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Sensitization of human K562 leukemic cells to TRAIL-induced apoptosis by inhibiting the DNA-PKcs/Akt-mediated cell survival pathway

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ABSTRACT

Despite the fact that many cancer cells are sensitive to TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis, human K562 leukemic cells showed resistance to TRAIL-induced apoptosis. Interestingly, K562/R3 cells, a stable TRAIL-sensitive variant isolated from K562 cells, showed down-regulation of DNA-PK/Akt pathway and a high responsiveness to TRAIL-mediated growth inhibition and apoptosis. We revealed that siRNA-mediated suppression of DNA-PKcs led to decreased phosphorylation of Akt and Bad, a target molecule of Akt, and increased expression of DR4/DR5. Also, we found that suppression of DNA-PKcs using siRNA down-regulated c-FLIP and sensitized K562 cells to TRAIL-induced apoptosis through activation of caspase-8, -9 and -3. In addition, we revealed that treatment with DMNB, a specific inhibitor of DNA-PK, resulted in an increase of DR4/DR5 mRNA levels and their surface expression and a decrease of c-FLIP mRNA levels in K562 cells. DMNB potentiated TRAIL-induced cytotoxicity and apoptosis through inhibition of DNA-PK/Akt pathway and activation of caspase-8, -9 and -3 in K562 cells. This study is the first to show that a protective role of DNA-PK/Akt pathway against TRAIL-induced apoptosis and thus TRAIL in combination with agents that inhibit DNA-PK/Akt pathway would have clinical applicability in treating TRAIL-insensitive human leukemic cells. This model may provide a novel framework for overcoming TRAIL resistance of other cancer cells with agents that inhibit DNA-PK/Akt pathway.

1. Introduction

TNF-related apoptosis-inducing ligand (TRAIL), a transmembrane protein that functions by binding to two closely related receptors (DR4 and DR5), is a promising cancer therapy that preferentially induces apoptosis in a wide variety of cancer cells [1–3]. However, some cancer cells show either partial or complete resistance to the pro-apoptotic effects of TRAIL [4–7]. Resistance to TRAIL-induced apoptosis might be an important therapeutic problem. TRAIL resistance is not solely regulated by differential expression of the receptors. Instead, it appears to be more likely that intracellular molecules acting downstream of the TRAIL receptors render some cells insensitive to TRAIL. This is supported by the findings that TRAIL resistance in certain types of cancer cells can be reversed by modulation of downstream molecules with various agents [8–11]. Human leukemic cells showed a resistance to TRAIL-induced apoptosis [12–14], and thus the study of the intracellular mechanisms that control TRAIL resistance of leukemic cells might enhance our knowledge of death receptor-mediated signaling and help to develop TRAIL-based approaches for the treatment of human leukemia and other types of cancer.

There are many factors contributing to the resistance to TRAIL-induced apoptosis. Among the cellular signaling pathways that promote cell survival, Akt, a serine/threonine protein kinase, is one of the important survival factors that contribute TRAIL resistance [15–18]. Previous studies have shown that Akt is implicated in mediating a variety of biological responses and plays an important role in survival, when cells are exposed to various kinds of apoptotic stimuli [19–21]. In fact, Akt has been shown to inhibit mitochondrial cytochrome c release and apoptosis induced by several pro-apoptotic Bcl-2 family members [22]. A recent report suggests that Akt phosphorylation on Ser473 (S473) is required for full activation of Akt [23], and S473 phosphorylation in the activation of Akt is mediated by DNA-dependent protein kinase,

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a member of the PI3K-related kinase subfamily of protein kinases (DNA-PK) [24]. DNA-PK is a three-protein complex consisting of a 470-kDa catalytic subunit (DNA-PKcs) and regulatory DNA binding subunits, Ku heterodimer (Ku70 and Ku80). DNA-PK plays an important role in DNA repair and protects cells from apoptosis induced by DNA damaging agents [25,26]. DNA-PKcs has been shown to colocalize with Akt and enhance Akt phosphorylation [24,27,28]. DNA-PK is the physiological Akt Ser473 kinase upon γ-irradiation-induced DNA damage [29]. Although Akt plays a critical role in cell survival, the involvement of DNA-PK in the protective role of Akt against TRAIL-induced apoptosis has not been investigated.

Here, we demonstrated a new TRAIL resistance mechanism that the DNA-PKcs/Akt pathway appears to play an essential role in the escape from TRAIL-induced apoptosis of leukemic cells, and found that 4,5-dimethoxy-2-nitrobenzaldehyde (DMNB), an inhibitor of DNA-PK, could sensitize K562 cells to TRAIL-induced apoptosis via inactivation of DNA-PKcs/Akt pathway. This study is the first to show that DNA-PKcs could interfere with TRAIL-induced apoptotic signaling in human leukemic cells, probably through activation of the Akt signaling pathway. This model might provide a novel framework for overcoming TRAIL resistance of other cancer cells with agents that inhibit DNA-PK.

2. Materials and methods

2.1. Cell culture

Human chronic myelogenous leukemia K562 cells and its TRAIL-sensitive K562/R3 cells were cultured in RPMI medium containing 10% (v/v) fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml). DNA-PKcs deficient SCID and its isogenic wild type murine embryonic fibroblast CB-17 cells were maintained in DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml).

2.2. Cell proliferation assays

Cell proliferation was measured by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma–Aldrich Co., St. Louis, MO) colorimetric dye-reduction method. Exponentially growing cells (5×10^3 cells/well) were plated in 96 wells and incubated in growth medium containing TRAIL and/or 4,5-dimethoxy-2-nitrobenzaldehyde (DMNB) at 37 °C. After five days, the medium was aspirated after centrifugation and MTT-formazan crystals were solubilized in 100 μl DMSO. The optical density of each sample was measured at 570 nm using an ELISA reader (Bio-Tec Instruments, VT, USA). The optical density of the media was proportional to the number of viable cells. Inhibition of proliferation was evaluated as a percentage of control growth (no drug in the sample). All experiments were repeated at least twice in triplicate.

2.3. Western blot analysis

Protein samples were separated by SDS-PAGE and blotted to nitrocellulose membrane (Hybond-ECL, GE Healthcare). The membrane was incubated with antibody as specified, followed by secondary antibody conjugated with horseradish peroxidase. Specific antigen–antibody complexes were detected by enhanced chemiluminescence (PerkinElmer, Life science). Western blot analysis was performed with the following antibodies: anti-Ku70/Ku80, Caspase-3 and PARP antibodies (Santa Cruz Biotechnology, CA), anti-Akt, phospho-Akt (p-Akt, S473), Bad, phospho-Bad (p-Bad, S136), Caspase-8 and -9 antibodies (Cell Signaling Technology, MA), anti-Hsp70 (Stressgen Biotechnologies Corp. Canada), anti-DNA-PKcs antibody (Thermo Fisher Scientific, CA) and β-actin antibodies (Sigma–Aldrich Co., St. Louis, MO). Secondary antibodies were obtained from GE Healthcare.

2.4. siRNA transfection

The siRNA used for targeted silencing of DNA-PKcs (5′-CAGUCUUAGUCCGGAUCAU dTdT-3′) was purchased from Bioneer Corporation (Daejeon, Korea). K562 cells were transfected with 0.2 μM siRNA for 48 h by oligofectamine according to the manufacture's protocol (InVitrogen, Carlsbad, CA). In brief, K562 cells (5×10^5 cells/well) were transfected with siRNA/oligofectamine complex in serum free RPMI medium at 37 °C in 6-well plates for 4 h. Thereafter, FBS was added for final 10% concentration. After 48 h, K562 cells were treated with TRAIL for additional 24 h and collected for western blot analysis to determine the levels of DNA-PKcs and other indicated proteins.

2.5. DNA-dependent protein kinase assay

The kinase activity of DNA-PK was determined using the Sigma TECT™ DNA-dependent Protein Kinase Assay System (Promega Corp., Madison, WI). In brief, 10 μg of nuclear extract was incubated with an activator DNA, a biotinylated p53-derived peptide substrate, and [γ-^{32}P] ATP at 30 °C for 5 min. The reaction

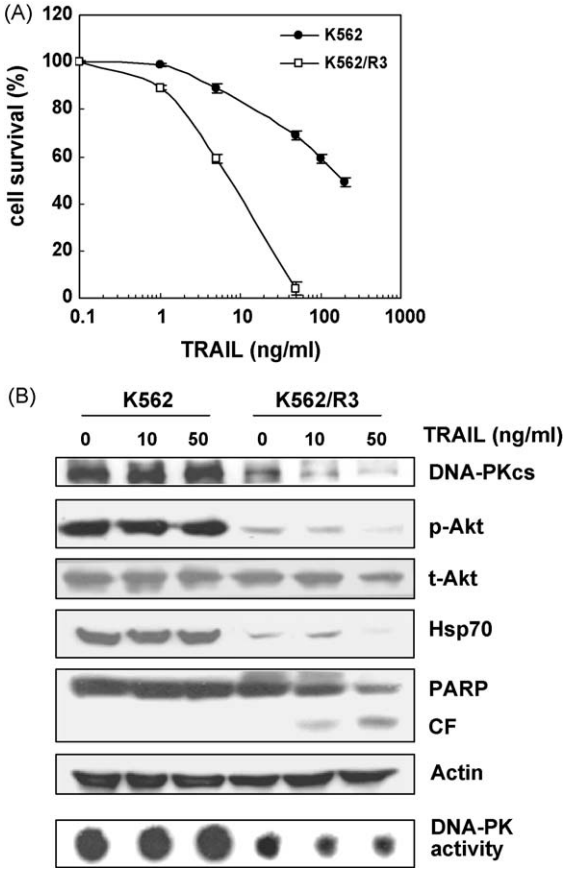

Fig. 1. Effect of TRAIL on DNA-PKcs/Akt signaling-related molecules in K562 and its TRAIL-sensitive variant K562/R3 cells. (A) K562 and K562/R3 cells were treated with graded single doses of TRAIL, and the percentage of cell survival was determined after 5 days of incubation using the MTT assay. Data points on the curve represent means ± S.E. of triplicate experiments. (B) K562 and K562/R3 cells were treated with indicated doses of TRAIL for 24 h. Thereafter, whole cell lysates from TRAIL-treated cells were subjected to western blot analysis to monitor the levels of DNA-PKcs, phosphorylated Akt on Ser473 (p-Akt), total Akt (t-Akt), Hsp70 and PARP. The level of (-actin (Actin) was used as a loading control. DNA-PK activity was measured by kinase activity assay of whole DNA-PK complex. CF; cleavage fragment.

was terminated by adding termination buffer. Each termination reaction sample was spotted onto SAM^{2TM} Biotin Capture Membrane and washed with 2 M NaCl and 2 M NaCl in 1% H_3PO_4. The SAM^{2TM} Membrane squares were analyzed using Molecular Imager System (Bio-Rad Laboratories, Inc., Model GS 525, Hercules, CA).

2.6. Flow cytometric analysis of TRAIL receptors

K562 and K562/R3 cells (2×10^6 cells) from the culture media were spun down at $500 \times g$, washed with phosphate-buffered saline (PBS) and resuspended in 500 μl PBS. The cells were then incubated with 5 μl of goat IgG2a, anti-DR4 or anti-DR5 polyclonal goat antibody (1:100, R&D, Minneapolis, MN) for 1 h. After washing with PBS, FITC-conjugated rabbit anti-goat polyclonal antibody (1:200, Sigma–Aldrich Co., St. Louis, MO) was added to the cell suspension and incubated for 1 h on ice. After rinsing with PBS, the samples were analyzed with a FACSort flow cytometer (Becton Dickinson, San Jose, CA). The data were analyzed using the CellQuest program.

2.7. RT-PCR analysis

Total cellular RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol and the levels of RNA transcripts were assessed with The Titan One Tube RT-PCR System (MJ research Inc., NV, USA). One microgram of total cellular RNA was reverse transcribed using Maloney murine leukemia virus reverse transcriptase (Invitrogen) with each dNTP and 1 μg oligo dT. Amplification of 1 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′-CTG-AAAGGCATC TGCTCAGGTG-3′ and (reverse), 5′-CAGAGTCTGCAT-TACCTTCTAG-3′; $FLIP_L$ (forward), 5′-GCTGAAGTCATCCATCAGGT-3′ and (reverse), 5′-CATACTGAGATGCAAGAATT-3′; $FLIP_S$ (forward), 5′-GCTGAAGTCATCCATCAGGT-3′ and (reverse), 5′-GATCAGGA-CAATGGGCATA G-3′; β-actin (forward), 5′-CAGAGCAAGAGAGG-CATCCT-3′ and (reverse), 5′-TTGAAGGTCTCAAACATGAT-3′. The resulting total cDNA was used in PCR performed in total volume of 20 μl using Taq polymerase (Solgent Co., Korea) at 94 °C for denaturation for 60 s, 60 °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 1.5% agarose gel and visualized with ethidium bromide staining.

2.8. Apoptosis assessment by Annexin V staining

K562 (2×10^5 cells/ml) were treated with TRAIL in the presence or absence of DMNB for 24 h. Then cells were centrifuged and resuspended in 500 μl of the staining solution containing Annexin V-fluorescein (FITC Apoptosis detection kit; BD ParMingen San Diego, CA) and propidium iodide in PBS. After incubation at room temperature for 15 min, cells were analyzed by flow cytometry for the discrimination of living cells (unstained with either fluorochrome) from apoptotic cells (stained only with Annexin V) and necrotic cells (stained with both Annexin V and propidium iodide).

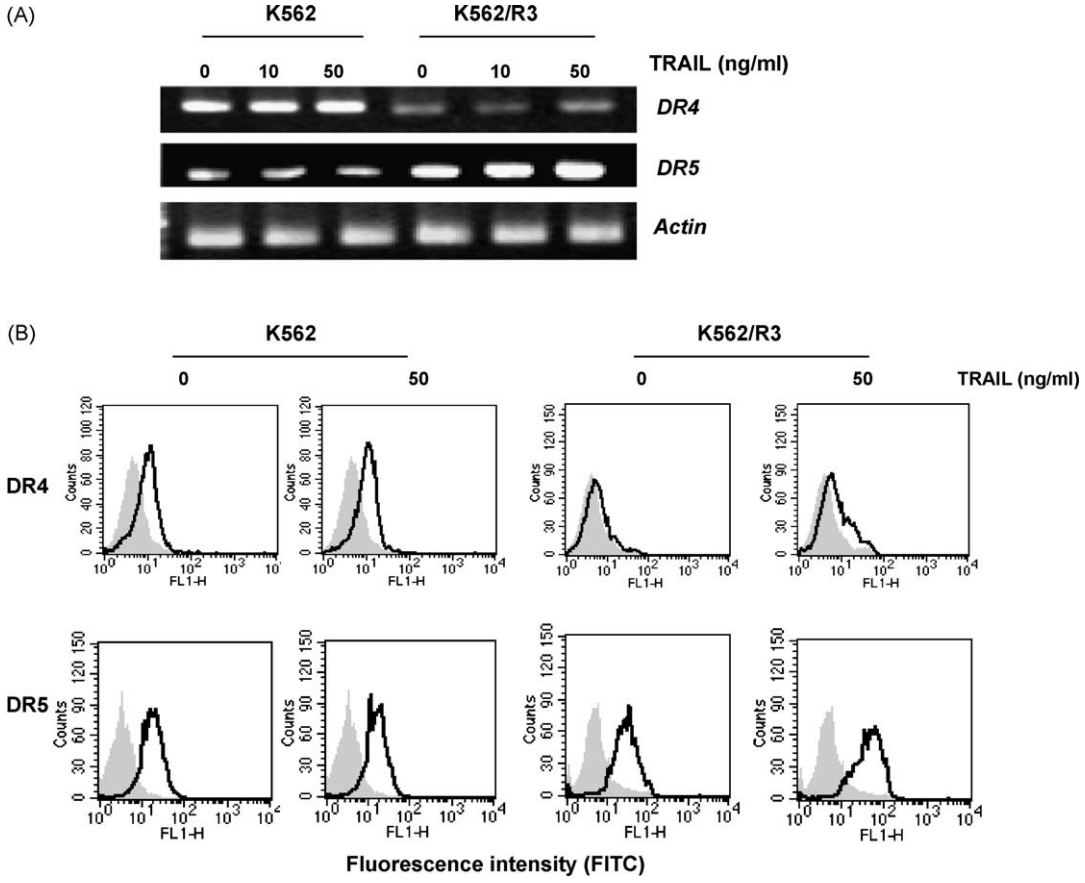

Fig. 2. Effect of TRAIL on mRNA and cell surface expression levels of DR4/DR5 in K562 and K562/R3 cells. (A) The mRNA levels of DR4 and DR5 in K562 and K562/R3 cells treated with or without TRAIL (10- and 50 ng/ml for 24 h) were determined by RT-PCR. (B) These cells were treated with 50 ng/ml TRAIL for 24 h. Thereafter, cells were stained with control IgG or anti-DR4 (or -DR5) antibody (1:100), and subsequently labeled with FITC-conjugated secondary antibodies (1:200) to determine the surface expression of DR4 and DR5. The cell surface expression was measured by a flow cytometer. Shaded and unshaded peaks correspond to control and specific stainings, respectively.

2.9. Statistical analysis

The results obtained were expressed as the mean ± S.E. of at least three independent experiments. The statistical significance of differences assessed using the Student's *t*-test and two-way ANOVA (GraphPad software; GraphPad, Santiago, CA) with Bonferroni post-tests. $p < 0.05$ was considered statistically significant in all experiments.

3. Results

3.1. Down-regulation of DNA-PKcs/Akt pathway is associated with the susceptibility to TRAIL-induced cytotoxicity

Primary or cultured leukemic cells are resistant to TRAIL-induced apoptosis [9,12]. Therefore, to investigate the potential mechanism of resistance to TRAIL in human leukemic K562 cells, differential *in vitro* sensitivity of K562 cells and their TRAIL-sensitive variant, K562/R3 cells, to TRAIL was determined. We found that K562/R3 cells exhibited about 18-fold more sensitive to TRAIL-induced cytotoxicity than parental K562 cells (Fig. 1A).

It has been reported that constitutively active Akt is an important regulator of TRAIL sensitivity and that activation of Akt inhibits TRAIL-induced apoptosis [15–18]. In addition, high level of phosphorylated Akt (p-Akt) is closely correlated with TRAIL resistance. Since it has been reported that DNA-PKcs acts upstream to Akt and directly phosphorylates and activates Akt [24,27,28], we investigated whether DNA-PK could modulate TRAIL sensitivity. To measure the different levels of DNA-PKcs, p-Akt, and total Akt (t-Akt) between K562 and K562/R3 cells in the presence or absence of TRAIL, western blot analysis was performed (Fig. 1B). As compared with K562 cells, K562/R3 cells showed profoundly reduced levels of DNA-PKcs and p-Akt. Moreover, when the cells were treated with TRAIL, the levels of DNA-PKcs and p-Akt were significantly decreased in K562/R3 cells but not in K562 cells. A similar result was obtained with the activity of DNA-PK. The inactivation of Akt was followed by down-regulation of Hsp70 in K562/R3 cells, supporting that the expression of Hsp70 is regulated by Akt activity [30]. We next determined whether treatment of K562/R3 cells with TRAIL would lead to proteolytic cleavage of PARP as a biochemical event during apoptosis. The increase of PARP cleavage yielding a characteristic 85 kDa fragment occurred in TRAIL-treated K562/R3 cells. However, K562 cells did not show PARP cleavage after TRAIL treatment. Our results suggest the possibility that down-regulation of DNA-PKcs/Akt pathway can be associated with the susceptibility to TRAIL-induced cytotoxicity.

Since TRAIL is known to trigger apoptotic signals via two types of death receptors, DR4 and DR5, the mRNA levels and cell surface

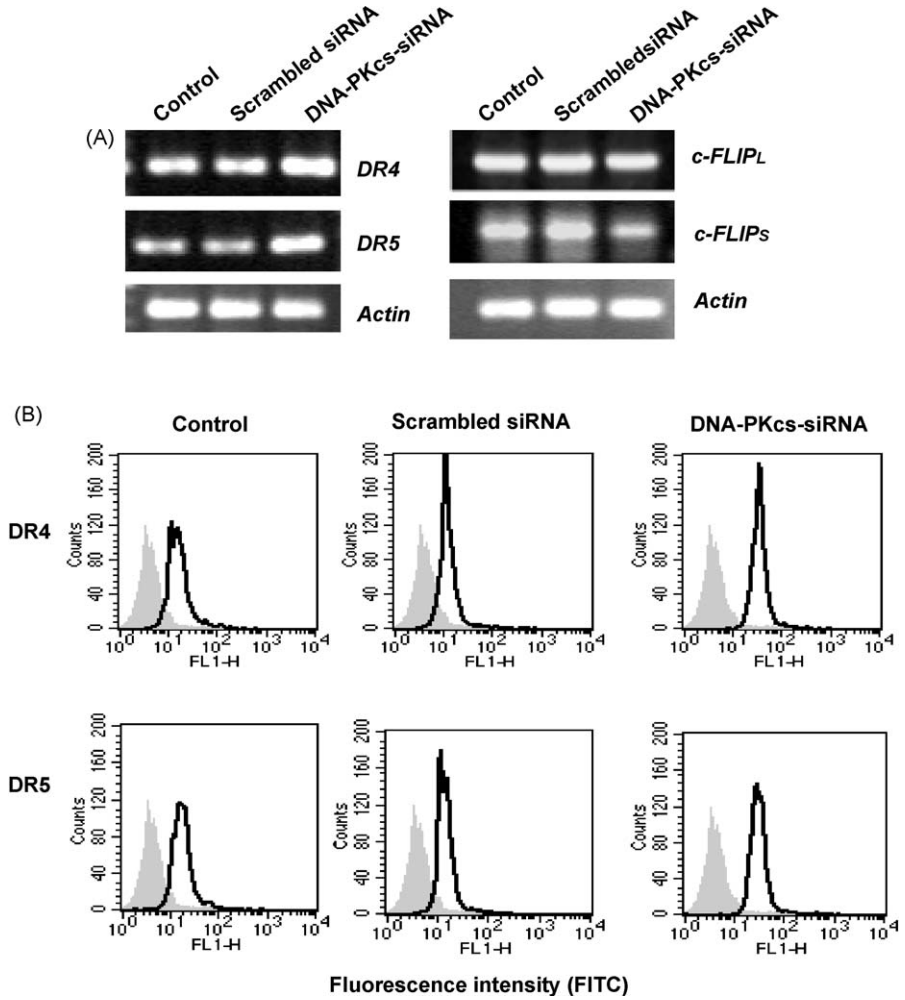

Fig. 3. Up-regulation of DR4/DR5 mRNA and its cell surface expression and down-regulation of c-FLIP$_{L/S}$ in DNA-PKcs-silenced K562 cells. (A) The mRNA levels of DR4/DR5 and c-FLIP$_{L/S}$ in K562 cells were transfected with DNA-PKcs-targeted siRNA or scrambled siRNA as control were performed by RT-PCR. (B) These transfectants were cells were stained with control IgG or anti-DR4 (or -DR5) antibody (1:100), and subsequently labeled with FITC-conjugated secondary antibodies (1:200) to determine the surface expression of DR4 and DR5. The cell surface expression was measured by a flow cytometer. Shaded and unshaded peaks correspond to control and specific stainings, respectively.

expression of DR4 and DR5 were compared between K562 and K562/R3 cells (Fig. 2). The mRNA levels and cell surface expression of DR4 and DR5 was decreased and increased in K562/R3 cells as compared with K562 cells, respectively. After treatment with TRAIL, mRNA levels and cell surface expression (~ 1.5- and 1.4-fold, respectively) of DR4 and DR5 was slightly increased in K562/R3 cells but not in K562 cells. These data suggest the possibility that the activity of DNA-PKcs/Akt pathway may regulate the expression of DR4 and DR5, which may affect the TRAIL sensitivity in K562/R3 cells.

3.2. siRNA-mediated suppression of DNA-PKcs leads to increased susceptibility to TRAIL-induced cytotoxicity through up-regulation of death receptors and down-regulation of c-FLIP

To understand the role of DNA-PKcs in expression regulation of DR4 and DR5, we silenced DNA-PKcs in K562 cells using small interfering RNA (siRNA) and determined the changed levels of TRAIL-responsive molecules using RT-PCR and flow cytometry analysis. RT-PCR analysis showed that the mRNA levels of both DR4 and DR5 were significantly increased in K562 cells transfected with DNA-PKcs siRNA compared to the cells transfected with scrambled siRNA (Fig. 3A, *left*). This result was followed by 2.5- and 2.1-fold increase of cell surface expression of DR4 and DR5, compared with those of the cells transfected with scrambled siRNA, respectively (Fig. 3B). Since the expression of c-FLIP as well as DR4/DR5 has been known to the major determinant of TRAIL sensitivity, we also evaluated the change of c-FLIP mRNA level in K562 cells transfected with DNA-PKcs siRNA (Fig. 3A, *right*). The mRNA level of c-FLIP, especially c-FLIP$_S$, in K562 cells was suppressed after transfection with DNA-PKcs siRNA. These results suggest that the activity of DNA-PK plays an important role in the regulation of both DR4/DR5 and c-FLIP expression, and considering the levels of DR4 and DR5 in K562/R3 cells with down-regulated level of DNA-PKcs, factors other than DNA-PKcs are also involved in determining the expression of DR4 and DR5.

Next, we examined whether siRNA-mediated suppression of DNA-PKcs affects TRAIL-induced cytotoxicity. The growth inhibitory effect of TRAIL in K562 cells was significantly increased after transfection with DNA-PKcs siRNA as compared with scrambled siRNA (Fig. 4A). This result was followed by increased susceptibility to TRAIL-induced apoptosis in K562 cells transfected with

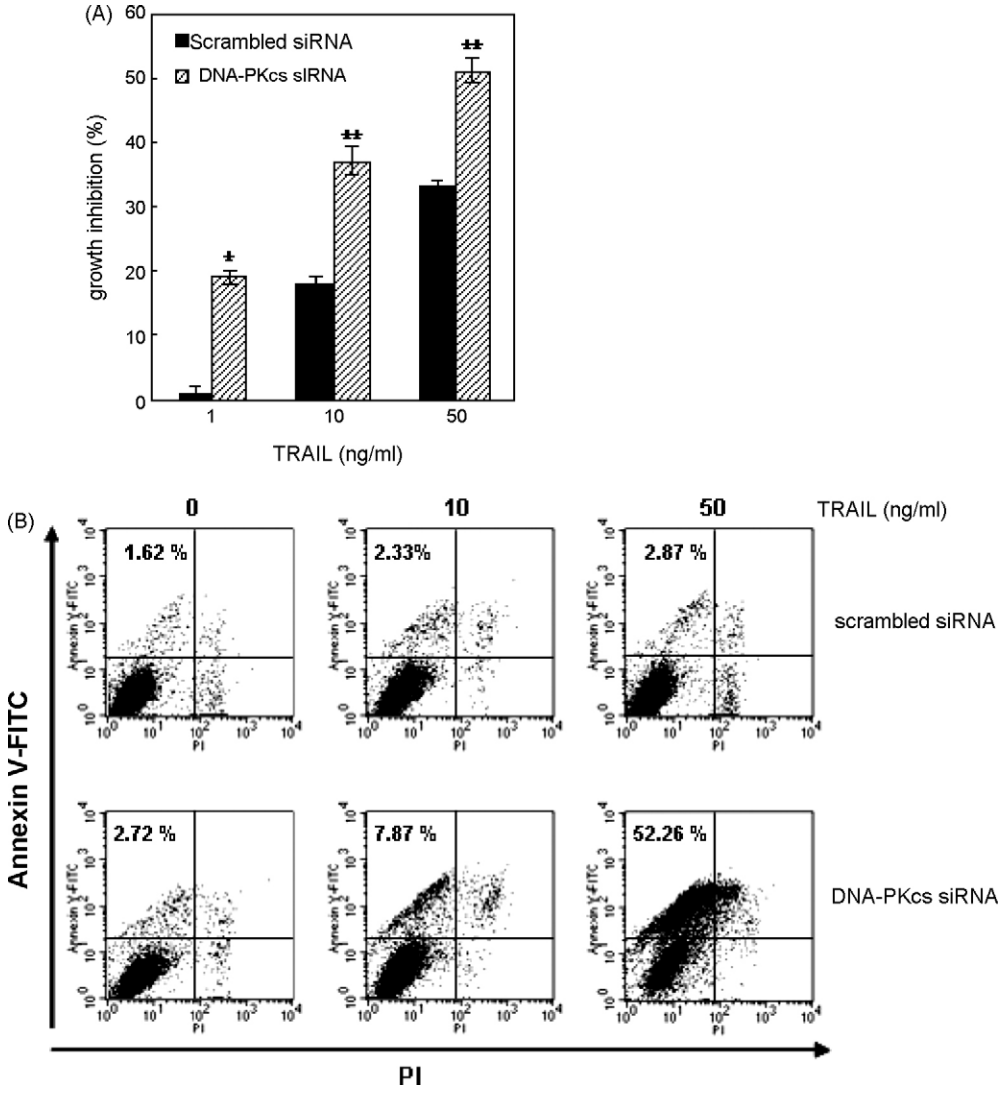

Fig. 4. Effects of DNA-PKcs-targeted siRNA on TRAIL-induced cytotoxicity and apoptosis. (A) K562 cells were transfected with a siRNA against DNA-PKcs or scrambled siRNA as a control. After 48 h, these transfectants were treated with graded single doses of TRAIL, the percentage of growth inhibition was determined after 5 days of incubation using the MTT assay. Data represent the means ± S.E. of triplicate experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ versus cells transfected with scrambled siRNA at the same dose point. (B) These transfectants were treated with TRAIL (10- and 50 ng/ml) for 24 h, and induction of apoptosis was performed by Annexin V analysis. Data are the means of triplicate determinations from three independent experiments; *bars*, S.E.

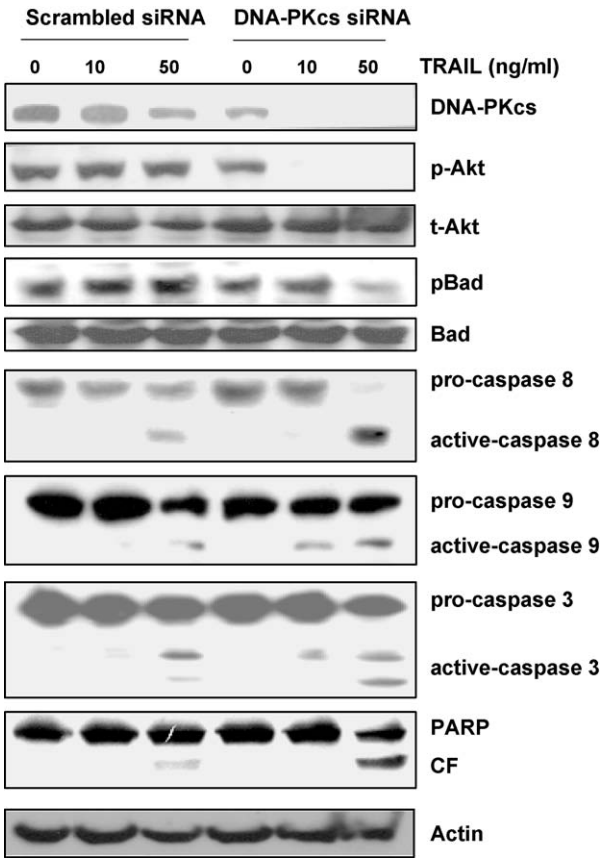

Fig. 5. Effects of DNA-PKcs-targeted siRNA on DNA-PKcs/Akt molecules and caspase activity. K562 cells were transfected with a siRNA against DNA-PKcs or scrambled siRNA as a control. These transfectants were treated with TRAIL (10- and 50 ng/ml) for 24 h and performed western blot analysis to monitor the levels of DNA-PKcs, p-Akt, t-Akt, p-Bad and t-Bad. Also, the transfectants were determined the activation of caspases and cleavage of PARP by western blot analysis.

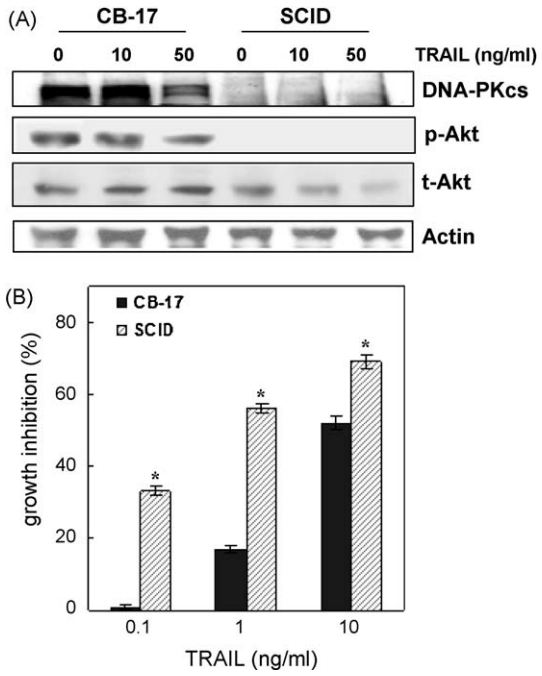

Fig. 6. Loss of DNA-PKcs leads to hypersensitivity to TRAIL. (A) Mouse DNA-PKcs deficient SCID and its parental CB-17 cells were treated with indicated doses of TRAIL for 24 h, and then subjected to western blot analysis to monitor the levels of DNA-PKcs, p-Akt and t-Akt. (B) CB-17 and SCID cells were treated with graded single doses of TRAIL, and the cell viability was determined after 5 days of incubation using the MTT assay. Data represent the means ± S.E. of triplicate experiments. $^{*}p < 0.05$ versus CB-17 cells at the same dose point.

DNA-PKcs siRNA compared with that in the cells transfected with scrambled siRNA (Fig. 4B). These results suggest that suppression of DNA-PKcs may lead to an enhancement of TRAIL sensitivity in K562 cells, probably through modulation of DR4/DR5 and c-FLIP expression.

3.3. siRNA-mediated suppression of DNA-PKcs enhances TRAIL-induced apoptosis dependent on activation of caspase cascade

In order to determine the involvement of DNA-PKcs/Akt pathway in caspase-dependent apoptosis induced by TRAIL, K562 cells transfected with DNA-PKcs siRNA or scrambled siRNA were exposed to TRAIL (Fig. 5). K562 cells transfected with DNA-PKcs siRNA showed a decreased Akt phosphorylation on S473 (p-Akt) in association with reduction of DNA-PKcs, even though t-Akt level was not altered. Moreover, in the presence of TRAIL, the levels of DNA-PKcs, p-Akt and p-Bad were remarkably decreased in K562 cells transfected with DNA-PKcs siRNA.

Since the expression of c-FLIP as an inhibitor of caspase-8 was significantly decreased in DNA-PKcs siRNA transfected K562 cells, we next investigated whether the sensitization of TRAIL-induced apoptosis by suppression of DNA-PKcs was associated with activation of caspase cascade. TRAIL-induced activation of caspase-8, which is located downstream to DR4/DR5, was more increased in K562 cells transfected with DNA-PKcs siRNA than in the cells transfected with scrambled siRNA. In addition, TRAIL-induced activation of caspase-3 as well as caspase-9 was also more increased in K562 cells transfected with DNA-PKcs siRNA than in the cells transfected with scrambled siRNA. These results were followed by an increased cleavage of PARP, an endogenous substrate of caspase-3 in K562 cells transfected with DNA-PKcs siRNA compared with the cells transfected with scrambled siRNA. These data suggest that inhibition of DNA-PKcs sensitize K562 cells to TRAIL-induced apoptosis probably by suppression of Akt pathway and c-FLIP, and up-regulation of DR4 and DR5.

To confirm the effect of DNA-PKcs/Akt pathway activity on the sensitivity to TRAIL, we compared the levels of t-Akt and p-Akt and the sensitivity to TRAIL between murine DNA-PKcs-deficient SCID cells and parental CB-17 cells (Fig. 6). p-Akt was undetectable in the presence or absence of TRAIL and t-Akt was sensitively decreased by TRAIL treatment in SCID cells, compared with the parental CB cells, which did not showed the alteration of levels of t-Akt and p-Akt after TRAIL treatment (Fig. 6A). In addition, the growth inhibitory effect of TRAIL was significantly higher in SCID cells than in CB-17 cells (Fig. 6B). These results strongly suggest that the activity of DNA-PKcs is strongly correlated with the phosphorylation status of Akt, and is one of the major determinants for the susceptibility to TRAIL-induced cytotoxicity.

3.4. Treatment with DMNB makes the TRAIL-resistant K562 cells sensitive to TRAIL-induced apoptosis

Since knock-down of DNA-PKcs with siRNA sensitized K562 cells to TRAIL, we determined if 4,5-dimethoxy-2-nitrobenzaldehyde (DMNB), a DNA-PK specific inhibitor [31], also can act as an effective sensitizer of TRAIL against K562 cells. RT-PCR analysis showed that both DR4 and DR5 mRNA levels were slightly increased by DMNB treatment in the K562 cells (Fig. 7A) and this result was followed by increased surface expression of DR4 (~1.7-fold) and DR5 (~1.3-fold) (Fig. 7B). Moreover, the mRNA levels of c-FLIP, especially c-FLIP$_S$, were significantly reduced by DMNB treatment in K562 cells (Fig. 7A). Since the modulation of these TRAIL-responsive molecules induced by DMNB was very similar with that observed in K562 cells transfected with DNA-PKcs siRNA,

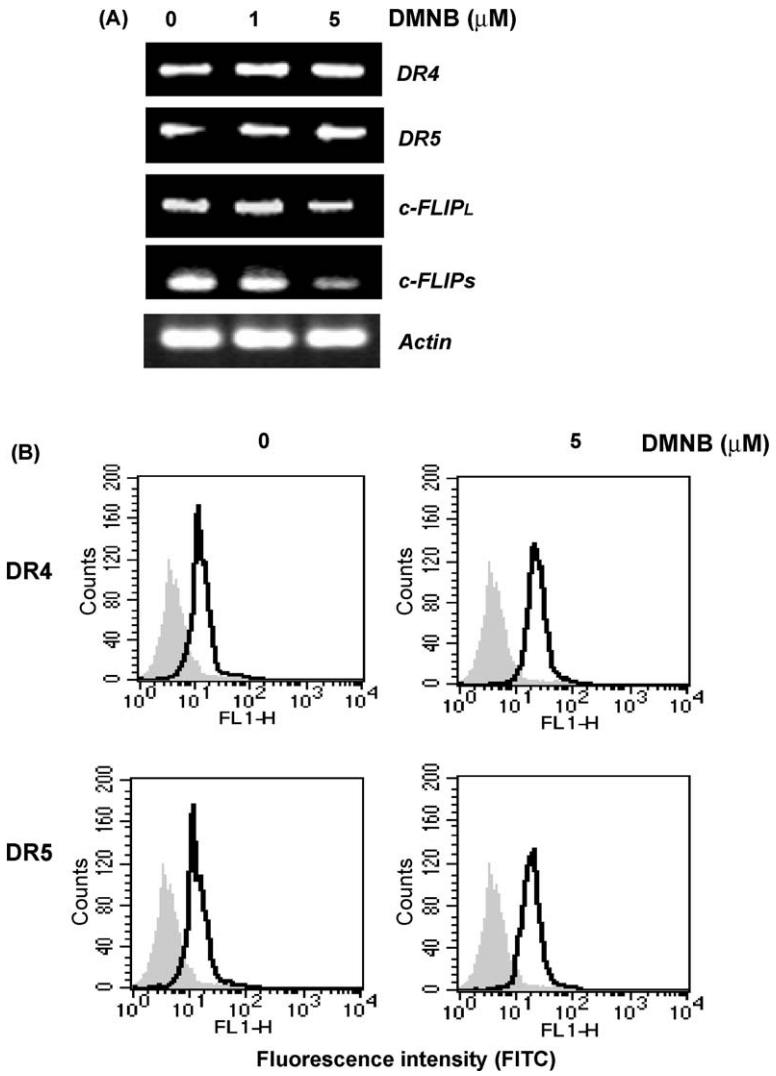

Fig. 7. Effect of DMNB on the expression of DR4/DR5 and c-FLIP. (A) K562 cells were treated with 1- or 5 μM DMNB for 24 h, and the changed mRNA levels of DR4/DR5 and c-$FLIP_{L/S}$ in K562 cells were determined by RT-PCR. (B) Cells treated with DMNB were stained with control IgG or anti-DR4 (or -DR5) antibody (1:100), and subsequently labeled with FITC-conjugated secondary antibodies (1:200) to determine the surface expression of DR4 and DR5. The cell surface expression was measured by a flow cytometer. Shaded and unshaded peaks correspond to control and specific stainings, respectively.

we determined whether DMNB potentiates TRAIL-induced cytotoxicity in K562 cells. DMNB in combination with TRAIL sensitized K562 cells to TRAIL-induced cytotoxicity in a dose-dependent manner (Fig. 8A). In addition, as shown in Fig. 8B, co-treatment of TRAIL with DMNB resulted in a significant increase in TRAIL-induced apoptosis, when compared to TRAIL alone.

To determine whether the sensitization to TRAIL-induced apoptosis by DMNB is accompanied by the same molecular changes observed in K562 cells transfected with DNA-PKcs siRNA, we assessed the TRAIL receptor signaling molecules as well as DNA-PK/Akt pathway (Fig. 9). During TRAIL-induced apoptosis in K562 cells, DMNB increased mRNA expression of both DR4 and DR5, decreased mRNA expression of c-$FLIP_S$ as well as c-$FLIP_L$, and suppressed the levels of DNA-PKcs, p-Akt and p-Bad. In addition, the combination of TRAIL and DMNB resulted in the decreased expression of Ku70/80 subunits of DNA-PK in the K562 cells. These proapoptotic molecular changes induced by DMNB were followed by increased activation of procaspase-8, -9 and -3, and PARP cleavage.

These results indicated that DMNB increased the TRAIL-induced apoptosis in K562 cells via enhancement of receptor-mediated and caspase-dependent apoptosis triggered by inhibition of DNA-PK/Akt pathway. Therefore, suppression of DNA-PKcs/Akt pathway may be a useful strategy to increase the susceptibility to TRAIL-induced cell death in TRAIL-resistant human leukemic cells.

4. Discussion

Induction of apoptosis in cancer cells by TRAIL is a promising therapeutic principle in oncology, although toxicity and resistance to TRAIL are limiting factors. Indeed, many tumors remain resistant to TRAIL-induced apoptosis, which related to the dominance of anti-apoptotic signals. Therefore, we studied to identify and target the anti-apoptotic molecules regulating the TRAIL resistance in human leukemic K562 cells. In the present study, K562/R3 cells, a stable TRAIL-sensitive variant isolated from K562 cells, showed down-regulation of DNA-PKcs/Akt signaling pathway and a high sensitivity to TRAIL-mediated growth inhibition and apoptosis as compared with K562 cells. In addition, DNA-PKcs-deficient SCID cells showed also the down-regulation of Akt phosphorylation and an increased susceptibility to TRAIL-induced cytotoxicity as compared with parental CB-17 cells, suggesting that the activity of DNA-PKcs/Akt signaling pathway may affect the sensitivity of cells to TRAIL-induced apoptosis.

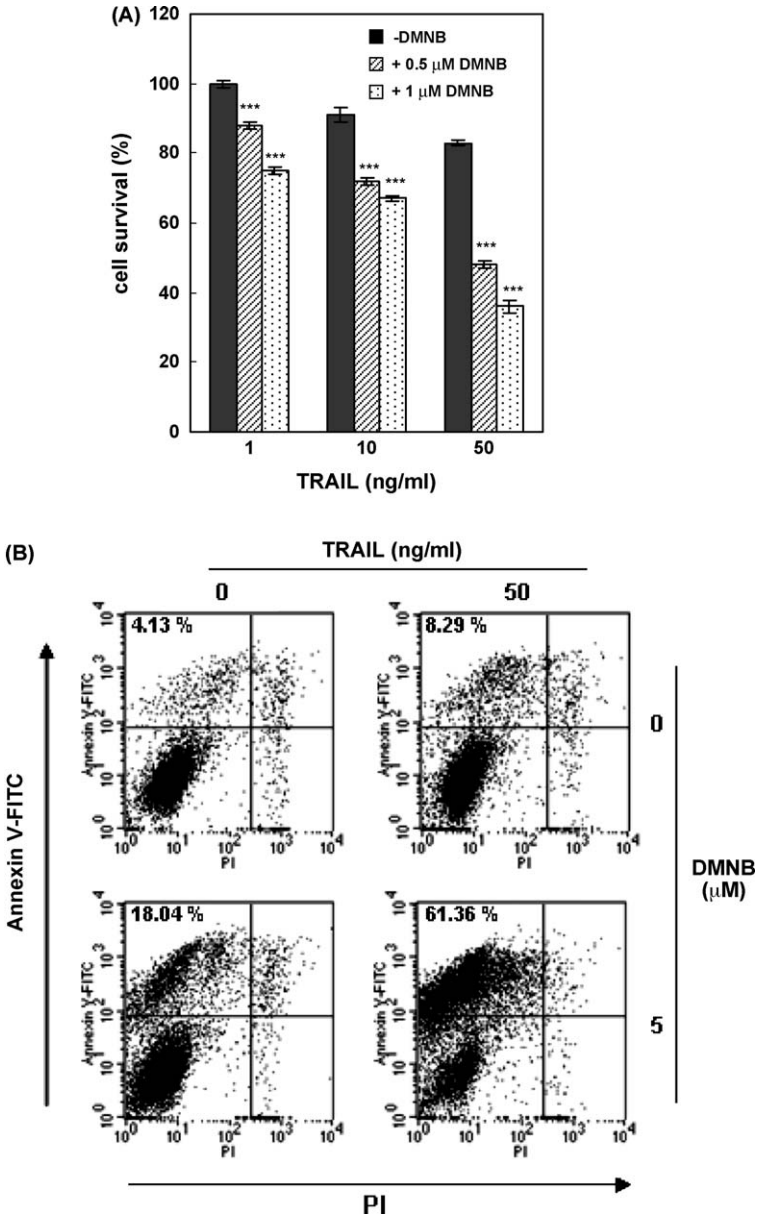

Fig. 8. Effect of DMNB on TRAIL-induced cytotoxicity and apoptosis. (A) K562 cells were treated with various concentrations of TRAIL (1, 10 and 50 ng/ml) in the presence or absence of DMNB (0.5- or 1 μM), and cell survival was determined after 5 days of incubation using the MTT assay. Data represent the means ± S.E. of triplicate experiments. $^{***}p < 0.001$ versus TRAIL alone-treated cells at the same dose point. (B) Cells were treated with 50 ng/ml TRAIL in the presence or absence of 5 μM DMNB for 24 h. Thereafter, the percentage of apoptotic cells in each cell population was determined by Annexin V staining and flow cytometry.

K562/R3 cells with a high sensitivity to TRAIL-induced cytotoxicity showed profoundly reduced levels of DNA-PKcs and p-Akt as compared with K562 cells. It has been reported that the constitutively active Akt inhibits TRAIL-induced apoptosis in various cancer cells such as prostate cancer, ovarian cancer, and acute leukemia cells [15–18], and that DNA-PKcs acts upstream to Akt and directly phosphorylates and activates Akt [24,27,28]. Therefore, the low activity of DNKA-PK and Akt may be responsible for the higher sensitivity of the K562/R3 cells to TRAIL as compared with K562 cells. It have been suggested that the induction of TRAIL receptors (DR4/DR5) is one of the major strategies to potentiate the TRAIL-induced apoptosis. Recently, it has been demonstrated that inhibition of PI3K/Akt by RNA interference sensitized resistant colon cancer cells to TRAIL-induced cell death through the induction of TRAIL receptors and activation of caspase-3 and caspase-8 [32]. Then we expected that DR4 and DR5 might be increased in K562/R3 cells. However, K562/R3 cells had an increased level of DR5 and a decreased level of DR4 as compared with K562 cells. Although reduction of DR4 levels in K562/R3 cells might cancel the increased sensitivity to TRAIL obtained from an increased level of DR5, this effect seemed to predominate over the cancelling effect from down-regulation of DR4, since the basal level of DR4 was lower than that of DR5 and TRAIL binds preferentially to DR5 [33]. Therefore, aup-regulation of DR5 might contribute to the increased susceptibility of K562/R3 cells to TRAIL-induced apoptosis. In addition, DR4 and DR5 were induced slightly in K562/R3 cell, but not in K562 cells after treatment with TRAIL. These changes in TRAIL receptors might determine the increased sensitivity to TRAIL in K562/R3 cells. Since DR4 and DR5 were induced after transfection with DNA-PKcs siRNA, some factors other than DNA-PKcs also might be involved in the expression regulation of TRAIL receptors and the determination of sensitivity to TRAIL in K562/R3 cells.

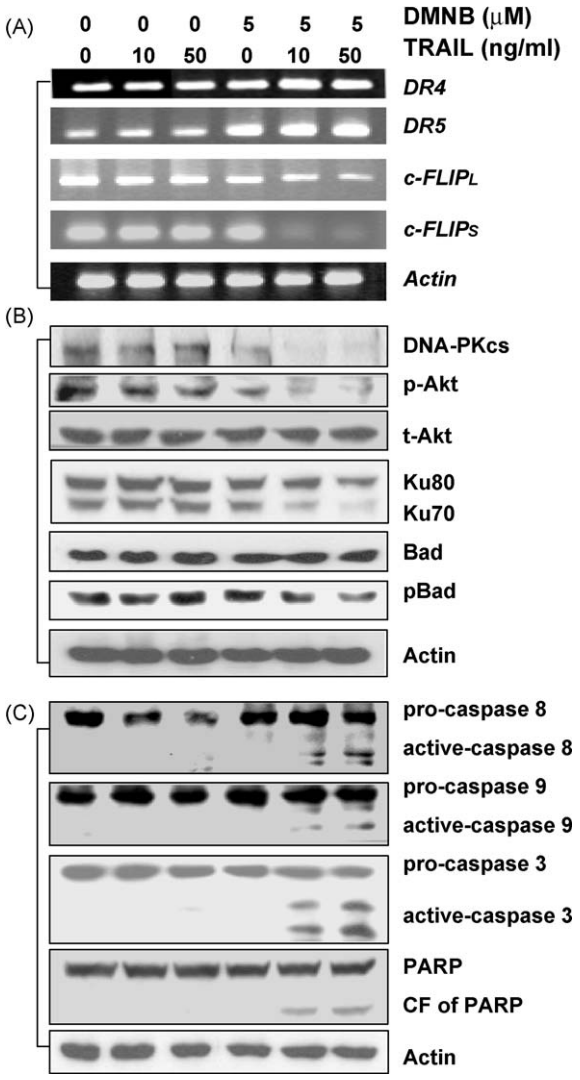

Fig. 9. The combination effect of DMNB and TRAIL on DNA-PKcs/Akt molecules and caspase activity. (A) K562 cells were treated with TRAIL (10 or 50 ng/ml) in the presence or absence of DMNB (5 μM) for 24 h and performed RT-PCR to monitor the mRNA levels of DR4/DR5 and c-$FLIP_{L/S}$. (B) The protein expression of DNA-PKcs, p-Akt, t-Akt and Ku70/80 was measured by western blot analysis in the cells co-treated with DMNB and TRAIL. (C) Activation of caspases and cleavage of PARP in the cells co-treated with DMNB and TRAIL were determined by western blot analysis.

To understand the role of DNA-PKcs in TRAIL-resistance, we silenced DNA-PKcs in K562 cells using small interfering RNA (siRNA). The targeted inhibition of DNA-PKcs led to up-regulation of DR4/DR5 and concurrent down-regulation of both c-$FLIP_L$ and c-$FLIP_S$, especially c-$FLIP_S$. The endogenous expression of c-FLIP, which has a sequence homology with caspase-8 and -10 but no protease activity, inhibits apoptosis by blocking the processing of caspase-8 [34]. A high level of c-FLIP is correlated with TRAIL resistance in some tumor types, and thus down-regulation of c-FLIP has been implicated in enhancement of TRAIL-induced apoptosis [35-37]. In addition, the level of p-Akt (S473) was also decreased by transfection with DNA-PKcs siRNA, which is reminiscent of K562/R3 cells with low levels of DNA-PKcs and p-Akt. It has been shown that the introduction of a dominant negative Akt adenoviral construct consistently reduced FLIP expression [38], and the reduction of Akt activity by LY294002 reduced the expression of $FLIP_S$ and the overexpression of constitutively active Akt in the TRAIL-sensitive cell line, SNU-668, rendered the cell line resistant to TRAIL [39]. Therefore, DNA-PK activity seemed to affect the expression of DR4, DR5 and c-FLIP via p-Akt (S473).

Recently, mTORC2 (mammalian target of rapamycin complex 2) was shown to be the elusive PDK2 responsible for phosphorylating Akt on S473 [40–42], which is also known to be phosphorylated by DNA-PKcs. In K562 cells, however, the phosphorylated status of Akt Ser473 was well-correlated with the activity of DNA-PKcs and could be suppressed almost completely by combination of DNA-PKcs siRNA and TRAIL. Therefore, DNA-PK, not mTORC2, may be a major determinant for Akt S473 phosphorylation in K562 cells.

The up-regulation of TRAIL receptors and concurrent down-regulation of c-FLIP induced by inhibition of DNA-PKcs was accompanied by increased sensitivity to TRAIL-induced apoptosis with increased activation of caspase-8, -9 and -3, which play a critical role in TRAIL-induced apoptosis [43]. Therefore, the targeted inhibition of DNA-PKcs would sensitize K562 cells to TRAIL-induced apoptosis via inactivation of DNA-PKcs/Akt pathway and subsequent increase of TRAIL receptor-mediated apoptotic pathway. Indeed, treatment with DMNB, a small molecule DNA-PK inhibitor [31], induced molecular changes reminiscent of the effects of DNA-PKcs siRNA in K562 cells, such as an increase in DR4 and DR5 and a decrease of c-$FLIP_{L/S}$ and p-Akt, and potentiated TRAIL-induced cytotoxicity and apoptosis.

Our study was the first study to provide evidence that the increased activity of DNA-PK/Akt pathway might play an important role in TRAIL resistance, and DNA-PK/Akt pathway might be a potential target for overcoming TRAIL-resistance in cancer cells with an increased activity of DNA-PK. It has been demonstrated that a new selective Akt inhibitor, 1L-6-hydroxymethyl-chiro-inositol 2(R)-2-O-methyl-3-O-octadecylcarbonate (Akt inhibitor), was as effective as Ly294002 in lowering the sensitivity threshold of HL60 cells to chemotherapeutic drugs, TRAIL, all-trans-retinoic acid, and ionizing radiation [44]. Therefore, TRAIL in combination with agents that inhibit DNA-PK/Akt pathway might have a clinical applicability for the treatment of TRAIL-insensitive human leukemic cells with an increased activity of DNA-PK. This model may provide a novel framework for overcoming of TRAIL resistance of other cancer cells such as prostate, lung, ovarian and breast cancer cells.

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