ovarian cancer cells to such combinations is not well known.

Recently, accumulating evidences have suggested that HDAC inhibitor (HDACI) can be used as a new class of anticancer drug due to their selective toxicity and synergistic activity with

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Abbreviations: EGFR, epidermal growth factor receptor; TSA, trichostatin A; HDACI, histone deacetylase inhibitor; PARP, poly(ADP-ribose) polymerase; ERK, extracellular signal-regulated kinase; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; DR4, death receptor 4; DR5, death receptor 5; c-FLIP, cellular FLICE-inhibitory protein; XIAP, X-linked inhibitor of apoptosis protein.

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chemotherapeutic drugs [11,12]. Several structural classes of HDACI have been identified as including derivatives of short-chain fatty acids, hydroxamic acids, cyclic tetrapeptides, cyclic peptides, and benzamides. Trichostatin A (TSA), a metabolite first isolated from cultures of *Streptomyces hygroscopicus*, is a kind of hydroxamic acids that is emerging as a new class of anticancer agents [13]. Recent studies suggested that TSA has a promising therapeutic effect on cancer cells when combined with radiotherapy or chemotherapy [14,15].

The epidermal growth factor receptor (EGFR) plays an important role in the regulation of cell proliferation, differentiation, development, and oncogenesis [16,17]. Amplification of the *EGFR* gene occurs frequently in breast, lung, ovary, and prostate cancer, which is associated with reduced patient survival [18,19]. As such, EGFR is an important therapeutic target in a number of human cancers and also confers reduced responses of cancer cells toward drug or radiation [20]. The EGFR regulates downstream signal such as PI3K/AKT [20], resulting in inhibiting the apoptosis induced by conventional or investigational cytotoxic agents [21]. Several targeted strategies have been developed to specifically inhibit aberrant EGFR signaling in tumor cells. The blockade of EGFR by inhibitors of EGFR tyrosine kinase leads to inhibition of cell cycle progression, induction of apoptosis, and enhancement of chemosensitivity, but EGFR-signaling antagonists alone do not appear to be curative in some instances [22–24]. Therefore, additional EGFR-targeted strategies or combination with other therapeutic approaches will be required. Recently, strong synergistic anticancer effects have been observed when EGFR-signaling antagonists were combined with activation of death receptors or their cognate ligands, including Fas and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) [25,26].

In this study, we demonstrated that TSA overcame resistance to TRAIL via EGFR pathway-dependent down-regulation of c-FLIP$_L$, consequently leading to induction of caspase-dependent mitochondrial apoptosis pathway in TRAIL-resistant SKOV3 cells. Therefore, we suggest that TSA in combination with TRAIL could be valuable in the treatment of TRAIL-resistant ovarian cancers.

2. Materials and methods

2.1. Cell culture, materials, and antibodies

The human ovarian cancer cell lines, SKOV3 cells, were obtained from American Type Culture Collection (ATCC, Manassas, VA) and Hey8 cells were obtained from Dr. I. J. Fidler (University of Texas M. D. Anderson Cancer Center, Houston, TX). Cells were maintained in RPMI 1640 medium (Invitrogen, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) and penicillin–streptomycin (50 U/ml) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. In this study the following inhibitors were used: caspase inhibitor, z-VAD-fmk (R&D Systems, Minneapolis, MN), EGFR1/2 inhibitor PKI-166 (Novartis, East. Hanover, NJ), PI3K/AKT inhibitor LY294002 (Sigma, St. Louis, MO), and ERK inhibitor PD98059 (Calbiochem, San Diego, CA). The inhibitors were dissolved in dimethylsulfoxide (DMSO; Sigma, St. Louis, MO) and the final concentration of DMSO was 0.1%. TRAIL was purchased from R&D Systems (Minneapolis, MN). TSA was purchased from Sigma–Aldrich (St. Louis, MO). Anti-EGFR1 clone 1005, anti-EGFR2 clone F-11, anti-p-ERK clone E-4, anti-Bcl-x$_L$ clone S-18, anti-Bcl-2 clone N-19, anti-Bax clone N-20, anti-PARP clone H-250, anti-caspase-3 clone H-277, and anti-cytochrome *c* clone H-104 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-caspase-8 clone 1C12, anti-p-AKT clone D9E, and anti-XIAP clone 2042 antibodies were purchased from Cell Signaling Technology (Danvers, MA). Anti-c-FLIP clone NF-6 antibody was purchased from Alexis Biochemicals (San Diego, CA). Anti-caspase-9 (AAP-109C) antibody was purchased from Stressgen Biotechnologies Corp (Victoria, BC Canada). Anti-Bid (AF860), anti-DR4 (AF347), and anti-DR5 (AF631) antibodies were from R&D systems (Minneapolis, MN). Anti-β-actin clone AC-74 antibody was purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Annexin V analysis for determining apoptosis

Cells were seeded in 6-well plates at a concentration of 1×10^6 cells/well and treated with increasing concentrations of TRAIL in the absence or presence of TSA for 16 h. The cells were resuspended in 100 μl of staining solution containing FITC-conjugated annexin V (BD Biosciences, San Jose, CA) and propidium iodide (Sigma, St. Louis, MO) in a HEPES buffer. After incubation at room temperature for 20 min, annexin V-positive cells were analyzed by FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). To determine whether caspase is involved in apoptosis induced by combined treatment with TSA and TRAIL, the cells were pre-treated with 20 μM of caspase inhibitor z-VAD-fmk for 3 h prior to addition of TSA and TRAIL followed by annexin V assay as described above. To evaluate that EGFR1/2 pathway and its downstream targets, PI3K/AKT and ERK are involved in TRAIL resistance in SKOV3 cells, 10 μM PKI-166, 10 μM LY294002, and 10 μM PD98059 were used. Cells were pre-treated with these inhibitors for 3 h prior to addition of TRAIL. The cells were incubated for a further 16 h at 37 °C and processed for annexin V analysis as described above.

2.3. Methylene blue analysis for determining cell survival

The methylene blue analysis was performed as previously described [27]. Cells were seeded in 96-well plates at a concentration of 1×10^3 cells/well and treated with increasing concentrations of TRAIL in the absence or presence of TSA for 16 h. After treatment, the cells were fixed with 70% ethanol, stained with methylene blue, and then the absorbance of dye eluted from the fixed cells in each well was measured on an automated scanning photometer at a wavelength of 630 nm. Results are presented as percentage of survival, taking control as 100%.

2.4. Flow cytometric analysis for determining DR4 and DR5 expression

The cells from the culture media were spun down at 500 × *g*, washed with phosphate-buffered saline (PBS), and resuspended in 500 μl PBS. Cells were then incubated with 5 μl of goat IgG2a, or anti-DR4 and anti-DR5 polyclonal goat antibody (1:100), respectively, for 1 h. After washing with PBS, FITC-conjugated rabbit anti-goat polyclonal antibody (1:200, Sigma, St. Louis, MO) was added to the cell suspension and incubated for 1 h on ice followed by washing with PBS. After rinsing, the samples were analyzed by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). The data were analyzed using the CellQuest program.

2.5. cDNA and siRNA transfection

The pcDNA3.1-CMV-FLIP$_L$ (kind gifts from Dr. Park SI, National Institute of Health, Seoul, South Korea) was transfected into SKOV3 cells using the Lipofectamine following the manufacturer's protocol (Invitrogen Life Technologies, Inc., Carlsbad, CA). The transfected cells were grown for 24 h before subjected to annexin V analysis and methylene blue analysis. The c-FLIP$_L$ (5′-AAGGAACAGCUUGGCG-CUCAAdT dT-3′), XIAP (5′-TGTAGACTGCGTGGCACTATTdTdT-3′), and control scrambled (5′-CUUCCCGAAAACUUGAGACdTdT-3′) siRNAs were synthesized. SKOV3 cells in exponential phase of growth were plated in 6-well plates at 5×10^5 cells/well, cultured for 24 h,

and then transfected with siRNA using oligofectamine and OPTI-MEM I reduced serum medium (Invitrogen Life Technologies, Inc., Carlsbad, CA), according to the manufacturer's protocol. The concentrations of siRNAs were chosen based on dose–response studies. Transcriptional silencing was examined 1–2 days after transfection.

2.6. RNA extraction and relative RT-PCR analysis

RNA extraction and relative RT-PCR analysis were performed as previously described [28]. Briefly, total cellular RNA was extracted from SKOV3 cells harvested at the indicated times using Tri reagent (Molecular Research Center, Cincinnati, OH). 1 μg of total cellular RNA from each sample was reverse transcribed using Maloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies, Inc., Carlsbad, CA) with 0.1 μM of each dNTP and 1 μg oligodT. Amplification of 2.5 μl of these cDNA by PCR was performed using the following gene-specific primers: DR4 (forward), 5′-CTGAGCAACGCAGACTCGCTGTCCAC-3′ and (reverse), 5′-AAGGACACGGCAGAGCCTGTGCCAT-3′; DR5 (forward), 5′-CTGAAAGGCATCTGCTCAGGTG-3′ and (reverse), 5′-CAGAGTCTGCATTACCTTCTAG-3′; c-FLIP$_L$ (forward), 5′-GCTGAAGTCATCCATCAGGT-3′ and (reverse), 5′-CATACTGAGATGCAAGAATT-3′; c-FLIP$_S$ (forward), 5′-GCTGAAGTCATCCATCAGGT-3′ and (reverse), 5′-GATCAGGACAATGGGCATAG-3′; XIAP (forward), 5′-GCAGATCTAGTGAATGCTCAGAAA-3′ and (reverse), 5′-TACTTGGTAGCAAATGCTAATGGA-3′; β-actin (forward), 5′-CAGAGCAAGAGAGGCATCCT-3′ and (reverse), 5′-TTGAAGGTCTCAAACATGAT-3′. The resulting total cDNA was used in PCR performed in total volume of 30 μl using TaKaRa Taq™ Kit (Takara) at 94 °C for denaturation for 60 s, 58 °C for annealing for 60 s, and 72 °C for amplification for 90 s for 30 cycles, followed by a final extension at 72 °C for 12 min. The amplified fragments were separated on 1% agarose gel and visualized ethidium bromide staining.

2.7. Western blot analysis

To extract whole cell lysates, cells were washed in ice-cold PBS and extracted for 30 min with a buffer containing 50 mM Tris-HCl, pH 7.5, 140 mM NaCl, 5 mM EDTA, 5 mM NaN_3, 1% Triton X-100, 1% NP-40, 1 mM EGTA, and protease inhibitor cocktail (Sigma, St. Louis, MO). Lysates were cleared by centrifugation at 13,000 × *g* for 30 min and protein concentrations were determined using Bradford protein assay. Proteins were denatured in 2% SDS containing sample buffer and same total protein amount was transferred onto a nitrocellulose membrane (Amersham Biosciences, Arlington Heights, IL). The membranes were probed with specific antibodies. Immunocomplexes were detected using horseradish peroxidase conjugated either with anti-mouse, anti-rabbit or anti-goat (Amersham Biosciences, Arlington Heights, IL) followed by chemiluminescence detection (ECL, Amersham Biosciences, Arlington Heights, IL).

2.8. Isolation of cytosolic fraction for detecting cytochrome c

SKOV3 cells (approximately 2×10^7 cells) were treated with TRAIL in the absence or presence of TSA for 16 h. Cells were harvested by centrifugation at 600 × *g* for 5 min at 4 °C, washed twice in ice-cold PBS, and then suspended in 5 × volume of ice-cold buffer [20 mM HEPES (pH 7.2), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 250 mM sucrose, and protease inhibitor mixture]. Cells were mechanically disrupted with a dounce homogenizer (30 strokes), which were centrifuged at 750 × *g* for 5 min at 4 °C. The supernatants were centrifuged at 10,000 × *g* for 15 min at 4 °C to pellet the cell debris. The supernatant, which contained mitochondria, was subjected to further centrifugation at 100,000 × *g* for 60 min at 4 °C to pellet the mitochondrial fraction. Samples were dissolved in SDS sample buffer, heated to 95 °C for 3 min, and subjected to Western blotting analysis.

2.9. Statistical analysis

The significance of differences between experimental conditions was determined using the student's *t* test for unpaired observations.

3. Results

3.1. Co-treatment with TSA and TRAIL induces apoptosis and inhibits cell viability in SKOV3 and Hey8 cells

To investigate the effect of combination of TSA and TRAIL on TRAIL-resistant ovarian cancer SKOV3 and Hey8 cells, we treated the cells with TRAIL (1–10 ng/ml) in the absence or presence of TSA (0.1–0.5 μM) for 16 h and performed annexin V analysis and methylene blue assay, as described in Section 2. As shown in Fig. 1A and C, TSA and TRAIL alone only moderately induced apoptosis in both SKOV3 and Hey8 cells. In contrast, co-treatment with TSA and TRAIL synergistically induced apoptosis in these cells. Moreover, cell viability was decreased in the combination of TSA and TRAIL compared to each reagent alone (Fig. 1B and D). These data suggest that the combination of TSA and TRAIL induces apoptosis and inhibits in TRAIL-resistant ovarian cancer cells.

3.2. TSA accelerates TRAIL-induced apoptosis by activating caspase-dependent mitochondrial pathway in SKOV3 cells

It is well known that TRAIL-induced apoptosis requires the activation of caspases, which in turn cleave key protein substrates [1]. TRAIL-induced activation of caspase-8 leads to activation of downstream caspases including caspase-9 and -3. To determine whether combined treatment with TSA and TRAIL leads to activation of caspases in TRAIL-resistant SKOV3 cells, the cells were treated with TRAIL (10 ng/ml) in the absence or presence of TSA (0.1 or 0.5 μM), and their caspase activity was determined using Western blot analysis. Combined treatment with TSA and TRAIL induced the proteolytic processing of procaspase-8, -9, and -3, but neither TSA nor TRAIL alone activated these caspases (Fig. 2A). Moreover, the combined treatment with TSA and TRAIL caused cleavage of PARP, a well known endogenous substrate of caspase-3, yielding a characteristic 85 KDa fragment, whereas TSA or TRAIL alone failed to induce cleavage of PARP (Fig. 2A).

To further confirm whether combined treatment with TSA and TRAIL induces caspase-dependent apoptosis, the cells were treated with a cell-permeable caspase inhibitor, z-VAD-fmk (20 μM) before addition of TSA and TRAIL. As shown in Fig. 2B, treatment with z-VAD-fmk significantly reduced the ability of TSA to sensitize SKOV3 cells to TRAIL-induced apoptosis. These results strongly suggest that the potentiation of TRAIL-induced apoptosis by TSA is mediated through a caspase-dependent signaling cascade.

Bcl-2 family proteins, which comprise both anti-apoptotic members such as Bcl-2 or Bcl-x_L and pro-apoptotic molecules such as Bax or Bid, play an important role in the regulation of the mitochondrial apoptotic pathway [5,29]. Therefore, we investigated whether the modulation of Bcl-2 family proteins is involved in the sensitization of SKOV3 cells to TRAIL-induced apoptosis by TSA. Fig. 2C revealed that co-treatment with TSA and TRAIL to SKOV3 cells resulted in the truncation of Bid and release of cytochrome *c* from mitochondria. Furthermore, combined treatment with TSA and TRAIL induced up-regulation of Bax, which led to prominent loss of Bcl-2 and Bcl-x_L (Fig. 2D). Collectively, these results suggest that sensitization of SKOV3 cells to TRAIL-induced

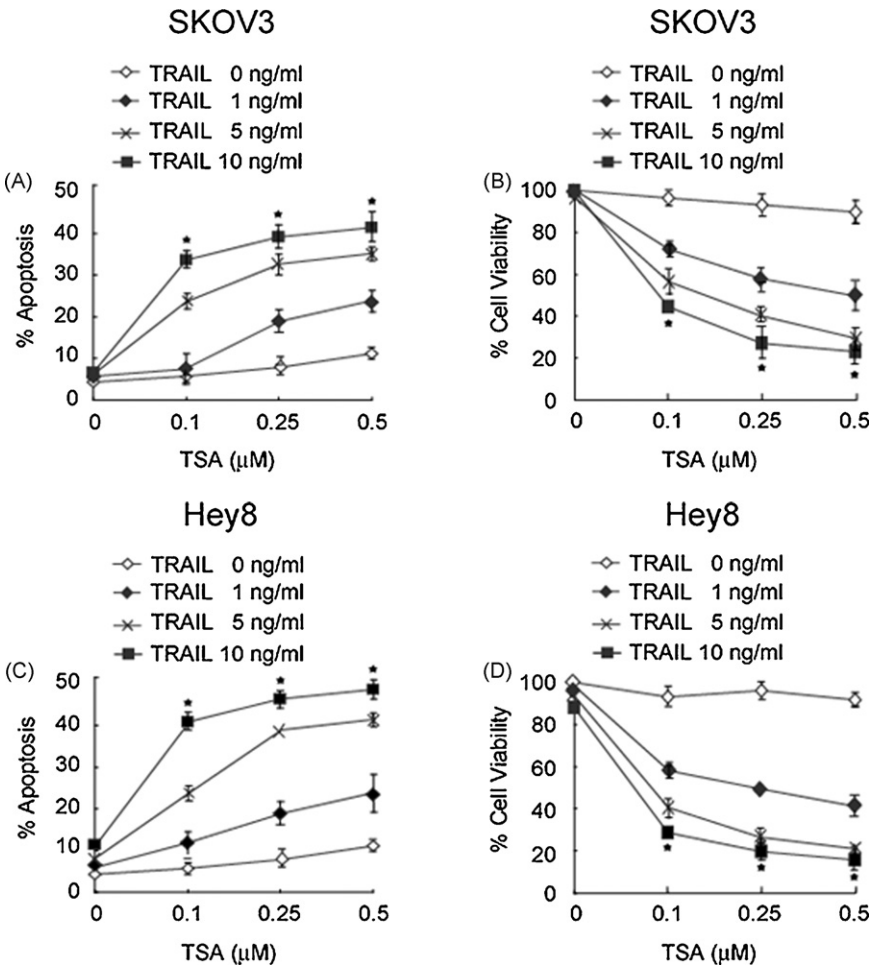

Fig. 1. Effect of TSA on TRAIL-induced apoptosis and cytotoxicity in human ovarian SKOV3 and Hey8 cancer cells. The cells were treated with TRAIL (1–10 ng/ml) in the absence or presence of TSA (0.1–0.5 μM) for 16 h. Thereafter, induction of apoptosis (A and C) or cell viability (B and D) was measured by annexin V binding analysis or methylene blue analysis, respectively, as described in Section 2. Each point is the average of triplicate determinants (*$P < 0.001$).

apoptosis by TSA occurs through caspase-dependent mitochondrial pathways.

3.3. The modulation of TRAIL receptors is not involved in sensitization of TRAIL-induced apoptosis by TSA

As noted above, the combined treatment with TSA and TRAIL resulted in caspase-8 activation, a key event in death receptor-mediated apoptosis. There are five receptors that can bind to TRAIL: death receptor 4 (DR4), DR5, decoy receptor 1(DcR1), DcR2, and osteoprotegerin. Only two of the TRAIL receptors, DR4 and DR5, contain functional death domains, thereby triggering apoptotic signals upon TRAIL binding [3]. Therefore, to investigate whether TSA and TRAIL change the level of TRAIL receptors, we treated SKOV3 cells with TRAIL (10 ng/ml) in the absence or presence of TSA (0.1 or 0.5 μM) and performed RT-PCR and Western blot analysis, respectively. Fig. 3A and B revealed that the levels of mRNA and protein of DR4 and DR5 were not changed after treatment with TSA and/or TRAIL. Moreover, the result from flow cytometry analysis of the cell surface expression of DR4 and DR5 revealed that the cell surface expression of DR4 was not altered, but DR5 was moderately down-regulated after treatment with TSA and/or TRAIL (Fig. 3C). These results suggest that the level of TRAIL receptors expression alone is not sufficient to account for sensitivity to TSA and TRAIL-induced apoptosis although both receptors are expressed, but other factors rather than DR4 and DR5 may be involved in the increased sensitivity to TSA and TRAIL-induced apoptosis in SKOV3 cells.

3.4. Down-regulation of c-FLIP$_L$ by TSA potentiates TRAIL-induced apoptosis in SKOV3 cells

To better understand the factors that contribute to TSA-stimulated sensitization of SKOV3 cells to TRAIL, we analyzed the status of c-FLIP and XIAP that are well known for interfering with the initial stages of caspase activation at the downstream of DR4 and DR5 [7,30]. To evaluate the combined effect of TSA and TRAIL on c-FLIP expression of SKOV3 cells, the cells were treated with TRAIL (10 ng/ml) in the absence or presence of TSA (0.1 or 0.5 μM), and the changed mRNA and protein levels of c-FLIP$_{L/S}$ and XIAP were assessed by RT-PCR and Western blot analysis, respectively. As shown in Fig. 4A, TSA alone reduced the level of c-FLIP$_L$ mRNA in a dose-dependent manner and in the presence of TRAIL TSA reduced further the level of c-FLIP$_L$ mRNA, while TRAIL alone did not alter the mRNA level of c-FLIP$_L$ (Fig. 4A). Consistent with this result of transcriptional change in c-FLIP$_L$, the protein level of c-FLIP$_L$ was reduced by TSA and co-treatment with TRAIL, but TRAIL alone did not alter the protein level of c-FLIP$_L$ (Fig. 4B). On the contrary, we did not observe any significant change in levels of mRNA and protein of c-FLIP$_S$ after treatment with TRAIL in the absence or presence of TSA (Fig. 4A and B). Interestingly, co-treatment with TSA and TRAIL led to reduction of level of XIAP protein, but not the level of mRNA (Fig. 4A and B).

In order to determine whether down-regulation of c-FLIP and XIAP plays an important role in TRAIL-induced apoptosis in SKOV3 cells, we first transfected SKOV3 cells with c-FLIP$_L$ siRNA and

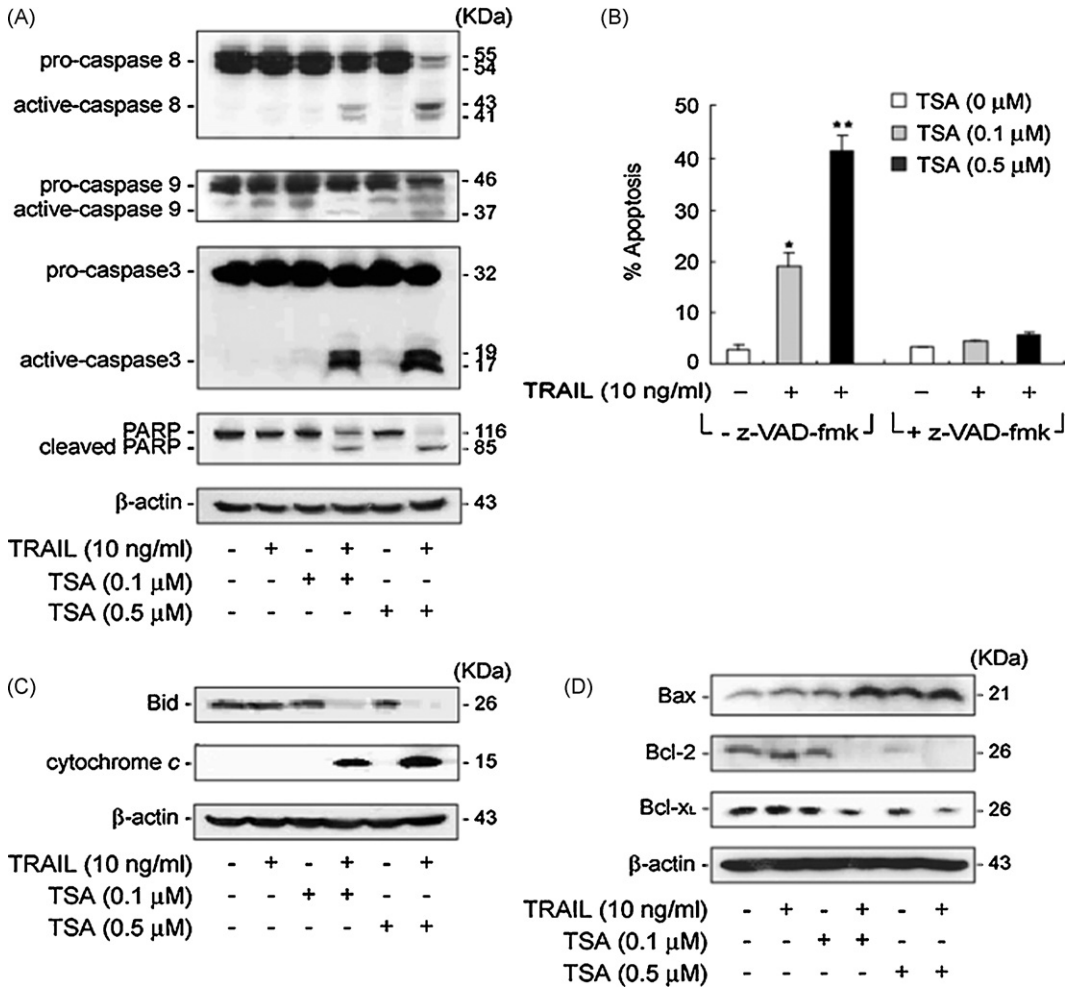

Fig. 2. Sensitization of TRAIL-induced apoptosis by TSA is mediated through caspase-dependent mitochondrial apoptotic pathway in SKOV3 cells. (A) Whole cell extracts from the SKOV3 cells treated with TRAIL (10 ng/ml) in the absence or presence of TSA (0.1 or 0.5 μM) for 16 h were used for Western blot analysis using caspase-8, caspase-9, caspase-3, and PARP antibodies. The levels of β-actin served as the loading control. (B) The cells were pre-treated with 20 μM of caspase inhibitor z-VAD-fmk for 3 h prior to addition of TSA (0.1 or 0.5 μM) and TRAIL (10 ng/ml). Thereafter, annexin V analysis was performed. Data are the means of triplicate determinations from three independent experiments; bars, SE. $^{*}P < 0.05$, $^{**}P < 0.01$. (C and D) The whole cell extracts from the cells treated with TRAIL (10 ng/ml) in the absence or presence of TSA (0.1 or 0.5 μM) for 16 h were used for Western blot analysis of the indicated proteins using Bid, cytochrome *c*, Bax, Bcl-2, and Bcl-x_L antibodies. To detect cytochrome *c* release from the mitochondria to cytosol, cytosolic fractions were prepared and used as described in Section 2. The levels of β-actin served as the loading control.

evaluated the sensitivity to TRAIL as using annexin V analysis and methylene blue assay. TRAIL-induced apoptosis and growth inhibition in the cells transfected with c-$FLIP_L$ siRNA were significantly more prominent than in the cells transfected with scramble siRNA (Fig. 5A and B) when the decrease in c-$FLIP_L$ protein was observed in the cells transfected with c-$FLIP_L$ siRNA but not in scramble siRNA (Fig. 5A, insert). Conversely, SKOV3 cells were transiently transfected with pcDNA3.1/$FLIP_L$ as c-$FLIP_L$ expression plasmid or pcDNA3.1 as mock control. The over-expression of c-FLIP protein after transient transfection with pcDNA3.1/$FLIP_L$ was confirmed in SKOV3 cells (Fig. 5C, insert). The transfectants were then treated with TRAIL (10 ng/ml) in the absence or presence of TSA (0.1 or 0.5 μM) and then annexin V analysis and methylene blue assay were performed, respectively. As shown in Fig. 5C and D, co-treatment with TSA and TRAIL decreased TRAIL-induced apoptosis and increased cell viability in SKOV3/$FLIP_L$ cells compared with SKOV3/pcDNA3.1 cells. Moreover, down-regulation of XIAP with specific siRNA led to increased susceptibility to TRAIL in SKOV3 cells (Fig. 5E), but the level of this increase was lower than that of c-$FLIP_L$ siRNA transfectants (Fig. 5A), suggesting that $FLIP_L$ may be more responsible for susceptibility to TRAIL than XIAP in SKOV3 cells. Although further studies will be required for this possibility, we could suggest that TSA-mediated down-regulation of c-$FLIP_L$ contributes to the increased susceptibility to TRAIL-induced apoptosis in SKOV3 cells.

3.5. Inhibition of EGFR pathway by TSA sensitizes TRAIL-induced apoptosis in SKOV3 cells

On the other hand, the EGFR family of receptor tyrosine kinases has been shown to play a key role in normal ovarian follicle development [31], and its over-expression in ovarian cancer cells has been suggested to be responsible for the resistance of ovarian cancer cells to many apoptotic agents, leading to a poor prognosis for patient survival [32]. To investigate the effect of TSA and/or TRAIL on EGFR1/2 expression, SKOV3 cells were treated with TRAIL (10 ng/ml) in the absence or presence of TSA (0.1 or 0.5 μM) for 16 h, and the level of EGFR1/2 was assessed by Western blot analysis as described in Section 2. Although TRAIL alone did not affect the expression level of EGFR1/2, exposure of SKOV3 cells to TSA for 16 h decreased the EGFR1/2 expression, and co-treatment with TRAIL (10 ng/ml) further reduced the EGFR1/2 expression. Consequently, these results were followed by similar changes in activities of downstream molecules including phosphorylated AKT (p-AKT) and phosphorylated ERK (p-ERK) (Fig. 6A). Next, we examined whether inhibition of EGFR pathway by TSA affects

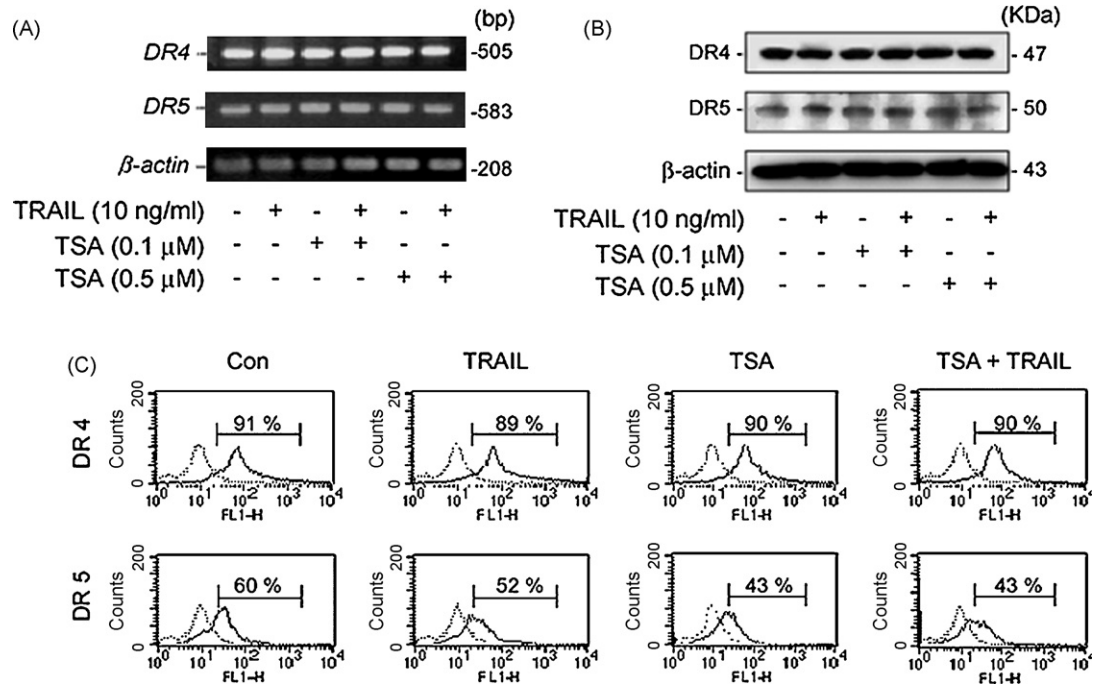

Fig. 3. Effect of TSA and/or TRAIL on the expression of TRAIL receptors in SKOV3 cells. SKOV3 cells were treated with TSA (0.1 or 0.5 μM) in the absence or presence of TRAIL (10 ng/ml) for 16 h. (A) RT-PCR and (B) Western blot analysis were performed to detect the level of mRNA and protein of DR4 and DR5, respectively, as described in Section 2. (C) SKOV3 cells were incubated with anti-DR4 and DR5 (1:100) antibodies, respectively, and subsequently labeled with FITC-conjugated secondary antibodies (1:200). Goat IgG2a was used as a control isotype antibody (dotted line). The cell surface expression is measured by flow cytometer.

TRAIL sensitivity of SKOV3 cells. The cells were treated with 10 μM PKI-166 (EGFR1/2 inhibitor), 10 μM LY294002 (PI3K/AKT inhibitor) and 10 μM PD98059 (ERK inhibitor), respectively, for 3 h before the addition of TRAIL and analyzed with annexin V analysis and methylene blue assay, respectively. Fig. 6B and C showed that exposure to PKI-166, LY294002, and PD98059 alone had no effect on apoptosis and cell viability, but in the presence of TRAIL, SKOV3 cells were sensitized to TRAIL-induced apoptosis and inhibited cell viability, as did TSA. From these results, it could be suggested that although suppression of EGFR-signaling pathway by TSA alone may be insufficient to fully induce apoptosis, but it may prime SKOV3 cells to sensitize TRAIL-induced apoptosis.

3.6. Inhibition of EGFR pathway by TSA down-regulates c-FLIP$_L$ in SKOV3 cells

Recently, it has been reported that the constitutive expression of c-FLIP$_L$ in tumor cells might be attributable to the activation of either PI3K/AKT kinase or MAP kinase pathways [33,34]. Therefore, we investigated whether the expression of c-FLIP$_L$ can be regulated by EGFR-signaling pathway to understand the mechanism of TSA-induced down-regulation of c-FLIP$_L$. SKOV3 and Hey8 cells were treated with 10 ng/ml TRAIL in the absence or presence of 10 μM PKI-166, LY294002, and PD98059 for 16 h, respectively, and the level of c-FLIP$_L$ was assessed by Western blot analysis. Fig. 7 revealed that exposure of SKOV3 and Hey8 cells to PKI-166, LY294002, and PD98059 decreased the c-FLIP$_L$ expression, and co-treatment with 10 ng/ml TRAIL further reduced the c-FLIP$_L$, as did TSA. These results suggest that EGFR-signaling pathway is associated with regulation of c-FLIP$_L$ expression, and inhibition of EGFR-signaling pathway by TSA sensitized TRAIL-induced apoptosis via down-regulation of c-FLIP$_L$ in ovarian cancer cells.

4. Discussion

In the present study, we demonstrated that the combination of HDAC inhibitor TSA and TRAIL synergistically induced apoptosis in TRAIL-resistant SKOV3 ovarian cancer cells. This effect

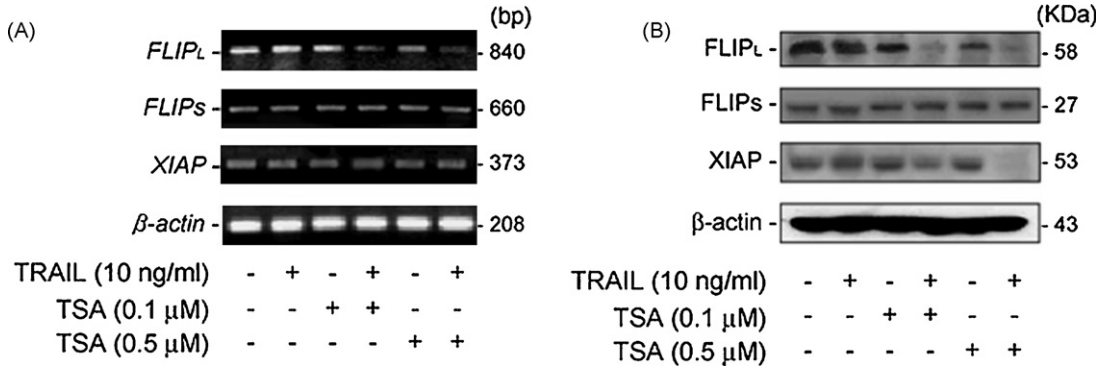

Fig. 4. The combination effect of TSA and TRAIL on c-FLIP$_{L/S}$ as well as XIAP expression in SKOV3 cells. SKOV3 cells were treated with TRAIL (10 ng/ml) in the absence or presence of TSA (0.1 or 0.5 μM) for 16 h. (A) RT-PCR and (B) Western blot analysis were performed to evaluate the level of c-FLIP $_{L/S}$ as well as XIAP in the mRNA and protein, respectively. The levels of β-actin served as the loading control.

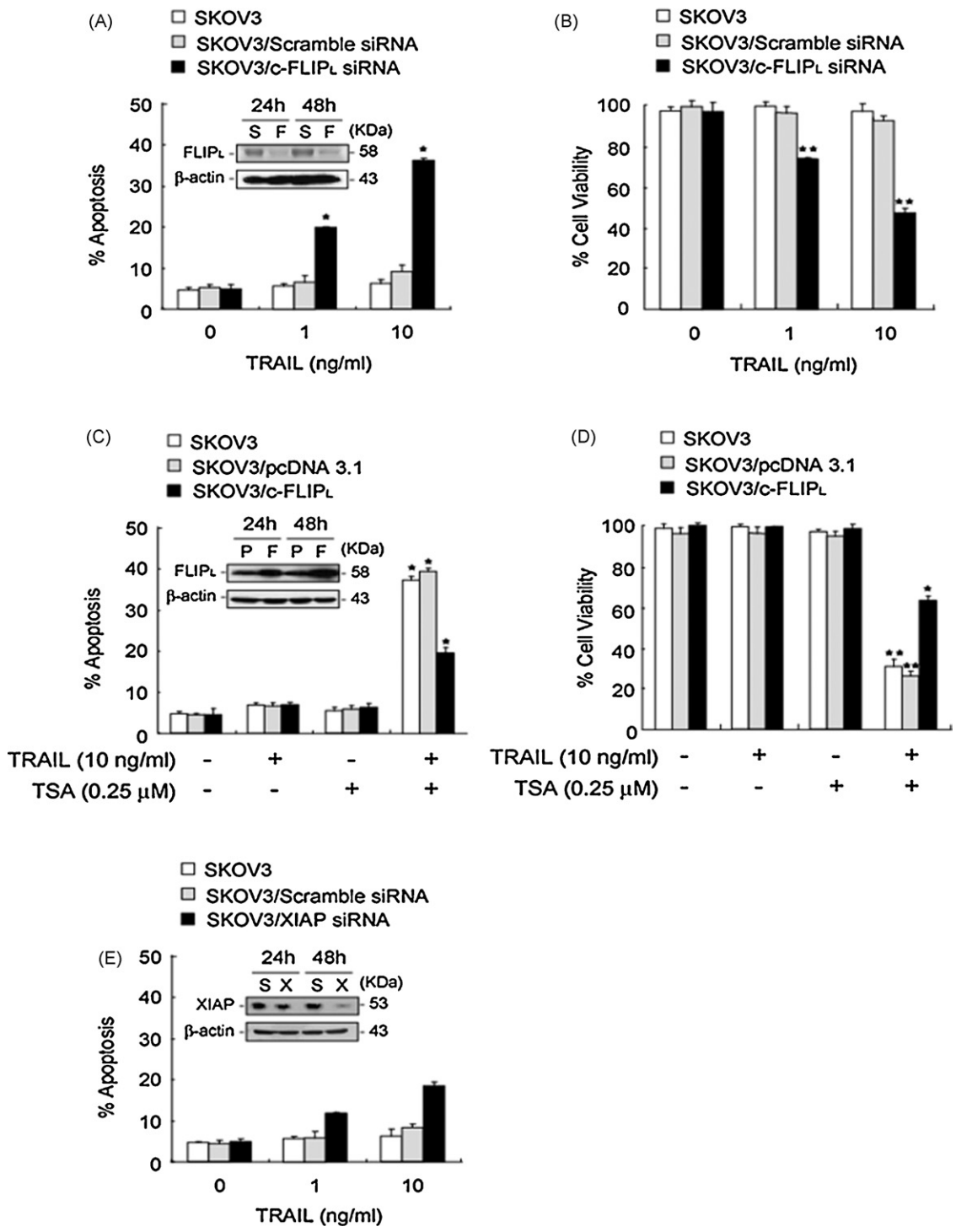

Fig. 5. Suppression of c-FLIP$_L$ accelerates TRAIL-induced apoptosis and growth inhibition in SKOV3 cells. SKOV3 cells transfected with scramble siRNA (S) or c-FLIP$_L$ siRNA (F) were treated with TRAIL (1 and 10 ng/ml) for 16 h, and analyzed for (A) apoptosis using annexin V (*$P < 0.01$) and (B) cell viability using methylene blue analysis (**$P < 0.001$), respectively. c-FLIP$_L$ level in the SKOV3 cells transfected with scramble siRNA or c-FLIP siRNA oligonucleotide was determined by Western blot analysis using the c-FLIP antibody (A, insert). SKOV3 cells were transiently transfected with pcDNA3.1/c-FLIP$_L$ (F) or pcDNA3.1 (P) as mock control. The transfectants were treated with TSA (0.25 μM) in the absence or presence of TRAIL (10 ng/ml) for 16 h and analyzed with (C) annexin V analysis and (D) methylene blue analysis, respectively. c-FLIP$_L$ level in the SKOV3 cells transfected with pcDNA3.1/c-FLIP$_L$ or pcDNA3.1 was determined by Western blot analysis using the c-FLIP antibody (C, insert). (E) SKOV3 cells were transfected with XIAP-targeted siRNA (X) or scrambled siRNA (S) as control. The transfectants were treated with TRAIL (1 and 10 ng/ml) and analyzed with annexin V analysis. XIAP level in the SKOV3 cells transfected with scramble siRNA or XIAP siRNA oligonucleotide was determined by Western blot analysis using the XIAP antibody (E, insert). Data are the means of triplicate determinations from three independent experiments; bars, SE.

of combination on apoptosis involved the activation of caspases including caspase-8, -9, and -3 and mitochondrial-dependent apoptosis pathway including Bid cleavage. Pre-treating SKOV3 cells with z-VAD-fmk efficiently prevented apoptosis that was induced by co-treatment with TSA and TRAIL, indicating that the apoptotic process occurred via caspase-dependent manner.

It has been reported that TRAIL induces apoptosis by binding to its two death receptors DR4 and DR5, and the extent of apoptosis

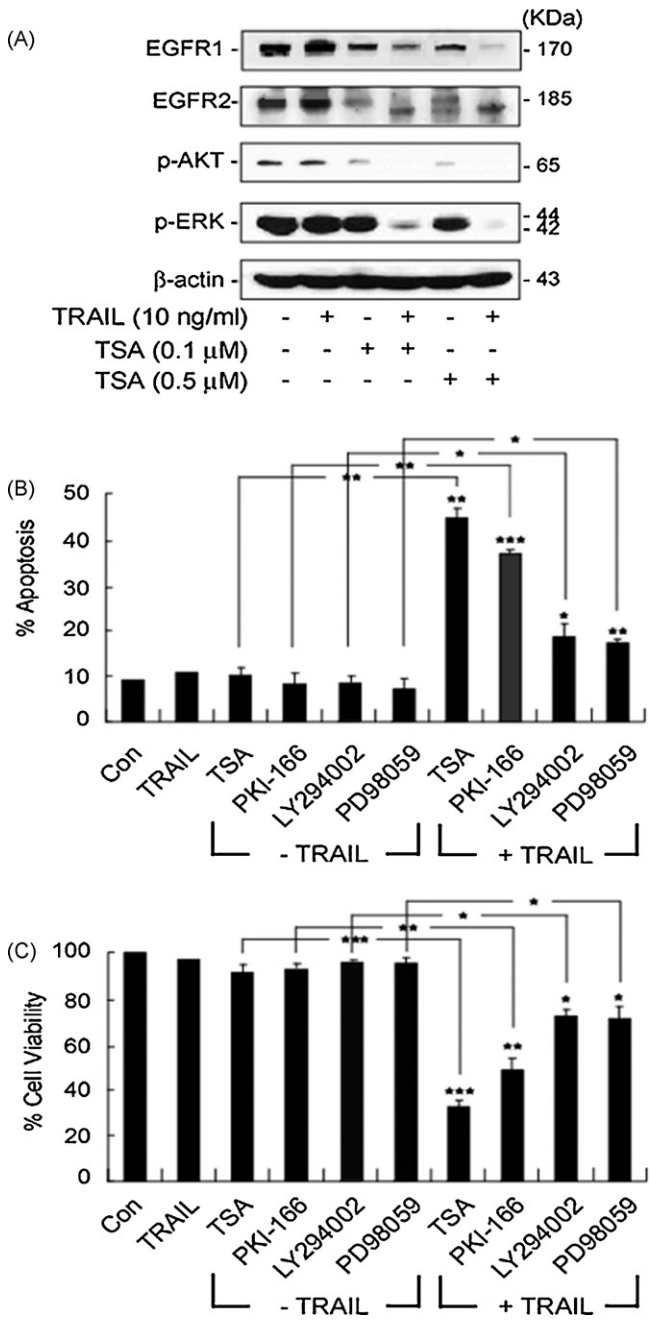

Fig. 6. TSA inhibits EGFR pathway including PI3K/AKT and MAPK (p42/44) in SKOV3 cells. SKOV3 cells were treated with TRAIL (10 ng/ml) in the absence or presence of TSA (0.1 or 0.5 μM) for 16 h. (A) Cells were lysed and proteins were used for Western blot analysis using EGFR1, EGFR2, p-AKT, and p-ERK antibodies. The levels of β-actin served as the loading control. (B and C) SKOV3 cells were treated with TRAIL (10 ng/ml) in the absence or presence of 0.5 μM TSA, 10 μM PKI-166, 10 μM LY294002, 10 μM PD98059. Annexin V analysis and methylene blue analysis were performed, respectively, as described in Section 2. Data are the means of triplicate determinations from three independent experiments; bars, SE. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

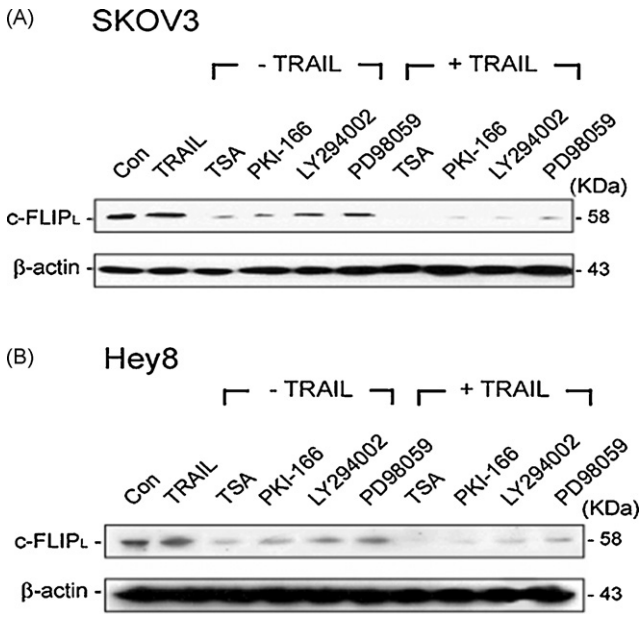

Fig. 7. TSA regulates c-FLIP$_L$ expression via inhibition of EGFR pathway in SKOV3 cells (A) SKOV3 and (B) Hey8 cells were treated with 10 ng/ml TRAIL in the absence or presence of 0.5 μM TSA, 10 μM PKI-166, 10 μM LY294002, and 10 μM PD98059. Cells were lysed and proteins were used for Western blot analysis using c-FLIP$_L$ antibody. The levels of β-actin served as the loading control.

by TRAIL is tightly regulated by the expression of these receptors [3]. Our results showed that co-treatment of SKOV3 cells with TSA and TRAIL did not affect the level of DR4 and DR5 in mRNA and protein level. Though TSA-mediated sensitization to TRAIL-induced apoptosis in SKOV3 cells was not correlated to changes of death receptor expression, recent studies have reported that the redistribution of death receptors within the plasma membrane into lipid rafts is essential for initiating signaling from receptors [35–38]. However, we did not observe the translocation of these receptors to lipid rafts in SKOV3 cells (data not shown), suggesting that other mechanisms, which can affect the efficiency of TRAIL receptor signaling, may be involved in TSA-mediated sensitization to TRAIL-induced apoptosis in SKOV3 cells.

In this study, we suggested that c-FLIP (FLICE-like inhibitory protein) is a regulator of TRAIL-induced apoptosis in SKOV3 cells. c-FLIP, an apoptosis inhibitory protein with homology to caspase-8 that lacks catalytic activity, is expressed at higher levels in cancer cells than in normal tissue cells [7], enabling cancer cells to overcome TRAIL-mediated apoptosis [39]. Our result showed that treatment with TSA alone as well as co-treatment with TSA and TRAIL resulted in the decrease of c-FLIP$_L$ but not c-FLIPs. We also revealed that inhibition of c-FLIP$_L$ expression using c-FLIP$_L$ siRNA sensitized SKOV3 cells to TRAIL-induced apoptosis and conversely transient over-expression of c-FLIP$_L$ inhibited apoptosis. Therefore, down-regulation of c-FLIP$_L$ by TSA contributes to sensitization of SKOV3 cells to TRAIL-induced cell death.

On the other hand, the epidermal growth factor receptor (EGFR) is a family of growth factor receptors which are frequently activated by amplification and mutation in malignant ovarian tumor tissue [32], and tissue content of EGFR has been directly associated with poor prognosis in patients with ovarian cancer [16]. Moreover, EGFR has distinct downstream signaling pathways including the PI3K/AKT cascade as well as MAPK pathway [17], which have been previously reported to play important roles in TRAIL resistance [40], but the molecular mechanisms linking EGFR pathway to TRAIL resistance in ovarian cancer cells are not well established. Our result showed that treatment with TSA alone as well as co-treatment with TRAIL induced down-regulation of EGFR1/2 and dephosphorylation of AKT as well as ERK, and treatment of PKI166 as EGFR1/2 inhibitor, LY294002 as PI3K/AKT inhibitor, and PD98059 as MAPK p42/44 inhibitor, respectively, sensitized SKOV3 cells to TRAIL-induced apoptosis, as did TSA, suggesting that inhibition of EGFR pathway is associated with TRAIL sensitivity in SKOV3 cells.

Since recent reports suggested that PI3K/AKT and MAP kinase signaling pathways are correlated with the regulation of c-FLIP$_L$ expression in cancer [41,42], we investigated that EGFR-signaling

pathway including PI3K/AKT and MAP kinase regulates the expression of c-FLIP$_L$ in SKOV3 cells. We observed that treatment of EGFR1/2 inhibitor, PI3K/AKT inhibitor, and MAPK inhibitor decreased c-FLIP$_L$ levels in SKOV3 cells, respectively, and co-treatment with TRAIL further reduced the c-FLIP$_L$ as did TSA. These results suggest that EGFR-signaling pathway is associated with regulation of c-FLIP$_L$ expression, and inhibition of EGFR-signaling pathway by TSA sensitizes TRAIL-induced apoptosis through down-regulation of c-FLIP$_L$ in SKOV3 cells.

In conclusion, we demonstrated that combination of TSA and TRAIL can effectively induce apoptosis in TRAIL-resistant SKOV3 cells. The TSA-mediated TRAIL sensitization in SKOV3 cells seems to be associated with down-regulation of c-FLIP$_L$ via inhibition of EGFR pathway, leading to caspase-dependent mitochondrial apoptosis. Therefore, our study provided a possible therapeutic application of TSA and TRAIL for treatment of ovarian cancers which are resistant to TRAIL.

Acknowledgment

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