A THESIS

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Piceatannol enhances TRAIL-induced apoptosis in human leukemia THP-1 cells through Sp1- and ERK-dependent DR5 up-regulates

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Piceatannol enhances TRAIL-induced apoptosis in human leukemia THP-1 cells through Sp1- and ERK-dependent DR5 up-regulation

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ABSTRACT

Although piceatannol (PIC) is known to mediate anti-cancer, anti-inflammatory, and anti-oxidant activities, little is known about the mechanism of PIC in terms of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis. In this study, we examined whether combined treatment with PIC and TRAIL synergistically induces apoptosis in THP-1 leukemia cells. Results indicate that PIC substantially enhances TRAIL-induced cell death including DNA fragmentation and poly(ADP-ribose) polymerase cleavage. Consistent with TRAIL-induced apoptosis, PIC significantly increased the mRNA and protein expression levels of DR5, a death receptor of TRAIL. Further, PIC enhanced DR5 promoter activity via Sp1 activation. Interestingly, the DR5 chimera antibodies significantly suppressed PIC and TRAIL-mediated apoptosis. The inhibitor of ERK also decreased PIC and TRAIL-induced apoptosis by blocking DR5 expression. In conclusion, our results suggest that PIC sensitizes TRAIL-induced apoptosis via Sp1- and ERK-dependent DR5 up-regulation.

Keywords: Piceatannol; TRAIL; Death receptor; Sp1; ERK

ABSTRACT IN KOREAN

Piceatannol (PIC)의 항 암, 항 염증, 항 산화 효과에 대해서는 알려져 있으나 PIC와 TRAIL의 병행 처리에 대한 mechanism은 거의 밝혀져 있지 않기 때문에 이번 연구를 진행 하였다. 이 연구에서 우리는 PIC와 TRAIL의 병행 처리가 사람의 백혈병 세포주인 THP-1 세포에서 synergistically 하게 세포사멸 유도 효과를 갖고 있음을 확인 하였다. 결과들을 보면, PIC는 TRAIL이 유도 하는 DNA fragmentation과 PARP의 cleavage의 형성을 아주 강하게 증가시켰음을 알 수 있다. 이러한 효과는 PIC가 TRAIL 수용체인 death receptor 5 (DR5)의 발현을 mRNA에서부터 발현을 증가 시켰기 때문이다. 이것은 PIC에 의해서 DR5의 주요 전사인자인 Sp1이 핵 안으로 translocation 활성이 증가하여 DNA 결합 활성이 증가 함에 따라 DR5의 promoter활성이 증가에 의한 것이었다. 흥미로운 것은, DR5와 TRAIL의 결합을 방해 하기 위하여 DR5 결합 항체를 제작 하여 처리 하게 되면 PIC와 TRAIL이 유도 하던 세포 사멸 효과가 강하게 억제가 되었다. 이에 따라 DR5의 증가가 세포사멸에 강하게 영향을 미치고 있음을 증명하였다. 뿐만 아니라, MAPK 중 하나인 ERK의 활성 억제제를 처리 하였을 때 역시 세포사멸 유도 효과가 억제 됨을 확인 하였고 이는 ERK 억제제가 DR5의 발현을 억제 하였기 때문이었다. 결론적으로 우리의 결과는 PIC가 Sp1과 ERK 의존적으로 DR5의 발현을 증가시키기 때문에 TRAIL의 세포 사멸 유도 효과를 강하게 한다는 것을 증명하였다.

1. INTRODUCTION

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a member of the TNF superfamily members, has been established to induce apoptosis in various human cancer cells *in vivo* and *in vitro*, without inducing any side-effects on normal cells (Wiley et al., 1995; Kischkel et al., 2000; Van Geelen et al., 2004). Previous studies have demonstrated that TRAIL induced-apoptosis is mediated by two cellular membrane proteins, TRAIL receptor, DR4 or DR5 (Sheridan et al., 1997). Binding of TRAIL to DR4 or DR5 leads to the activation of Fas-associated death domain and procaspase-8 (Chaudhary et al., 1997; Pan et al., 1997a; Pan et al., 1997b), which then forms death-inducing signaling complex, thereby inducing death signaling and the apoptosis pathway (Muzio et al., 1996; Sprick et al., 2000). Nevertheless, a recent study has demonstrated that many tumor cells acquire resistance to TRAIL-induced apoptosis through down-regulation of their death receptors (Jin et al., 2006). Thus, it is important to develop an agent that could regulate the DR4 and/or DR5 expression and overcome TRAIL resistance. Recent evidence suggests that treating leukemia cells with a combination of TRAIL and dihydroflavonol BB-1 or honokiol enhances TRAIL-induced apoptosis (Hasegawa et al., 2006; Raja et al., 2008). Consequently, a natural product with strong synergistic activity in TRAIL-induced apoptosis may be

a novel tool in developing anti-cancer therapy.

Piceatannol (PIC; 3,5,3',4'-tetrahydroxy*trans*-stilbene), a phenolic compound and an analogue of resveratrol naturally occurring in grapes and red wine (Roupe et al., 2006), has been shown to possess anti-cancer properties in leukemia, melanoma, and other cancer cell lines. The anti-cancer activity of PIC appears to occur primarily through reduction of anti-apoptotic protein Bcl-2 and inhibitors of apoptosis (IAP) family expression, and through caspase activation (Kuo and Hsu 2002; Larrosa et al., 2004; Chowdhury et al., 2005; Kim et al., 2008b). In addition, PIC has also been shown to possess anti-inflammatory activity via down-regulation of nuclear transcription factor (NF-κB) (Islam et al., 2004; Jin et al., 2006). However, the precise mechanism of PIC on TRAIL-mediated cancer cell death has not been fully elucidated thus far.

In this study, we examined whether the combined treatment of PIC and TRAIL (PIC/TRAIL) induces apoptosis in human leukemia THP-1 cells. We found that the combined treatment with PIC/TRAIL increases cell death via up-regulated DR5 expression through activation of extracellular signal-regulated protein kinase (ERK) and Sp1. Together, findings suggest that PIC is an ideal candidate for TRAIL-induced apoptosis.

2. MATERIALS AND METHODS

2.1. Reagents and antibodies

PIC, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and propidium iodide (PI) were purchased from Sigma (St. Louis, MO). PIC was dissolved in DMSO (vehicle control). PD98059, SB203580, and SP600125 were obtained from Calbiochem (San Diego, CA). RPMI 1640 medium and fetal bovine serum (FBS) was purchased from Invitrogen Corp. (Carlsbad, CA) and GIBCO-BRL (Gaithersburg, MD), respectively. Antibodies against caspase-3, caspase-8, caspase-9, cytochrome-*c*, DR4, DR5, and PARP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against Sp1, nucleoline, p-JNK, JNK, p-ERK, ERK, p-p38 and p38 were purchased from Cell Signaling (Beverly, MA). Antibody against β-actin was obtained from Sigma. Peroxidase-labeled rabbit and mouse polyclonal immunoglobulins were purchased from KOMA Biotechnology (Seoul, Republic of Korea).

2.2. Cell culture and treatment with drugs

Human monocytic leukemia cells (THP-1 and U937) and human acute myeloblastic leukemia cells (HL60) were obtained from the American Type Culture

Collection (Manassas, VA). Cells were cultured in RPMI 1640 supplemented with 10% FBS and antibiotics (Sigma). Cells were seeded at 1×10^5 cells/ml and treated with the indicated concentrations of PIC, TRAIL or PIC/TRAIL. After treatment for 24 h, cell viability was determined by MTT assays and by under microscope, respectively.

2.3. Flow cytometric analysis

Cells were fixed with 1 U/ml RNase A (DNase free) and 10 μg/ml propidium iodide (Sigma) overnight at room temperature in the dark. A FACSCalibur flow cytometer (Becton Dickenson; San Jose, CA) was used to analyze the level of apoptotic cells containing sub-G_1 DNA content. For annexin-V staining, live cells were incubated with annexin-V (R&D Systems, Minneapolis, MN) according manufacturer's instructions.

2.4. In vitro *caspase-3 activity assay*

A caspase-3 activation kit was used according to the manufacturer's protocol (R&D systems, Minneapolis, MN) to measure the activity of caspase-like protease.

2.5. DNA fragmentation assay

Cells were lysed in a buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, and 0.5% Triton X-100 for 30 min on ice. Lysates were vortexed and cleared by centrifugation at 10,000 *g* for 20 min. Fragmented DNA in the supernatant was extracted with an equal volume of phenol/chloroform/isoamyl alcohol mixture (25:24:1) and analyzed.

2.6. Western blot analysis

Total cell extracts were prepared using the PRO-PREP protein extraction solution (iNtRON Biotechnology; Sungnam, Republic of Korea). Total cell extracts were separated on polyacrylamide gels and standard procedures were used to transfer them to the nitrocellulose membranes. The membranes were developed using an ECL reagent (Amersham; Arlington Heights, IL).

2.7. RT-PCR analysis

Total RNA was extracted from THP-1 cells using the TRIzol reagent (Invitrogen; Carlsbad, CA). RT-PCR was performed following the manufacturer's protocol (One-Step RT-PCR Premix; Daejeon, Republic of Korea). The sense primer 5'-GTC TGC TCT GAT CAC CCA AC-3' and the anti-sense primer 5'-CTG CAA CTG TGA CTC

CTA TG-3' were used to amplify human DR5 mRNA. The sense primer 5'- CTG AGC AAC GCA GAC TCG CTG TCC AC-3' and the anti-sense primer 5'- TCA AAG GAC ACG GCA GAG CCT GTG CCA T-3' were used to amplify human DR4 mRNA. For glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the sense primer 5'-CGT CTT CAC CAT GGA GA-3' and the anti-sense primer 5'-CGG CCA TCA CGC CCA CAG TTT-3' were used.

2.8. Luciferase assays

The pDR5/-605 plasmid [containing DR5 promoter sequence (-605/+3)] was kindly provided by Dr. T. Sakai (Kyoto Prefectural University of Medicine, Kyoto, Japan) (Yoshida et al., 2001). Briefly, THP-1 cells were seeded onto 6 well plates at a density of 5×10^4 cells/ml and grown overnight. The cells were co-transfected with 1 μg of pDR5/-605 plasmid construct and 0.2 μg of the pCMV-β-galactosidase plasmid by the LipofectAMINE Plus reagent (Life Technologies), in accordance with the manufacturer's instructions. Luciferase activity was normalized by β-galactosidase activity in cell lysates and expressed as an average of three independent experiments.

2.9. Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was performed using the EZ-Chip^{TM} assay kit according to the manufacturer's protocol (Upstate Biotechnology; Lake Placid, NY). The primers used for the amplification of the Sp1 binding site of DR5 promoter region were as follows: 5'-GCC AGG GCG AAG GTT A-3' (sense) and 5'-GGG CAT CGT CGG TGT AT-3' (antisense) (276-bp DNA product).

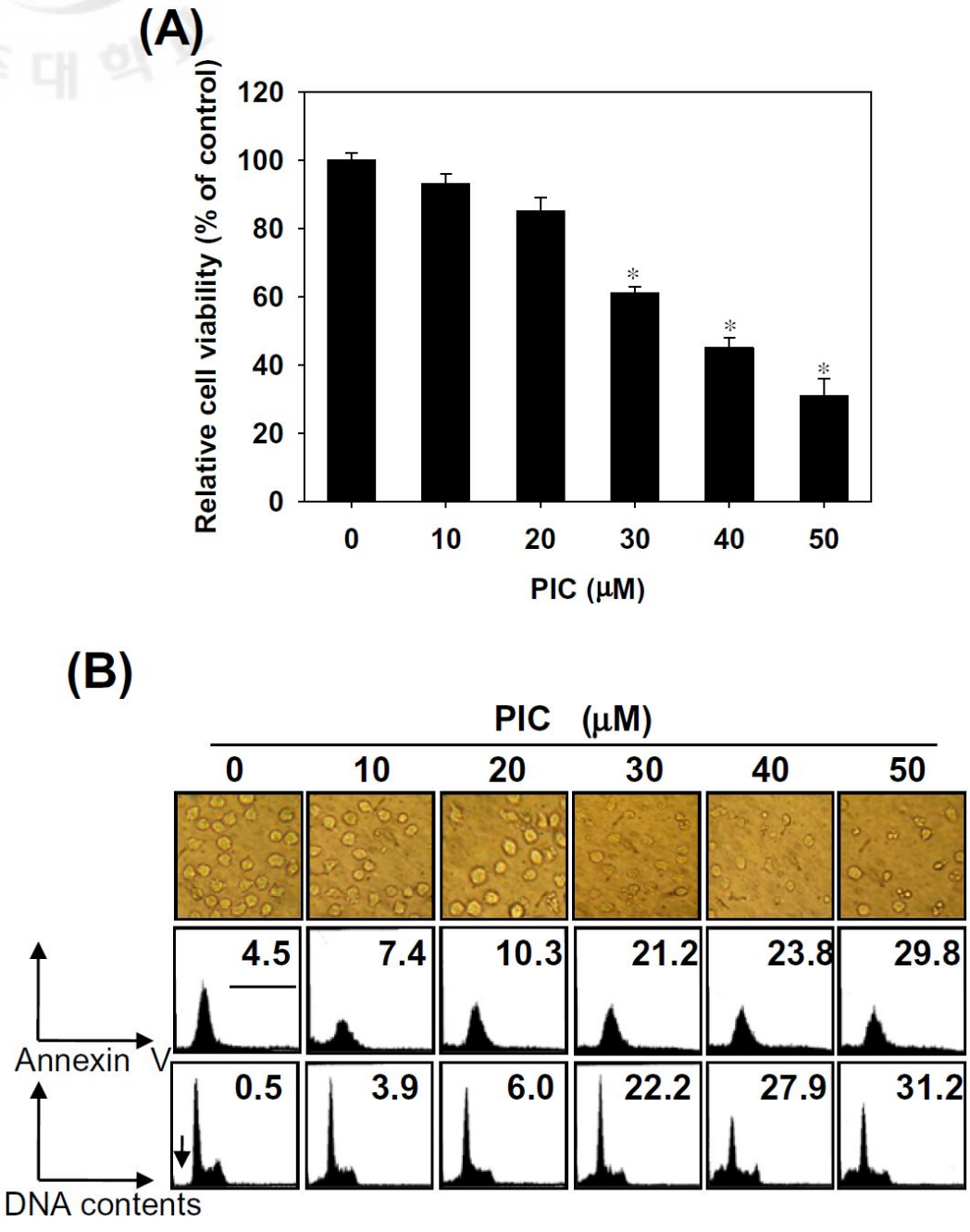
2.10. Statistical analysis

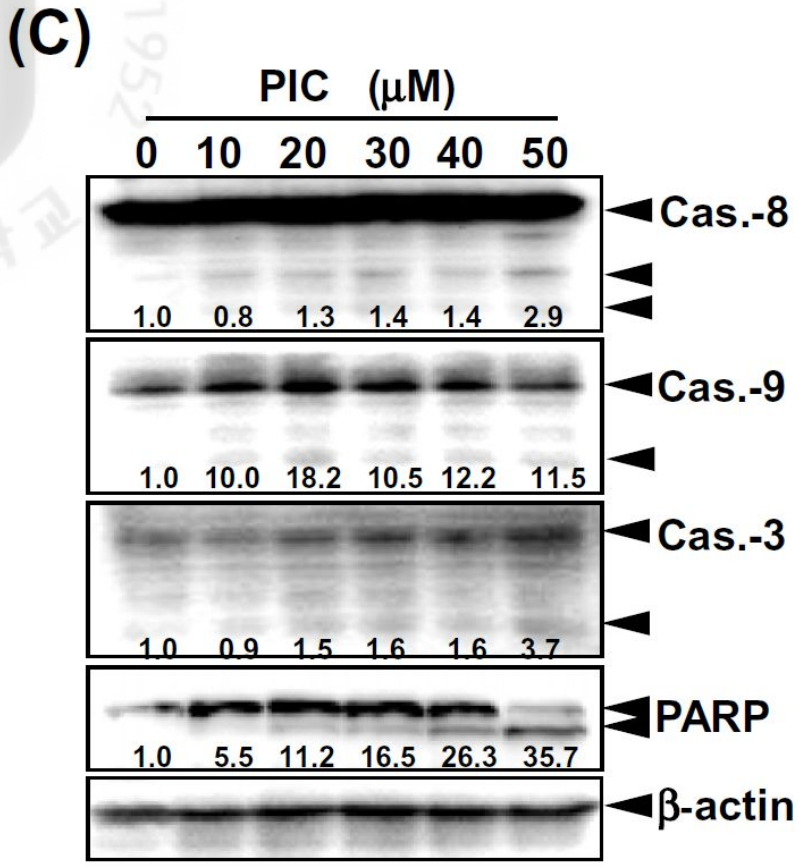
All data were derived from at least three independent experiments. The images were visualized with Chemi-Smart 2000 (Vilber Lourmat, Marine; Cedex, France). Images were captured using Chemi-Capt (Vilber Lourmat) and transported into Photoshop. All bands were quantified by Scion Imaging software (http://www.scioncorp.com). Statistical analyses were conducted using SigmaPlot software (version 6.0). Values were presented as mean ± SD. Significant differences between the groups were determined using the unpaired Student's *t*-test. Statistical significance was regarded at $P < 0.05$.

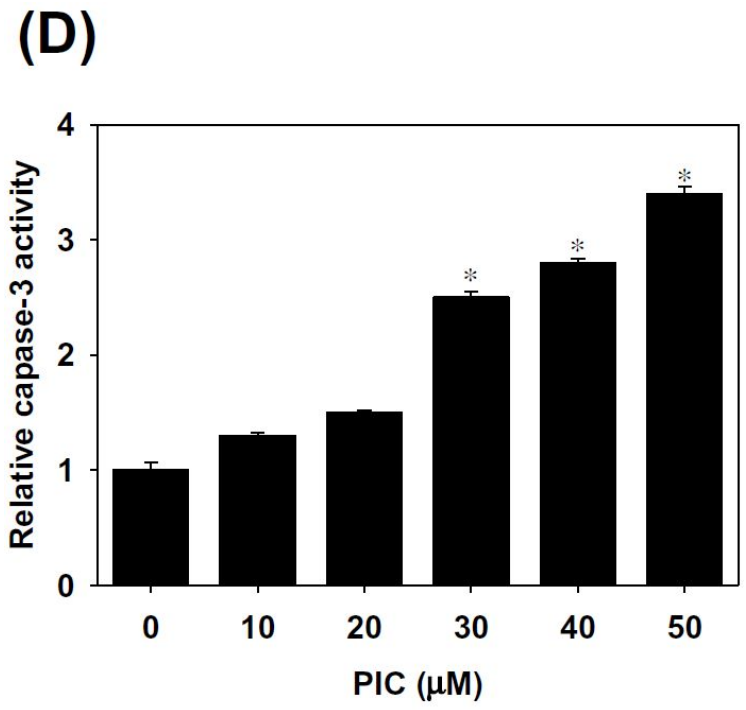
3. RESULTS

3.1. PIC induces apoptosis in THP-1 cells via mitochondrial dysfunction

In order to investigate whether PIC induces anti-proliferative activity, we treated the indicated concentrations of PIC in THP-1 cells for 24 h. Treatment with PIC resulted in dose-dependent inhibition of cell viability (Fig. 1A). PIC also inhibited cell growth in HL60 and U937 leukemia cells (data not known). These results were further confirmed by light microscopic and FACS analysis. We found that cell shrinkage (upper panel), annexin-V+ populations (middle panel), and sub-G_1 phases (lower panel) were significantly increased at more over 30 μM PIC (Fig. 1B). Furthermore, we attempted elucidate the putative apoptotic effect of PIC by mitochondrial dysfunctions using Western blot analysis. Treatment with PIC resulted in a significant increase in the cleavage of capase-3, -8, -9, and PARP (Fig. 1C). In consistent with the cleavage of effector molecules, PIC highly increased caspase-3 activity (Fig. 1D). PIC treatment also decreased mitochondrial potential in a dose-dependent manner (Fig. 1E). Thus, we also found that cytochrome-*c* is released into cytosol by treatment with PIC (Fig. 1F). These results indicate that PIC triggers apoptosis in human leukemia cells via mitochondrial dysfunctions.







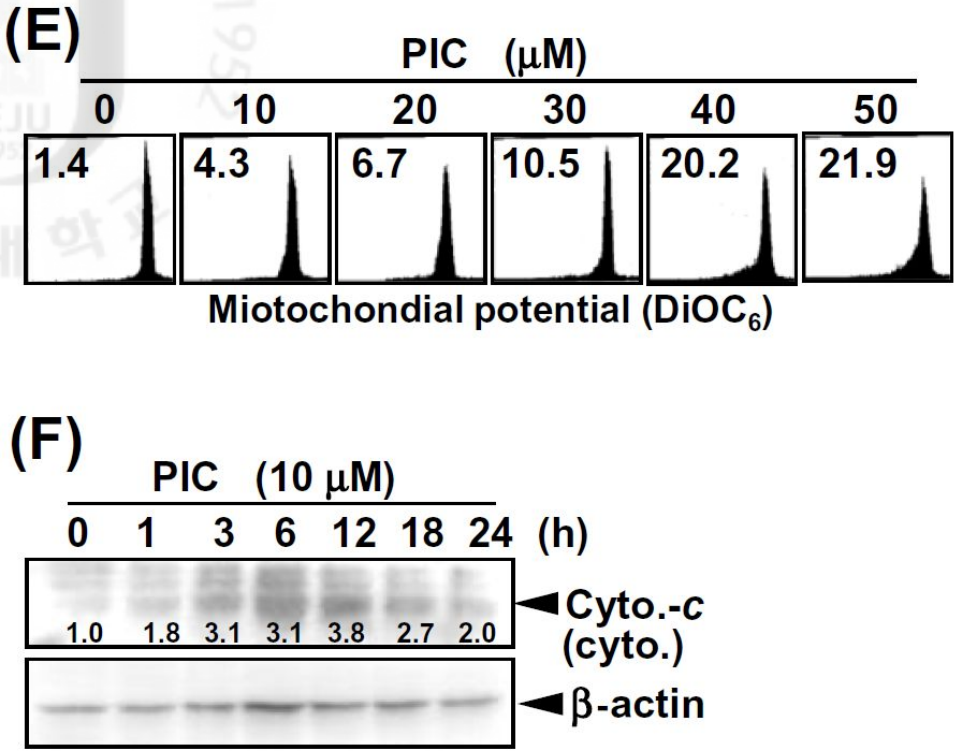


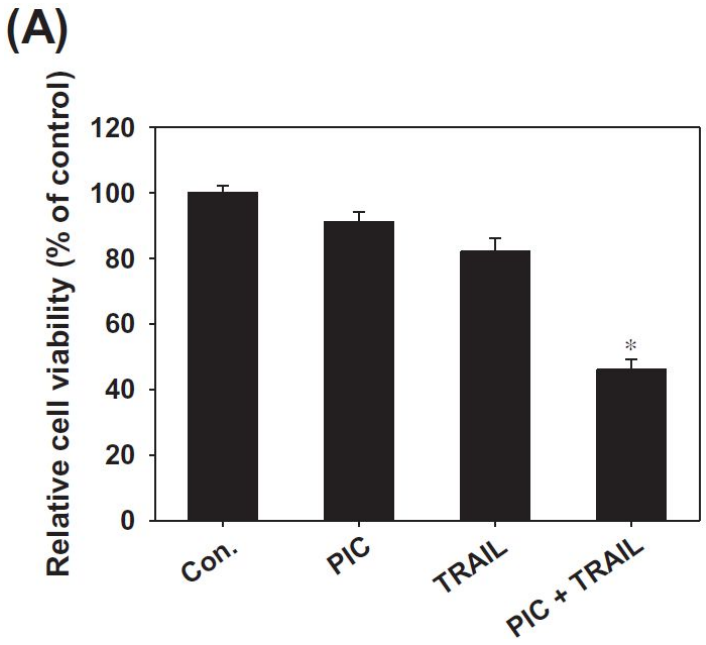
Fig. 1. PIC reduces a dose-dependent viability in THP-1 cells. Cells were seeded at 1 × 10^5 cells/ml and treated with the indicated concentrations of PIC for 24 h. (A) Cell viability was determined by MTT assay. (B) The cellular morphology of cells with or without PIC for 24 h was examined under light microscopy (× 400) (upper panel). Annexin-V^+ population (middle panel) and sub-G_1 cell distribution (lower panel) of THP-1 cells were analyzed using flow cytometer. (C) For analyzing expression of apoptotic proteins, cells were treated with PIC for 24 h and lysed. Samples (30 μg protein/lane) were subjected to 10% SDS-PAGE, transferred to nitrocellulose membrane, and probed with specific antibodies. (D) Caspase-3 activity was

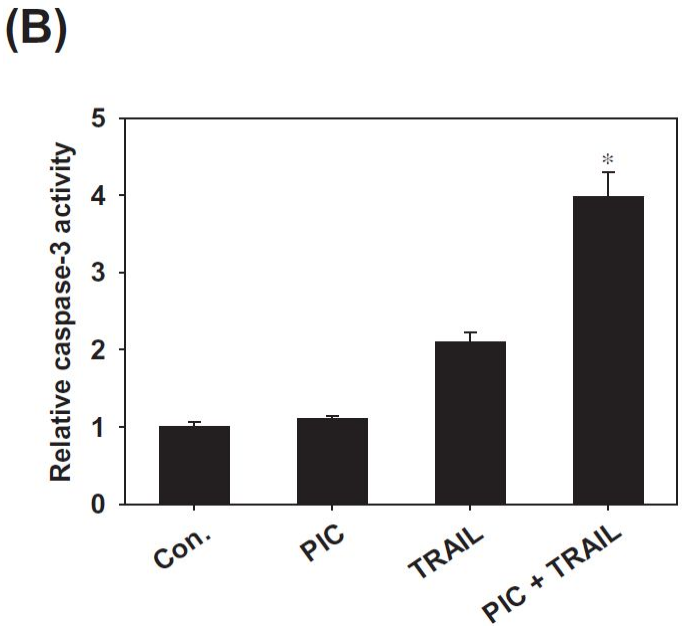
determined following the manufacturer's protocol. (E) Mitochondrial membrane potential was measured by $DiOC_6$ dye using flow cytometry. (F) The translocation of cytochrome-*c* into cytosol was analyzed by Western blot analysis. β-Actin was used as a loading control. Data are representative of three independent experiments. Each point represents the mean ± SD of three independent experiments. The significance was determined by Student's *t*-test (*, $p < 0.05$ *vs.* vehicle control). Cas.-8, caspase-8: Cas.-3, caspase-3; Cas.-9, caspase-9; Cyto-*c*, cytochrome-*c*.

3.2. PIC triggers TRAIL-induced apoptosis in THP-1 cells

To investigate the effects of PIC on TRAIL-mediated apoptosis, THP-1 cells were treated with sub-cytotoxic 10 μM PIC or 20 ng/ml TRAIL alone, and PIC/TRAIL. We did not find any cytotoxicity in treatment with PIC or TRAIL alone (Fig. 2A). However, treatment with both PIC and TRAIL remarkably triggered THP-1 cell death to approximate 50%. During this process, the combined treatment significantly increased caspase-3 activity (Fig. 2B). Next, we examined the cytotoxic effects in combination with PIC and TRAIL in other human leukemia cells U937 and HL60. In consistent with the date in THP-1 cells, PIC or TRAIL alone did not significantly induce cytotoxicity, however the cytotoxicity was remarkably increased by treatment with a combination of PIC and TRAIL (Fig. 2C). To assess the effects more detail, we analyzed the cell populations of annexin-V^+ and sub-G_1 phase using flow cytometer. Treatment with PIC or TRAIL alone induced limited cell death (< 10%) at 24 h (Fig. 2D). However, PIC/TRAIL significantly increased the accumulation of annexin-V staining (upper panel) and sub-G_1 phase (lower panel) in THP-1 cells. Western blot analysis also revealed that PIC/TRAIL increases PARP cleavage to a greater extent than do PIC or TRAIL alone (Fig. 2E). Furthermore, DNA fragmentation analysis showed a typical ladder pattern of internucleosomal DNA

fragmentation treated with PIC/TRAIL, but not in cells treated with PIC or TRAIL alone (Fig. 2F). These findings suggest that PIC treatment enhances apoptosis induced by TRAIL in human leukemia cells.





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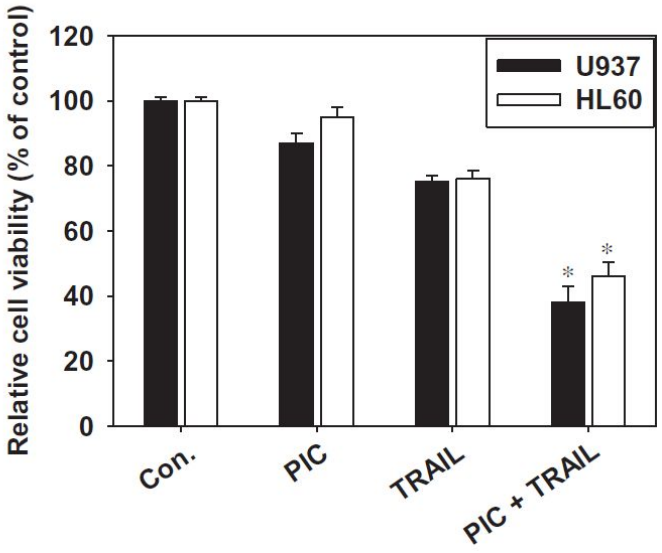

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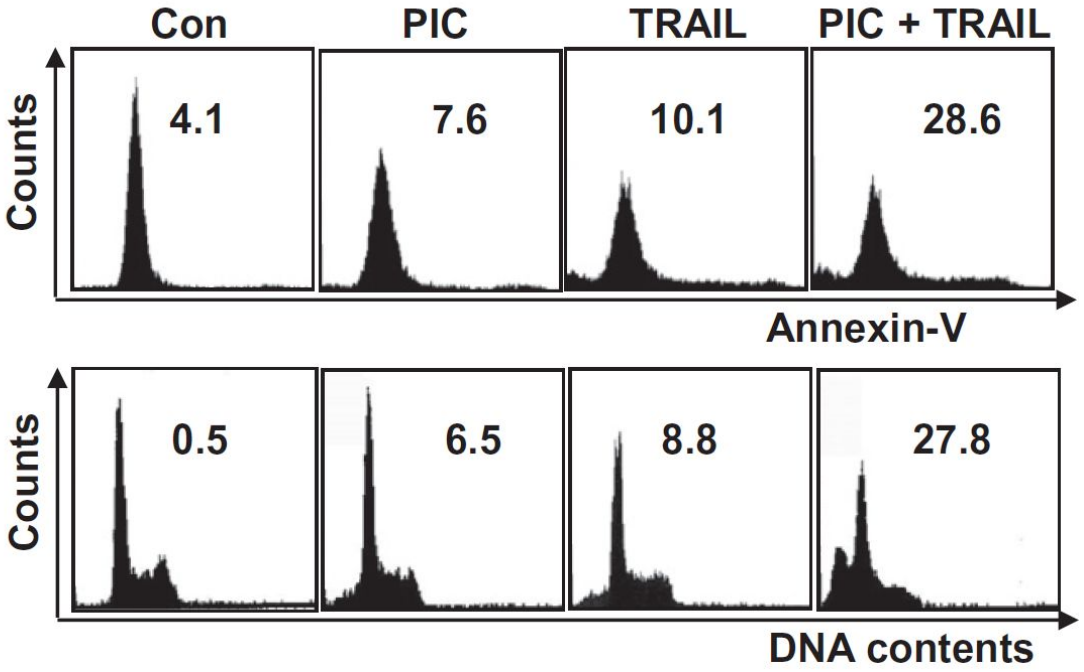


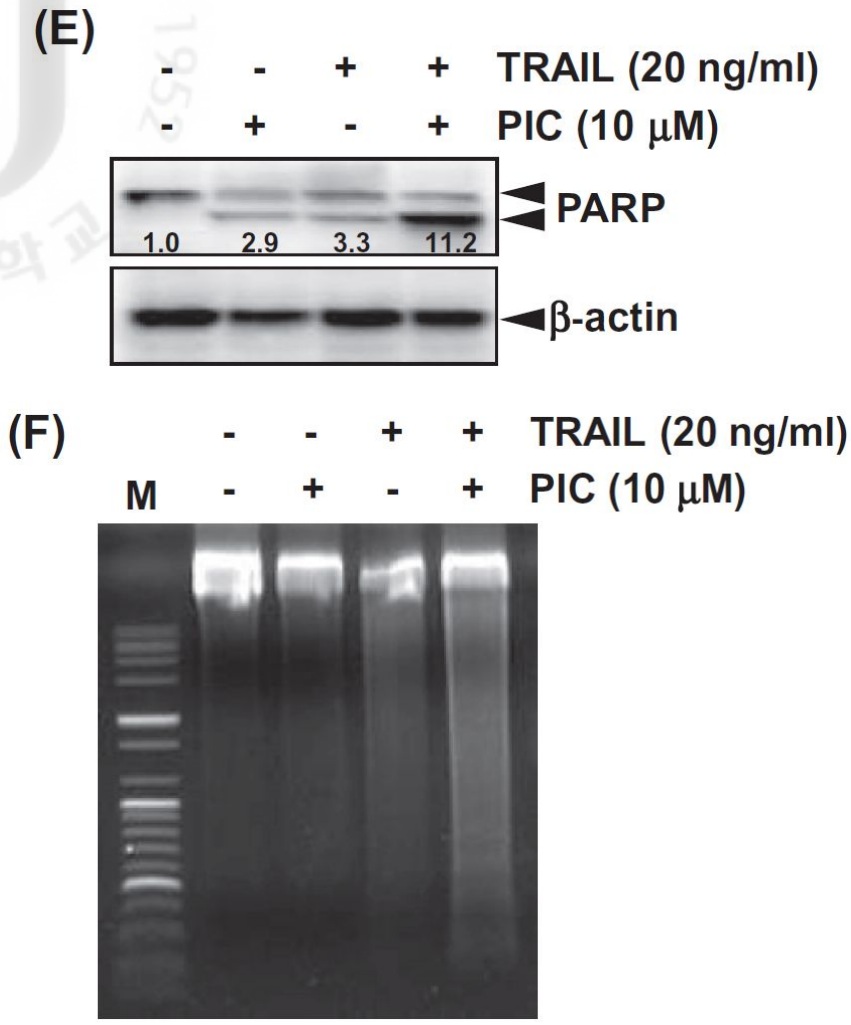
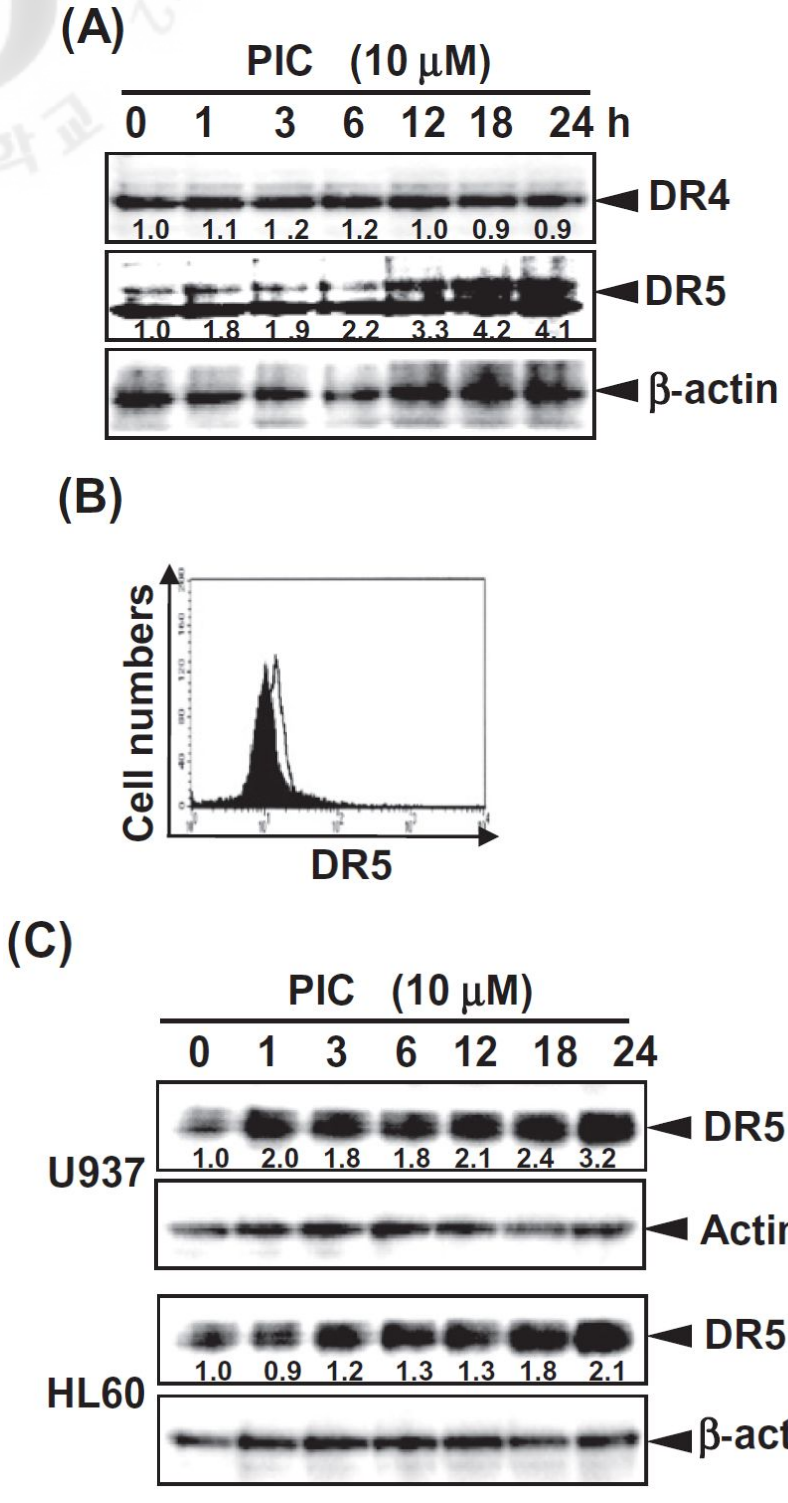


Fig. 2. PIC sensitizes TRAIL-induced apoptosis in human leukemia cell. THP-1 cells were treated with 10 μM PIC alone, 20 ng/ml TRAIL alone, or PIC/TRAIL for 24 h. (A and C) Cell viability was determined by MTT assay [(A); THP-1 and (C); U937 and HL60]. (B) Caspase-3 activity was determined following the manufacturer's protocol. (D) Annexin-V^+ and sub-G_1 populations were analyzed using flow cytometry. (E) For the effect of PIC/TRAIL on levels of PARP, equal amounts of cell

lysates were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with specific antibodies to PARP. β-Actin was used as an internal control. (F) After the indicated treatment for 24 h, fragmented DNAs were extracted from the treated cells and analyzed on 1.5% agarose gel. Data are representative of three independent experiments. Each point represents the mean ± SD of three independent experiments. The significance was determined by Student's *t*-test (*, $p < 0.05$ *vs.* vehicle control).

3.3. PIC enhances PIC/TRAIL induced-apoptosis via up-regulation of DR5 expression

Given that TRAIL is known to induce apoptotic signals through two types of death receptors, DR4 and DR5, we examined whether PIC treatment regulates the expression levels of DR4 and/or DR5. Treatment with PIC increased expression levels of DR5 in a time-dependent manner, but does not affect the levels of DR4 in THP-1 cells (Fig. 3A). FACS analysis also showed that the PIC-induced surface expression of DR5 was significantly increased in THP-1 cells (Fig. 3B). Consistent with these results, PIC treatment also increased DR5 protein levels in other human cancer cell lines, U937 and HL60 (Fig. 3C). Next, we assessed whether DR5 expression is associated with PIC/TRAIL-induced apoptosis. Treatment with DR5 chimera antibodies restored PIC/TRAIL-induced apoptosis in THP-1 cells (Fig. 3D). Similarly, addition of DR5 chimera antibodies decreased the DNA fragmentation induced by PIC/TRAIL treatment (Fig. 3E). These results indicate that PIC-induced up-regulation of DR5 is important for the enhancement of TRAIL sensitivity.



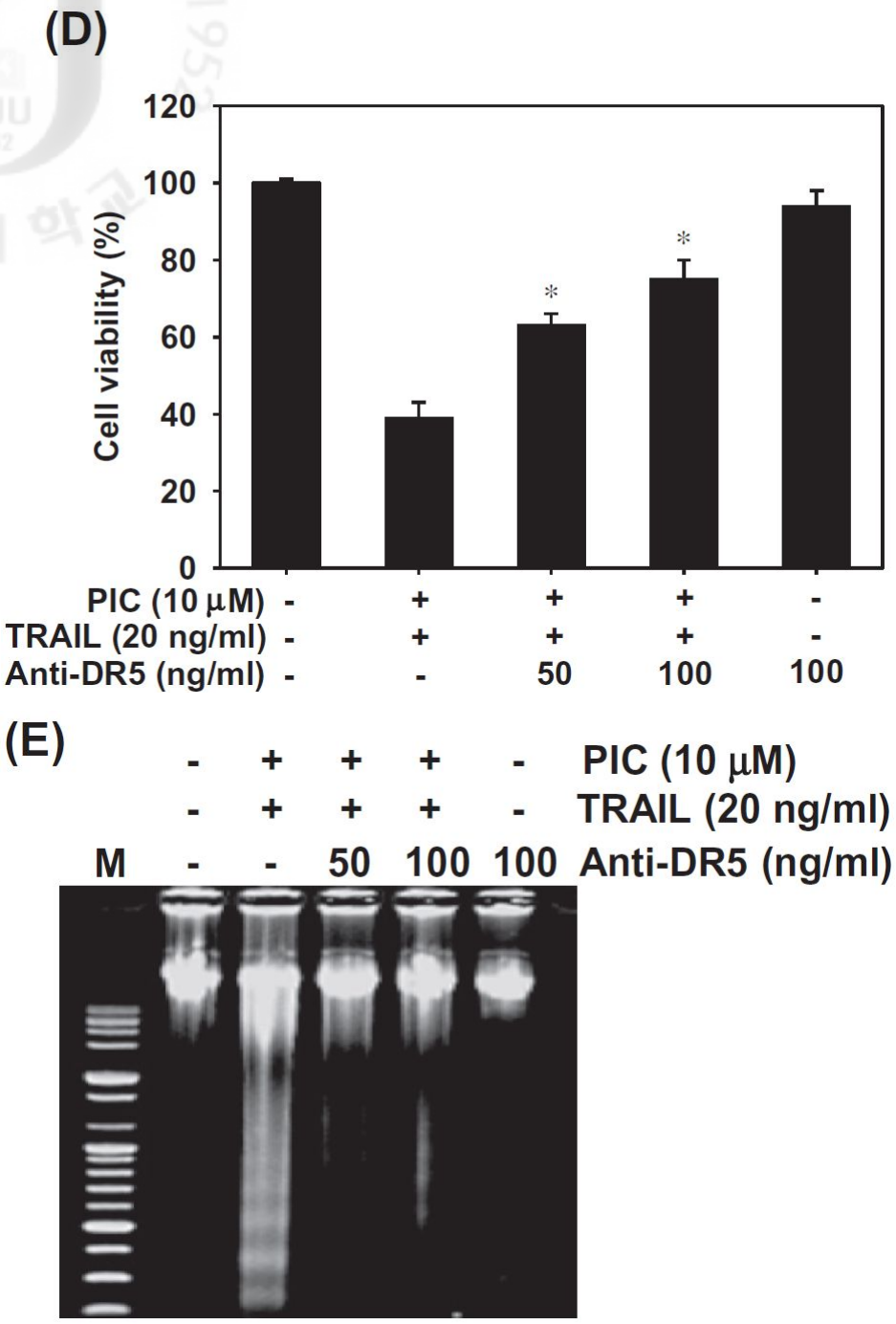
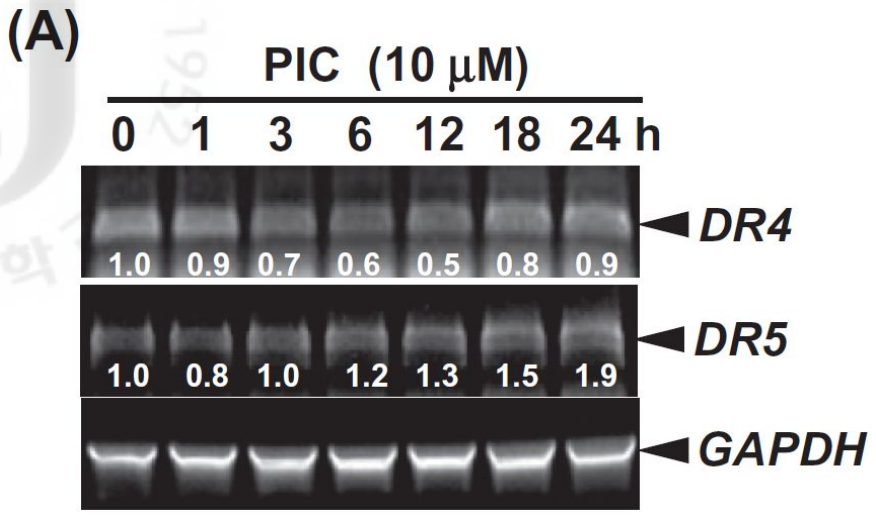


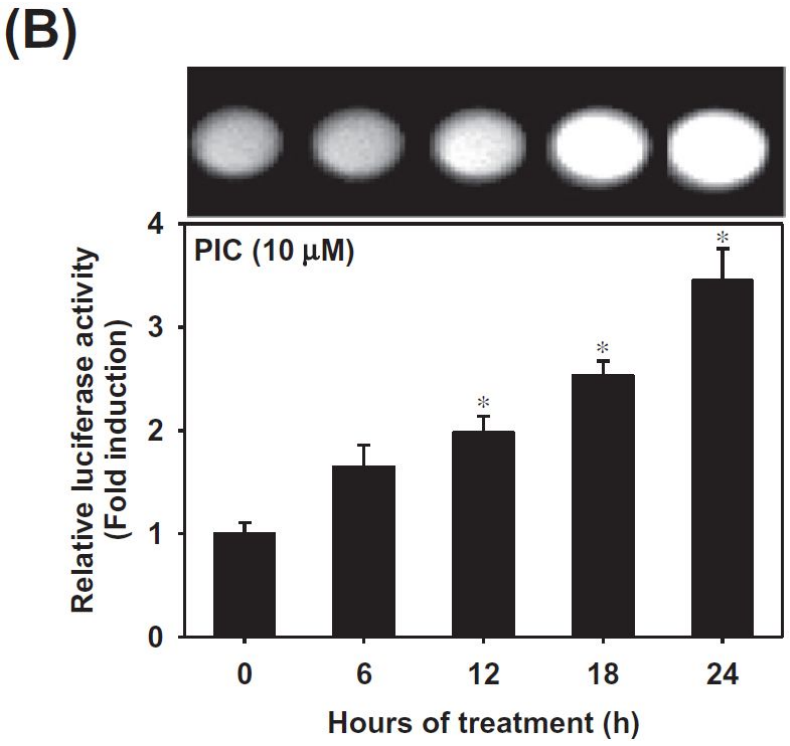
Fig. 3. PIC sensitizes TRAIL-induced apoptosis via up-regulation of DR5. (A) THP-1 cells were treated with 10 μM PIC for the indicated time points. Total protein was

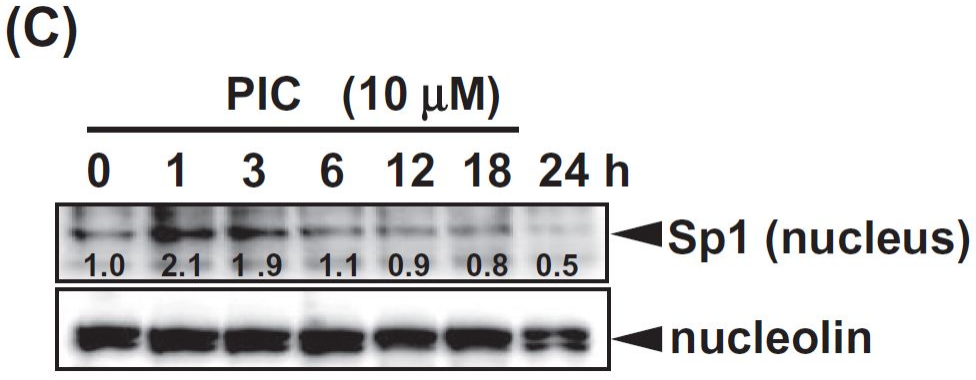
isolated and Western blot analysis for DR4 and DR5 was performed. β-Actin was used as an internal control. (B) Flow cytometry was used to analyze the surface expression of DR5. Black shade histograms, untreated control; black line, treated cells with PIC. (C) U937 and HL60 cells were treated with 10 μM PIC for the indicated times and cell extracts were prepared for Western blot analysis for DR5. β-Actin was used as an internal control. (D and E) THP-1 cells were pre-treated with 10 μM PIC and 20 ng/ml TRAIL in the presence of the indicated concentrations of DR5 chimera antibodies. After treatment for 24 h, cell viabilities were measured by MTT assay (D) and fragmented DNA was extracted and analyzed on 1.5% agarose gel containing EtBr (E). Data are representative of three independent experiments. Each point represents the mean ± SD of three independent experiments. The significance was determined by Student's *t*-test (*, $p < 0.05$ *vs.* vehicle control).

3.4. PIC up-regulates DR5 expression via Sp1 binding on DR5 promoter region

To access whether PIC-induced DR5 up-regulation is tightly controlled at transcriptional level, we analyzed expression of DR5 mRNA by RT-PCR. We found that PIC treatment increases DR5 mRNA expression in a time-dependent manner, but not DR4 mRNA (Fig. 4A). Next, the effects of PIC on the promoter activities of reporter constructs containing 605-bp fragments of the DR5 gene promoter region (pDR5/-605) in THP-1 cells were examined by luciferase assay. Results indicated that PIC significantly increases the promoter activities of pDR5/-605 in a time-dependent manner (Fig. 4B). In particular, 10 μM PIC enhanced the DR5 promoter activity approximately three-fold greater than control values at 24 h, supporting the idea that PIC-induced DR5 up-regulation is controlled at the transcriptional level. To further confirm the precise mechanism by which PIC regulates DR5 expression, Western blot and ChIP analysis was performed to quantify Sp1 activity on the DR5 promoter regions. Nuclear translocation of Sp1 protein increased from at 3 h and reclined at 12 h in the presence of PIC (Fig. 4C). PIC also increased Sp1 binding to the promoter regions of DR5 and significant Sp1 binding levels were observed at 6 h after PIC treatment (Fig. 4D). These results show that PIC at least up-regulates DR5 expression via Sp1 binding and activation on the DR5 promoter region.







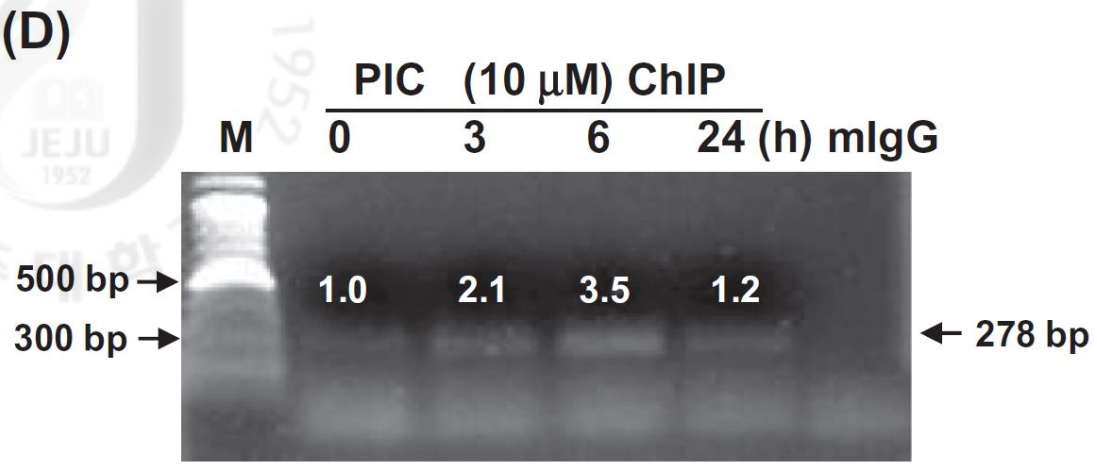
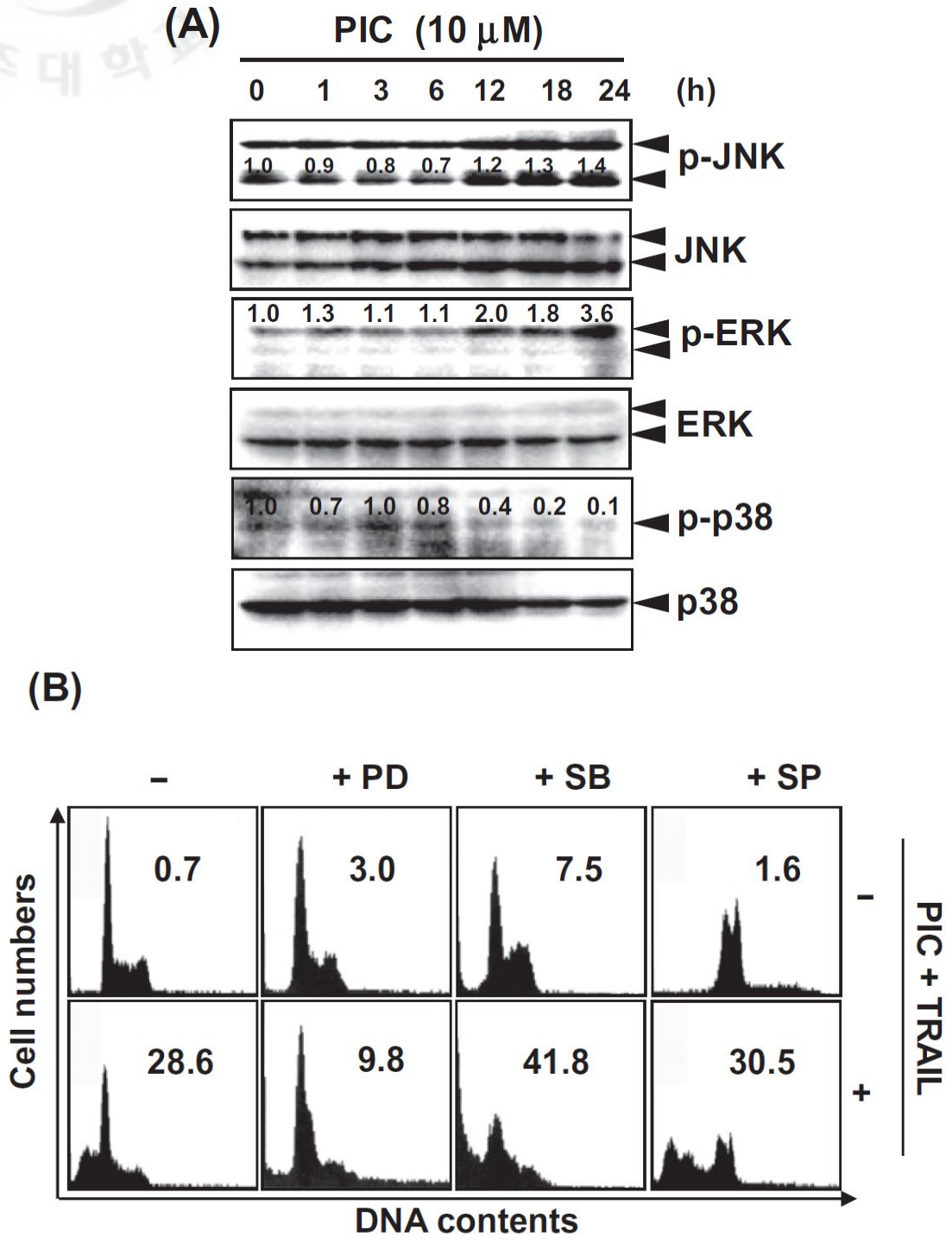


Fig. 4. PIC enhances DR5 expression via activation of Sp1. (A) THP-1 cells were treated with 10 μM PIC for the indicated time points. (A) Total RNA was isolated and RT-PCR analysis for DR4 and DR5 was performed. GAPDH was used as an internal control. (B) pDR5/–605 promoter was transfected into THP-1 cells, which were than treated with 10 μM PIC for the indicated time points, lysed and assayed luciferase activity. (C) Nucleus protein was prepared and Western blotting analysis of Sp1 was conducted. Nucleolin was used as an internal control. (D) ChIP assay was performed using antibodies against Sp1. Negative controls were performed using antibody against rabbit IgG. Data are expressed as overall mean ± SD from three independent experiments. Statistical significance was determined by Student's *t*-test (*, $P < 0.05$ *vs*. vehicle control.

3.5. Activation of ERK is required for the enhanced DR5 expression

Recent studies have revealed that MAPKs are considered important regulators in DR5 expression as the TRAIL sensitizing signal pathway. To investigate the role of MAPKs in PIC-induced DR5 expression, Western blot analysis was used to assess the change in phosphorylations of JNK, ERK, and p38. PIC time-dependently increased the phosphorylations of JNK, ERK, and p38 (Fig. 5A). Thus, THP-1 cells were incubated with PIC in the presence of PD98059, SB239063 and SP600125 to investigate the functional roles of these phosphorylations. Only inhibition of ERK by PD98059 significantly restored induction of sub-G_1 population induced by treatment with PIC and TRAIL (Fig. 5B). Pre-treatment with SB203580 or SP600125 slightly increased sub-G_1 population under the same experimental conditions. We also investigated whether ERK phosphorylation directly contributes to the increase in DR5 expression. Consistent with the decrease in sub-G_1 populations, pre-treatment with PD98059 significantly decreased PIC-induced DR5 up-regulation (Fig. 5C). Therefore, we assessed the effect of PD98059 on DR5 promoter activity. Pre-treatment with PD98059 significantly decreased PIC-induced DR5 promoter activity suggesting that PIC up-regulates DR5 expression via ERK-dependent activation (Fig. 5D). These data indicate that the ERK signal pathway is one of the main regulators in

PIC/TRAIL-induced apoptosis.



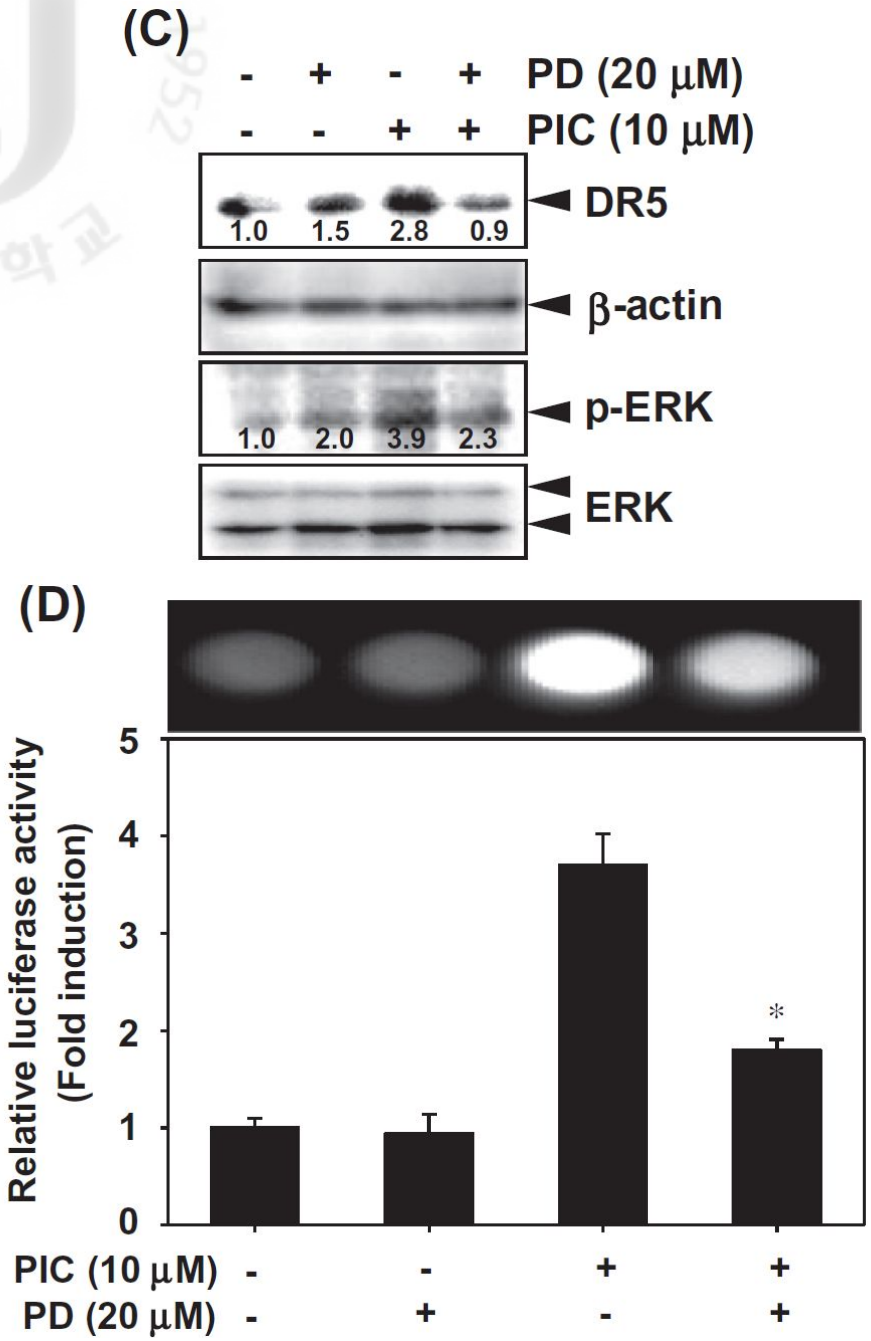


Fig. 5. PIC-induced pDR5/–605 promoter activation is mediated by the ERK signal pathway. (A) THP-1 cells were treated with 10 μM PIC for the indicated times. Equal amounts of cell lysates were resolved on SDS-polyacrylamide gels, transferred

to nitrocellulose membranes, and probed with antibodies against p-JNK, JNK, p-ERK, ERK, p-p38 and p38. (B) Flow cytometric analysis of sub-G_1 population by PIC (10 μM)/TRAIL (20 ng/ml) after pre-treatment with 20 μM PD98059 (PD), 10 μM SB203580 (SB) or 20 μM SP600125 (SP) for 1 h was performed using flow cytometry. (C) PD98059 (20 μM) was treated 1 h before treatment with PIC. Western blot analysis was performed using specific antibody against DR5 and ERK. β-Actin was used as an internal control. (D) pDR5/–605 was transfected into THP-1 cells, which were then pretreated with 20 μM PD98059 for 1 h and further treated with 10 μM PIC for 24 h; thereafter, they were lysed and assayed for luciferase activity.

4. Discussion

PIC has been previously known as an active component of *Melaleuca leucadendrom* (white tea tree), *Cassia garretiana* (Asian legume), and *Rheum undulatum* (Korean rhubarb), which are considered to be useful in traditional herbal medicine (Roupe et al., 2006). Recent studies have demonstrated that PIC induces anti-cancer activity in several cancer cell lines (Kuo and Hsu 2002; Larrosa et al., 2004; Chowdhury et al., 2005; Kim et al., 2008b). Ashikawa et al. (2002) also investigated whether PIC sensitizes TNF-induced apoptosis through inhibition of IKK activity, thereby blocking NF-κB activation. However, the information on the effect of PIC in TRAIL-induced apoptosis is limited. Here, we demonstrated that PIC may be an ideal candidate for novel TRAIL enhancer in some human leukemia cells. Further, on the basis of these findings, we propose that various other cancer cells will be tested for elucidating the molecular mechanisms as a TRAIL sensitizer.

Several studies have suggested that TRAIL may be a potential candidate for treating different types of cancer cells, because it lacks the ability to induce side effects in normal cells (Wiley et al., 1995; Kischkel et al., 2000; Van Geelen et al., 2004). Although the mechanism by which cancer cells successfully develop resistance to TRAIL is unknown, recent studies have reported many cancer cells

acquire resistance against TRAIL-induced apoptosis (Dida et al., 2008). Therefore, it is essential to discover TRAIL sensitizers to overcome this resistance. Many flavonoids have been proposed as candidate novel strategies to induce synergistic activity with TRAIL but have minimal toxicity. A number of studies have reported that flavonoids, especially curcumin and resveratol, significantly sensitize TRAIL-induced apoptosis in its resistant cancer cells through DR5 up-regulation (Andrezejewski et al., 2008; Ivanov et al., 2008). Regulation of DR5 expression by flavonoids may be an important target for sensitizing TRAIL-induced apoptosis. As has been reported previously, PIC enhanced TRAIL-induced apoptosis via DR5 up-regulation expression in this study. These effects with PIC were significantly blocked by DR5 chimera antibodies. Further studies will be needed to precisely elucidate the molecular mechanisms of DR5 expression by PIC.

Many reports have investigated that overexpression of p53 transactivates DR4 and DR5 expression, confirming that DR5 is regulated by p53 (Wu et al., 1997; Takimoto and El-Deiry 2008). However, our results rule out this possibility because all types of cells used in this study are p53-deficeint. Recent studies have suggested that binding of Sp1 transcription factor in the promoter regions tightly regulates DR5 transcription in a variety of cancer cells (Xu et al., 2008). Extensive evidence

demonstrated that Sp1 activation by TRAIL sensitizers increases sensitivity in its resistant cancer cells through enhancement of DR5 expression (Sun et al., 2008). Thus, Sp1 up-regulation is a useful target in TRAIL-resistant cancer cells to augment its sensitivity through DR5 up-regulation. In this study, we also found that PIC sensitizes up-regulation of DR5 *via* activation of Sp1. Additionally, Sp1 regulators have been also investigated because of their importance in Sp1 up-regulation in DR5 expression. Previous studies suggest that a JNK inhibitor completely abrogates bile acid-induced DR5 expression via involvement of Sp1, whereas ERK and p38 MAPK inhibitors failed to block DR5 induction (Higuchi et al., 2004). In contrast, recent studies demonstrated that ERK or p38 MAPK are also associated with TRIAL-induced apoptosis *via* up-regulation of DR5 (Kim et al., 2008a). Our data showed that PIC induces DR5 up-regulation for TRAIL sensitivity via Sp1 activation which can be regulated by ERK. Nevertheless, further studies are needed to confirm how Sp1 activity is regulated by the ERK signal pathway. In addition, the role of other transcriptional factors in the regulation of DR5 expression remains to be determined, because NF-κB (Ammann et al., 2009) and CHOP (Yamaguchi and Wang 2004) have been implicated in regulating DR5 expression. Additionally, it was recently reported that reactive oxygen species (ROS) are essential for sensitizing TRAIL (Kim et al.,

2006; Chen et al., 2010), though many antioxidants trigger TRAIL-induced apoptosis through inhibition of NF-κB or activation of CHOP to up-regulate DR5 expression (Lirdprapamongkol et al., 2010; Kim et al., 2010). Nevertheless, we also found that ROS is not important to regulate DR5 expression (Moon et al., 2010). To settle this discrepancy, further studies are needed more details how antioxidant PIC enhances TRAIL-induced apoptosis.

Taken together, this study demonstrates that PIC/TRAIL potentiates the triggering of an apoptotic cascade via up-regulation of DR5 expression in THP-1 cells. We also showed that DR5 expression is tightly regulated by Sp1 activity in the promoter regions of the gene via the phosphorylation of ERK.

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감사의 글

사실 공부와는 거리가 멀었던 제가 대학원에 진학을 하고 이렇게 석사과정을 마친다는 것이 아직 실감이 나지 않습니다. 아직 부족하고 배워야 할 것이 많지만 그런 것들은 박사 과정에 들어가서 더욱 열심히 하여 채우도록 하겠습니다. 이토록 제가 석사 과정을 마치고 더 나아가 박사 과정을 밟을 수 있도록 많은 격려와 도움을 주신 모든 분들에게 지면으로 나마 감사의 마음을 전하고 싶습니다.

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모든 분들에게 항상 건강하시고 웃는 일들만 가득하길 바라며 언제나 행복했으면 좋겠습니다.

그리고..... 사랑합니다.