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Aspirin enhances TRAIL-induced apoptosis via regulation of ERK1/2 activation in human cervical cancer cells

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ABSTRACT

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) triggers tumor-specific apoptosis. However, some tumors and cancer cell lines are resistant to TRAIL. Here, the effect of the non-steroidal anti-inflammatory drug aspirin on sensitization of human cervical cancer cells to TRAIL and the underlying mechanism(s) of the effect were explored. Combination treatment with aspirin and TRAIL markedly enhanced apoptotic cell death, as assessed by lactate dehydrogenase (LDH) assay and analysis of cell cycle sub-G1 phase. The two agents together activated the several caspases and mitochondrial signaling pathway. Whereas Mcl-1 protein level was increased and extracellular signal-related kinase (ERK)1/2 was activated in cells treated with TRAIL alone, combination treatment dramatically inhibited ERK1/2 activation and down-regulated Mcl-1 protein level. An inhibitor of ERK1/2 activation, PD98059, also augmented TRAIL-induced apoptosis. Combination treatment with PD98059 and TRAIL showed the activation of caspases and mitochondrial pathway, and the down-regulation of Mcl-1 level. These results suggest that cancer cells can be sensitized to TRAIL-induced apoptosis by pre-treatment with aspirin via suppression of ERK1/2 activation. These findings provide a basis for further exploring the potential applications of this combination approach for the treatment of cancer, including cervical cancer.

1. Introduction

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a member of the tumor necrosis factor (TNF) gene superfamily and a promising anticancer cytokine because of its preferential toxicity in cancer cells [1]. TRAIL interacts with so-called death receptors on the cell surface, TRAIL receptor-1 (DR4) and/or TRAIL receptor-2 (DR5), to activate the extrinsic apoptosis signaling pathway. Upon binding of TRAIL to its receptor, initiator caspase-8 is recruited to the receptor and activated via adaptor proteins such as fas-associated death domain (FADD) or TNFRSF1A-associated death domain (TRADD) [2]. Strong caspase-8 activity may in turn directly activate effector caspases (caspase-3, -6, and -7). TRAIL can also activate the intrinsic pathway of apoptosis through mitochondrial events. The intrinsic pathway involves regulation of the activity of Bcl-2 family members, including Bcl-2 and Mcl-1, which control the integrity of the mitochondrial membrane. The release of proapoptotic factors such as cytochrome *c* from the mitochondria into the cytoplasm promotes the activation of initiator caspase-9, which then activates the effector caspases; this may also require signal amplification via cleavage of the Bcl-2 family protein Bid [3].

Recombinant TRAIL induces apoptosis in a diverse set of cancer cell lines *in vitro* and suppresses primary tumor growth and metastasis in preclinical *in vivo* models. It is currently being tested as a cancer therapeutic agent in clinical trials [4,5].

The potential of TRAIL as an antitumor agent, however, is limited by the emergence of resistance to TRAIL-induced apoptosis in many human tumors and cancer cells, including human cervical cancer cells, HeLa. TRAIL resistance can occur through any number of defects in TRAIL signaling pathways, including down-regulation of expression of the TRAIL death receptors, loss of function of the proapoptotic Bcl-2 family member Bax, over expression of anti-apoptotic Bcl-2 family members [6–8]. In addition, TRAIL itself could induce anti-apoptotic pathways [9,10]. Although several groups have reported various potential agents [6,7,11–13], and chemotherapeutic agents in combination with radiation [14,15] by which TRAIL resistance may be overcome, efficient sensitizing strategies still remain to be explored.

Aspirin (acetylsalicylic acid) is a non-steroidal anti-inflammatory drug (NSAID) that suppresses the activity of the cyclooxygenase enzymes (COX-1 and COX-2). Aspirin has additional effects such as antitumor activity in several cancer cell lines, and has been shown to reduce the risk associated with various types of malignancies [16–18]. In addition to its cytotoxic effect on many types of cancer cells, aspirin can sensitize cancer cells to TRAIL-induced apoptosis [19–21]. However, the detailed mechanisms involved

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in sensitizing effect of aspirin to TRAIL treatment still remains unclear. We tested whether aspirin could enhance the sensitivity to TRAIL and investigated the underlying mechanism(s) of TRAIL sensitization in human cervical cancer cells. We found that pretreatment with aspirin augmented TRAIL-induced apoptosis in the cells through inhibition of ERK1/2 activation and a subsequent increase in caspase activity and down-regulation of Mcl-1. These results show that suppression of ERK1/2 activation may be an important means to sensitize cervical cancer cells to TRAIL. Further research on this pathway may result in an efficient cancer treatment modality in the future.

2. Materials and methods

2.1. Cell culture

Human adenocarcinoma (HeLa) cells were obtained from the American Type Culture Collection. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco-BRL) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (Gibco-BRL). Cells were maintained in a 37 °C humidified incubator with a mixture of 95% air and 5% CO_2.

2.2. Reagents

Aspirin purchased from Sigma–Aldrich was dissolved in DMSO and the pH was adjusted to 7.0 using 10 N NaOH. Recombinant human TRAIL/Apo2 ligand (10 non-tagged 19 kDa protein, amino acids 114–281) was obtained from KOMA Biotech (Seoul, South Korea). The ERK1/2 inhibitor PD98059 and caspase inhibitor zVAD-fmk were purchased from Cell Signaling.

2.3. Antibodies

Anti-caspase-3, anti-caspase-8, anti-caspase-9, anti-PARP, anti-Bid, anti-Bak, anti-Mcl-1, anti-phospho-p44/42 MAPK (p-ERK1/2) (Thr202/Tyr204), and anti-ERK1/2 antibodies were purchased from Cell Signaling; anti-Cox-4 and anti-cytochrome *c* antibodies were purchased from Clontech; anti-Bcl-2 antibody was from Epitomics; anti-DR5 and the horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG polyclonal antibodies were from AbCam; anti-DR4 and anti-α-tubulin antibodies were from Upstate and Abfrontier, respectively.

2.4. Cytotoxicity assay (LDH assay)

Lactate dehydrogenase (LDH) is a stable cytosolic enzyme that is released upon membrane damage. LDH activity was measured using a commercial cytotoxicity assay kit, according to the manufacturer's instructions (Promega). Briefly, LDH released into culture supernatants was measured using a coupled enzymatic assay in which conversion of tetrazolium salt results in the formation of red formazan product. Cells were treated as indicated and then LDH released into the culture medium was measured. Absorbance at 490 nm was recorded using a Model 680 Microplate Reader (Bio-Rad).

2.5. Analyses of sub-G1 population of cell cycle

Propidium iodide (PI) staining and flow cytometry were used to assess cell cycle distribution and apoptosis. Cells (2×10^5) were washed with 1 ml of phosphate-buffered saline (PBS) and then treated with 1 ml of 0.05% trypsin–EDTA at 37 °C for 3 min. Cells were washed with 1 ml of PBS and then resuspended in 50 μl of PBS. Resuspended cells were added dropwise into 1 ml of cold 70% ethanol with gentle vortexing and then kept at −20 °C overnight. The fixed cells were centrifuged at 400*g* at 4 °C for 10 min and then the pellets were washed with 1 ml of PBS and resuspended in 50 μl of PI (10 μg/ml in PBS) and RNase (300 μg/ml). Cells were incubated at 37 °C for 60 min before being analyzed by flow cytometry (FACS Aria III model) using Diva program (Becton Dickinson).

2.6. Annexin-V/PI staining

Apoptotic cell death was assessed by double staining using an FITC-labeled annexin V/PI Apoptosis Detection kit (BD Bioscience), according to the manufacturer's instructions. Briefly, cells (2×10^5) were washed with PBS and collected by trypsinization. Cells were sedimented by centrifugation (1500 rpm for 5 min), washed with PBS, resuspended in 200 μl of 1X binding buffer containing 5 μl of annexin V-FITC and 5 μl of PI (10 μg/ml in PBS) for 15 min. Cells were analyzed immediately using the flow cytometer. Cells in early stages of apoptosis are represented by annexin V single-positive cells.

2.7. Immunoblotting

Cells were washed in PBS and then lysed in 2X Laemmli buffer (126 mM Tris–HCl [pH6.8], 4% SDS, 20% glycerol, 0.02% Bromophenol blue and 2% β-mercaptoethanol). Following boiling for 5 min, samples were separated on 8–15% SDS–polyacrylamide gels and then transferred to an Immobilon membrane (Millipore). The membranes were blocked with 5% non-fat dry milk in TBS/Tween20 (0.05% v/v) for 1 h followed by incubation at 4 °C overnight with the indicated primary antibodies. The membranes were washed three times with TBST buffer (10 mM Tris–HCl [pH 7.4], 150 mM NaCl, 0.05% Tween 20) and then incubated for 1 h with HRP-conjugated anti-rabbit or -mouse secondary antibodies. Visualization of protein bands was accomplished using enhanced chemiluminescence (Amersham Life Science).

2.8. Measurement of mitochondrial membrane potential (MMP)

The potential or integrity of the mitochondrial membrane ($\Delta\Psi m$) was evaluated using the cationic dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimi-dazolylcarbocyanineiodide (JC-1) (Invitrogen). Cells (2×10^5) were trypsinized and washed with PBS twice. The cell pellets were incubated at 37 °C for 30 min in 1 ml of complete medium containing 1 μl of a JC-1 stock solution (5 mg/ml). The cells were washed with PBS and then resuspended in 300 μl of PBS. Mean green fluorescence for FITC (FL-1 channel) and orange-red fluorescence for PE (FL-2 channel) were recorded and quantified by flow cytometry. Data were expressed as the ratio of green (JC-1 monomer)/red (JC-1 aggregates) signals. Unstained control cells were evaluated to establish baseline fluorescence.

2.9. Analysis of cytochrome c release

To assess mitochondrial cytochrome c release, cytosolic protein extracts were obtained according to instructions of a commercial cell fractionation kit (Clontech). Briefly, 1×10^6 cells in a dish of 60 mm in diameter were washed twice with cold PBS and then centrifuged at 600*g* for 5 min at 4 °C. Cells were suspended in 0.1 ml of ice-cold fractionation buffer and then allowed to swell on ice for 10 min before being homogenized with a syringe needle. The homogenates were centrifuged at 700*g* for 10 min at 4 °C, after which the supernatants were collected and centrifuged at 10,000*g* for 25 min at 4 °C. The supernatant was again collected as the cytosolic extract free of mitochondria and analyzed for cytochrome *c* release.

2.10. Statistical analysis

All results of bar graphs are expressed as the mean ± S.D. obtained from three independent experiments in duplicate. Statistical differences were evaluated using the Student's *t*-test and a $P < 0.05$ was considered statistically significant.

3. Results

3.1. Aspirin triggers TRAIL-induced apoptosis through caspase activation

To test cytotoxicity, the cells were treated with aspirin (5 mM) for 32 h (24 + 8 h) or TRAIL (100 ng/ml) for 8 h. For the combination treatment, cells were pre-treated with aspirin for 24 h and additionally treated with TRAIL for 8 h without removing aspirin. The relative cytotoxicity was measured by LDH assay (Fig. 1A). Treatment with aspirin or TRAIL alone was not significantly toxic to the cells. When the cells were combination-treated, the rate of cell death increased remarkably. To determine whether enhanced TRAIL-mediated apoptosis by aspirin involved caspase activation, immunoblot analysis was performed. Combination treatment markedly induced the activation of caspase-8, -9, and -3 and the cleavage of poly (ADP-ribose) polymerase (PARP) (Fig. 1B). In addition, Bid was disappeared in the combination treated cells. To confirm the involvement of caspases in the enhanced apoptosis, combination-treated cells were pre-treated with a pan-caspase inhibitor, zVAD-fmk (50 μM) for 24 h, and sub-G1 population of cell cycle and PARP cleavage were analyzed. Increases in sub-G1 population and PARP cleavage, which were induced by combination treatment, were abrogated by pre-incubation with zVAD-fmk (Fig. 1C and D). These results suggested that aspirin promotes TRAIL-induced apoptotic cell death through enhanced caspase activation.

3.2. Aspirin enhances TRAIL-induced apoptosis through the caspase-dependent mitochondrial pathway

As shown in Fig. 1B, combination treatment resulted in decrease of Bid and caspase-9 activation. These results suggest involvement of the mitochondrial pathway of apoptotic cell death. To confirm this, MMP change and cytochrome *c* release from mitochondria to the cytosol in response to treatment were measured. We also investigated whether changes in MMP and cytochrome *c* release were dependent on caspase activation. As shown in Fig. 2A, the MMP, presented as green (monomers)/red (aggregates) ratio of the dye JC-1 in the cells, was clearly increased in the combination-treated cells compared to aspirin or TRAIL alone. Furthermore, pre-incubation with zVAD-fmk completely blocked the effect of combination treatment on MMP loss. These results indicated that the loss of MMP occurred in response to combination treatment, and it was caspase-dependent. The release of cytochrome *c* from mitochondria to the cytosol was assessed in cytosol fractions by immunoblot analysis. Cytochrome *c* was clearly detected in the cytosolic fraction of combination-treated cells, but not in single-treated or untreated cells (Fig. 2B). Cox-4, which is a marker protein of mitochondria, was used as a negative control and not detected in the cytosolic fractions. Cytochrome *c* release from mitochondria can be either dependent on or independent of caspase activation [22,23]. To determine whether the release of cytochrome *c* in combination-treated cells was dependent on caspase activation, cells were pretreated with zVAD-fmk. The presence of cytochrome *c* in the cytosolic fraction of combination-treated cells was completely abrogated by zVAD-fmk. Collectively, these results suggested that pretreatment with aspirin potentiates the caspase-dependent mitochondrial apoptotic pathway in response to TRAIL.

3.3. ERK1/2 inactivation and decreased Mcl-1protein level were induced by combination treatment with aspirin and TRAIL

To investigate further the mechanism(s) by which aspirin enhanced TRAIL-induced apoptosis, we examined changes in the expression levels of various apoptotic regulators by immunoblot analysis. Since TRAIL receptors can be up-regulated by some chemotherapeutic agents [1,6,7], we analyzed the effect of aspirin on DR4 and DR5 expression. At the protein level, DR4 and DR5 expression was not affected by aspirin alone, even after 32 h of treatment (Fig. 3A), TRAIL alone, or combination treatment with two agents (Fig. 3B). In addition, changes in the levels of the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bak were not significant in single- and combination-treated cells (Fig. 3A and B). On the other hand, aspirin changed the level of ERK1/2 phosphorylation.

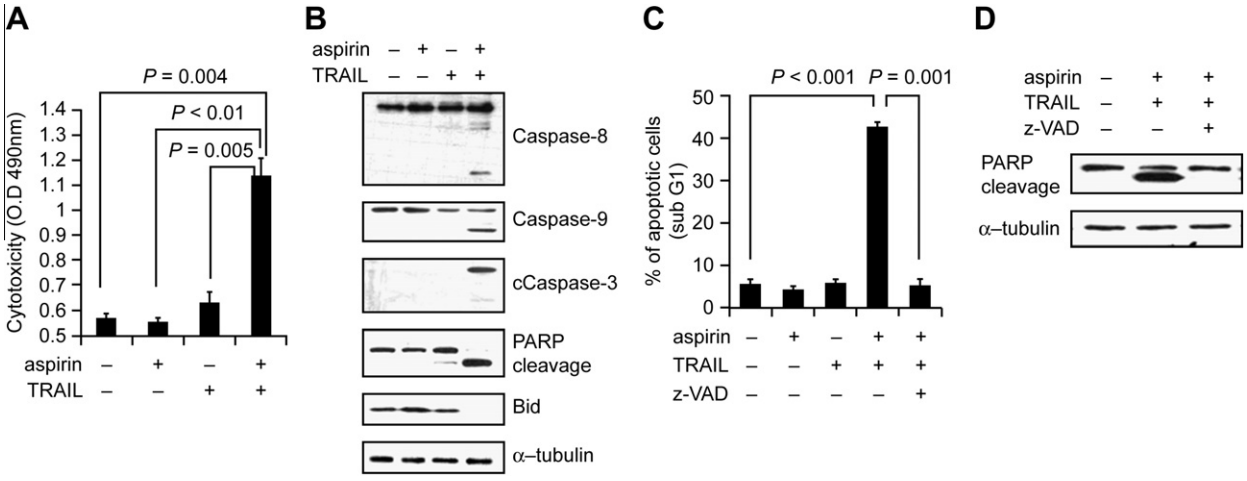

Fig. 1. Enhancement of TRAIL-induced apoptosis by aspirin through caspase activation. (A) LDH assay for measuring cytotoxicity (B) Changes in protein expression in cell lysates after treatment were assessed by immunoblot analysis (C) Flow cytometric analysis for measuring percentage of cells with sub-G1 cell cycle. Cells seeded at a density of 2×10^5 cells per well in 6-well plates were stabilized for 20 h, and incubated with 5 mM aspirin for 24 h or 100 ng/ml TRAIL for 8 h. For combination treatment, cells were pretreated with aspirin for 24 h and then treated with TRAIL for 8 h continuously. For analysis of cell cycle by flow cytometry, cells were stained with PI after treatment. Effects of zVAD-fmk on percentage of cells with sub-G1 phase (C) and PARP cleavage (D) were analyzed. Cells were pretreated with 50 μM zVAD-fmk for 1 h before each treatment. Data in bar charts (A and C) represent the means ± SD of three independent experiments. In (B and D), α-tubulin was used as loading controls.

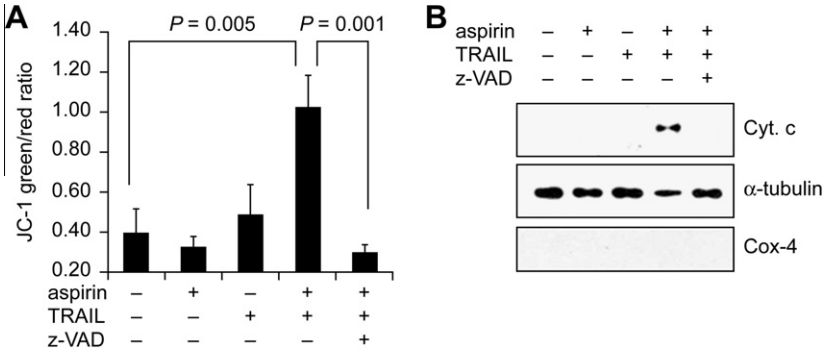

Fig. 2. Effects of combination treatment with aspirin and TRAIL on MMP change and cytochrome *c* release. (A) Measurement of MMP change induced by single- or combination-treated in the presence or absence of 50 μM of zVAD-fmk. Data represent the means ± SD of three independent experiments. (B) Analysis of cytochrome c release from mitochondria to cytosol. Cytosolic fractions from 1×10^6 cells were prepared and analyzed by immunoblot. α-Tubulin was used as a loading control, and Cox-4 was analyzed to monitor the separation of cytosolic from mitochondrial fractions.

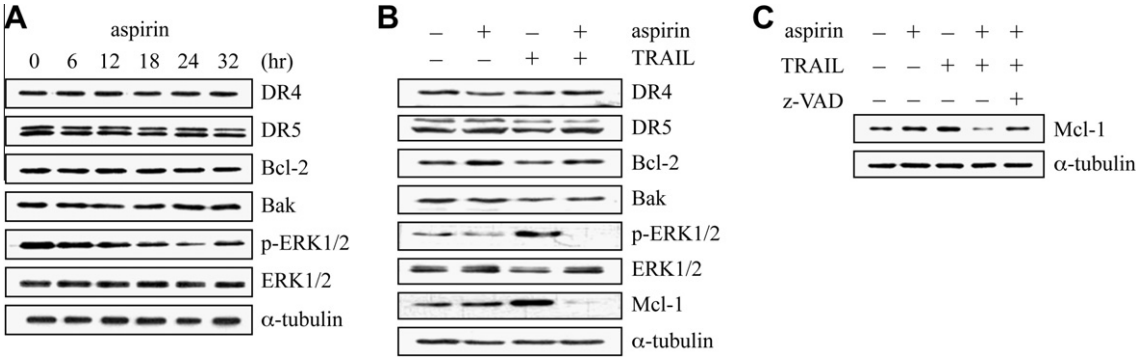

Fig. 3. Inhibition of ERK1/2 activation and Mcl-1 down-regulation by combination treatment with aspirin and TRAIL. Cells were treated with aspirin for the indicated periods (A) or single- and co-treated with aspirin and TRAIL (B and C), and then total protein extracts were subjected by immunoblot analysis. In (C), 50 μM of zVAD-fmk was pretreated for 1 h. α-Tubulin was used as loading controls in the immunoblot experiments.

Phosphorylation of ERK1/2, up-regulated by TRAIL alone (Fig. 3B), was decreased by aspirin alone in a time-dependent manner up to 24 h and combination treatment (Fig. 3A and B). The protein level of Mcl-1, also up-regulated by TRAIL alone, was also decreased in combination-treated cells (Fig. 3B). The decrease of Mcl-1 by combination-treatment was blocked by pre-treatment of zVAD-fmk, indicating Mcl-1 protein level was regulated by caspases (Fig. 3C). It is suggested that ERK1/2 inactivation and Mcl-1 down-regulation by aspirin are involved in enhancing TRAIL-induced apoptosis.

To confirm the critical role of aspirin as suppressor of ERK1/2 activation, PD98059, an ERK inhibitor, was pre-treated instead of aspirin in TRAIL-treated cells, and the results were assessed by several methods. Based on the results of annexin V-FITC/PI staining analysis, sub-G1 population analysis, and MMP change analysis, the rate of apoptosis was clearly increased by the combination of PD98059 and TRAIL in comparison to TRAIL alone (Fig. 4A). In the results of MMP change, increment of JC-1 monomer released from mitochondria to cytosol was obvious, resulting in increased ratio of FITC/PE. Combination treatment with PD98059 and TRAIL also showed increased TRAIL-induced cytotoxicity in LDH assay (data not shown). However, the increased rate of apoptosis induced by combination of PD98059 and TRAIL (11.3% of sub G1) was lower in comparison to that by combination of aspirin and TRAIL (42.9% in Fig. 1C), suggesting that ERK1/2 activation may not be only target of aspirin for sensitizing TRAIL-treated cells. Inhibition of ERK1/2 activation by PD98059 promoted TRAIL-induced activation of caspases and PARP cleavage, as well as down-regulation of Mcl-1 (Fig. 4B). Bcl-2 level, however, was not changed by combination treatment. Collectively, these results indicate that pre-treatment with aspirin results in ERK1/2 inactivation, and at least the blockade of ERK1/2 activation is a critical mechanism for sensitizing cells to TRAIL-induced apoptosis by promoting the activation of caspases, Mcl-1 down-regulation, and mitochondrial signaling pathway.

4. Discussion

Apoptosis is a complex process of cell death involving many cellular factors and patho-physiologic pathways. The abnormal regulation of apoptosis underlies a number of diseases, including cancer. Because of its ability to induce cancer cell-specific apoptosis, TRAIL represents a promising new anticancer therapeutic [1,4,5]. However, many human cancer cells are resistant to TRAIL-mediated apoptosis. Thus, for therapy to be effective, approaches to circumvent tumor cell resistance to TRAIL, such as sensitization by other agents, is needed. Here, we demonstrated that aspirin could sensitize cancer cells to TRAIL-induced apoptosis, and the sensitizing effect occurs at least through modulation of ERK1/2 activity.

ERK1/2 is the canonical member of the MAPK family and is implicated in the regulation of a variety of cellular processes. Phosphorylation and activation of ERK1/2 can facilitate cell survival or death. For example, ERK1/2 activation is required for cisplatin-induced apoptosis [24]. By contrast, ERK1/2 activation promotes cell survival in pancreatic cancer cells and melanoma cells [25,26]. We showed that activation of ERK1/2 was induced in TRAIL-resistant HeLa cells, when treated with TRAIL alone, suggesting that ERK1/

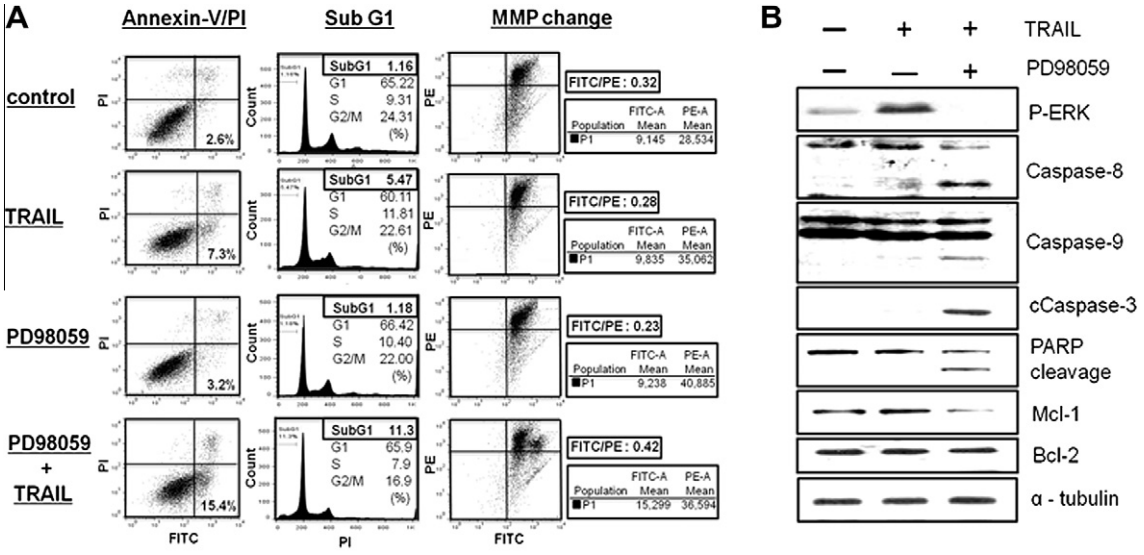

Fig. 4. Effects of an inhibitor of ERK1/2 phosphorylation on TRAIL-induced apoptosis. For inhibiting ERK1/2 phosphorylation, cells were pretreated with PD98059 (20 μM) for 2 h before treatment. Annexin-V-FITC/PI staining and FACS analysis, analysis of sub-G1 population, analysis of MMP change (A), and immunoblotting (B) were performed as described in Section 2.

2 activation in response to TRAIL is important in cellular resistance to apoptosis. Interestingly, the combination of aspirin and TRAIL significantly suppressed ERK1/2 activation, in company with the increased apoptotic cell death and the induction of caspase activation and the caspase-dependent mitochondria pathway. When we tested cytotoxic effects induced by an inhibitor of ERK1/2 and TRAIL, the effects were lower than those by aspirin and TRAIL, suggesting that blocking of ERK1/2 activation was necessary, but not sufficient to sensitize TRAIL-treated cells by aspirin. The sum of these findings point to ERK1/2 inactivation by aspirin being one of the critical mechanisms of sensitization of HeLa cells to TRAIL.

Mcl-1 is an anti-apoptotic Bcl-2 family protein. It has been suggested that down-regulation of Mcl-1 might induce apoptosis via the translocation of tBid, which is cleaved by caspase-8, from the cytosol to the outer mitochondrial membrane, resulting in triggering a mitochondrial pathway and amplifying caspase activation [27]. We showed combination treatment with aspirin and TRAIL down-regulated Mcl-1, the expression of which was actually enhanced by treatment with TRAIL alone. It is suggested that Mcl-1 is also involved in aspirin-induced sensitization to TRAIL, although we did not address the detailed link between ERK1/2 inactivation and Mcl-1 down-regulation. We also demonstrated that the down-regulation of Mcl-1 induced by combination treatment with aspirin and TRAIL was prevented by pan-caspase inhibitor, indicating that Mcl-1 cleavage was dependent on caspases, which were activated by combination treatment. The protein levels of Bcl-2 and Bak were not affected by combination of the two agents, indicating that their actions are not necessary in aspirin-induced sensitization of HeLa cells to TRAIL.

In summation, it is suggested that ERK1/2 inactivation by pretreatment with aspirin can promote TRAIL-induced caspase activation and activate caspase-dependent cleavage of Mcl-1, triggering the mitochondrial pathway of apoptosis. Inhibition of TRAIL-mediated ERK1/2 activation by aspirin might be an effective strategy to sensitize cancer cells to TRAIL-mediated apoptosis.

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