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Downregulation of Mcl-1 by daunorubicin pretreatment reverses resistance of breast cancer cells to TNF-related apoptosis-inducing ligand

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ABSTRACT

The tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promising cancer therapeutic agent. However, tumor cells often develop resistance to TRAIL, limiting its therapeutic potential. To study the mechanism underlying TRAIL-resistance in breast cancer cells, we performed a high-throughput compound screen in MCF-7 cells. We identified daunorubicin as a potent sensitizer of TRAIL-induced apoptosis in MCF-7 cells. Daunorubicin in combination with subtoxic concentrations of recombinant human TRAIL induced massive apoptosis in MCF-7 cells. This combination was effective in TRAIL-resistant MDA-MB-231 and T47D breast cancer cells. By immunoblotting, we found that daunorubicin treatment induced loss of the anti-apoptotic protein, Mcl-1, in breast cancer cells. RNA interference experiments revealed that reduced expression of Mcl-1 sensitized MCF-7 cells to TRAIL. Together, these data suggest that Mcl-1 is a major contributor to TRAIL-resistance in breast cancer cells, and that reduction of Mcl-1 protein levels using DNA damaging agents is a promising approach for cancer therapy.

1. Introduction

The tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces apoptotic cell death by signaling through TRAIL receptors including TRAIL-R1(DR4) and TRAIL-R2(DR5) [1,2]. Human TRAIL recombinant protein (rhTRAIL) induces cell death in cancer cells, but shows little or no toxicity in normal cells [3]. This fact has led to the initiation of several clinical trials testing TRAIL for cancer therapy, using agonistic TRAIL receptor antibodies and recombinant TRAIL protein. However, most tumors, including breast cancer cell lines and primary tumors from patients become resistant to TRAIL, highlighting the need for research to understand this resistance mechanism [4–6].

To date there have been some reports on the mechanism of resistance to TRAIL in cancer cells. Various mechanisms have been proposed, including abnormalities in TRAIL receptors [7–10], cell death signaling [11–14], and assembly of death inducing signaling complexes [15,16].

Numerous chemotherapeutic agents have been reported to potentiate TRAIL cytotoxicity in cancer cells. Proposed mechanisms of sensitization include increase of TRAIL receptor expression and elevation of pro-apoptotic proteins [17–19]. In particular, DNA damaging agents, including doxorubicin, have been proposed as sensitizing chemotherapeutic agents of TRAIL in cancer cells [20–24]. Doxorubicin potentially increases the expression level of death receptor 5 (DR5) in leukemia cells. However, this effect was not reproduced in breast cancer cells, suggesting that it may be cell line-dependent [20,21]. Clustering of DR5 has been proposed as a possible mechanism for sensitization of cancer cells to doxorubicin, but this was only observed in lymphocytes and splenocytes [22].

In this study, we performed a compound screen to identify novel mechanisms of resistance developed by breast cancer cells. We identified daunorubicin, a structural analog of doxorubicin, as a strong sensitizer of TRAIL in MCF-7 cells. Daunorubicin enhanced TRAIL cytotoxicity in TRAIL-resistant breast cancer cells by downregulating the anti-apoptotic protein Mcl-1. Our results suggest that Mcl-1 is a major contributor of TRAIL resistance. The combination of TRAIL and doxorubicin compounds has been reported to reverse drug resistance in various cancer cells. However, our study is the first to identify the involvement of Mcl-1 in the reversal of TRAIL-resistance.

2. Materials and methods

2.1. Cell culture and materials

MCF-7, T47D and SK-BR-3, human breast cancer cells were maintained in RPMI-1640. HeLa human cervical carcinoma cells, HCT-116 human colorectal carcinoma cells, and MDA-MB-231 human breast carcinoma cells were maintained in Dulbecco's Modified Eagle Medium. All media were supplemented with 10% fetal bovine serum (Life Technologies, Grand Island, NY), L-glutamine,

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and penicillin–streptomycin (100 U/ml). TRAIL and Lipofectamine 2000 reagent were purchased from Life Technologies. CellTiterGlo luminescence assay solution was purchased from Promega (Madison, WI).

2.2. Compound screening

MCF-7 cells were recovered from cultures and seeded in 96-well plates at a density of 7×10^3 cells per well in 80 μL media. The plates were then incubated for 40 h at 37 °C. Using an automated liquid handler (Perkin Elmer model AJM8M01), 10 μL test compound was added to wells to achieve final concentrations of 5 μM. Ten microliters of culture media were added to columns 1 and 12. After 2 h, 10 μL TRAIL-containing medium was added to each well (for final 25 ng/ml concentration), except for the wells in column 1, to induce cell death. Ten microliters of culture medium was added to the wells in column 1 to yield the same volume of reaction as TRAIL-treated wells. After 20 h, cell viability was assessed using a cellular ATP content assay (CellTiterGlo) with a Victor multi-label counter (Perkin Elmer). Raw values were transferred to Excel software to evaluate relative cell survival. The quality of each assay plate was assessed by calculating the Z′ factor [25]. The Z′ factor for the total screen was 0.755, implying that the screening was performed with high resolution. Using this system, we screened a library of 1200 marketed drugs (Prestwick-1200™). To identify effective compounds, a standard score for each tested sample was calculated using the equation, σ = (raw value of well − mean of total tested wells in a plate)/(standard deviation of total tested wells).

2.3. Immunoblotting

Cell extracts were prepared by adding lysis buffer (50 mM Tris–Cl, pH 7.4, 1% Igepal, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM Na_3VO_4, 2.5 mM β-glycerophosphate, and protease inhibitor mixture) to collected cells. After determining the protein concentration, 30 μg cell extract was analyzed by sodium dodecyl sulfate-poly acrylamide gel electrophoresis (SDS–PAGE), followed by immunoblotting using polyvinylidene difluoride membranes (Bio-Rad). The membranes were incubated in specific antibodies, which were diluted 1:1000. Antibodies used for immunoblotting include the following: anti caspase-8 (CS-9746), caspase-7 (CS-9494), Bid (CS-2002), PARP (CS-9532), Bcl-2 (CS-2872), Bcl-X_L (CS-2764), phospho ERK (CS-4370), ERK (CS-4695), and Mcl-1 (CS-5453) from Cell Signaling (Danvers, MA). Antibody-antigen complexes were detected with horseradish peroxidase-conjugated immunoglobulin G (Bio-Rad), and a chemiluminescent substrate (SuperSignal West Pico-Pierce). The membranes were then stripped and re-probed with an antibody to α-tubulin, which served as a loading control.

2.4. Cell viability analysis

Cell viability was determined using a cellular ATP content evaluating luminescence assay (CellTiterGlo assay-Promega), propidium iodide uptake, or trypan blue exclusion assay. For ATP assays, cells were seeded in 96-well plates at a density of 5×10^3 cells/well in 80 μL culture media. Cells were pre-treated with 10 μL daunorubicin-containing medium to achieve the indicated concentrations. After 2 h of incubation, cells were treated with various concentrations of rhTRAIL in 10 μL medium. After 20 h of incubation, 10 μL assay solution was added to each well, and the corresponding luminescence value was acquired. Raw values were analyzed using GraphPad Prism software (GraphPad, La Jolla, CA) to evaluate relative cell survival.

For flow cytometric analysis, 5×10^5 cells were cultured in 6-well plates. Cells were pre-incubated with 2.5 μM daunorubicin and treated with rhTRAIL. After 20 h, cells were recovered from the wells, washed with cold phosphate-buffered saline (PBS) and resuspended in 500 μl binding buffer containing propidium iodide (50 μg/ml). The percentage of dead cells was determined by flow cytometry, using a FACS Calibur instrument (BD Biosciences, San Jose, CA).

2.5. DNA fragmentation assay

After treatment, 3×10^6 cells were washed with cold PBS and directly lysed with 500 μl genomic DNA extraction buffer (0.1 M NaCl, 0.01 M EDTA, 0.3 M Tris–HCl pH 7.5, and 0.2 M sucrose). 25 μL of 10% SDS was added, and cells were incubated for 30 min at 65 °C. Samples were neutralized using 120 μL of 5 M KoAC and stored on ice for 1 h. After centrifugation, samples were mixed with 500 μL Tris EDTA and 2 μL RNAase (10 μg/ml), and incubated for 30 min at room temperature. DNA was extracted using phenol–chloroform and resolved in a 1% agarose gel.

2.6. siRNA transfection and cell viability assay after Mcl-1 knockdown

Mcl-1 siRNA (5′-aaguaucacagacguucuctt-3′) and non-silencing siRNA (5′-acgugacacguucggagaauu-3′) were synthesized by Genolution (Seoul, Korea). RNAi max reagent™ was used for reverse transfection of breast cancer cells using the manufacturer's protocol. Briefly, cells were seeded in 24-well dishes at a density of 5×10^4 cells/well in 0.5 ml culture media and incubated with 0.1 ml transfection mixture containing 6 pmol siRNA and 1 μl transfection reagent. After 2 days of incubation, cells were treated with rhTRAIL, with or without daunorubicin. Following 20 h of incubation, cell viability was assessed using trypan blue exclusion assays. To confirm knockdown efficiency, cell extracts were prepared and subjected to SDS–PAGE, followed by immunoblotting.

3. Results

3.1. Daunorubicin is a sensitizer of TRAIL in MCF-7 cells

Breast cancer cells are generally resistant to TRAIL-induced cell death. We first compared the viability of human breast cancer cells upon exposure to rhTRAIL in a panel of six human cancer cell lines. HeLa, a human cervical carcinoma cell line, and HCT-116, a human colon carcinoma cell line showed dose-dependent cell death when treated with rhTRAIL and evaluated by cellular ATP assays (Fig. 1A). In contrast, breast cancer cells (MCF-7, MDA-MB-231, T47D, and SK-BR-3) were strongly resistant to rhTRAIL-induced cell death. At a concentration as low as 25 ng/ml, rhTRAIL induced cell death in 52% of HeLa and 91% of HCT-116 cells, relative to control cells (Fig. 1A). However, at concentrations as high as 100 ng/ml TRAIL, all breast cancer cells showed relative viability greater than 60% (Fig. 1A).

To identify novel mechanisms of resistance to TRAIL in breast cancer cells, we performed a compound screen using a library consisting of marketed drugs (Prestwick-1200) to isolate sensitizing compounds for rhTRAIL (Fig. 1B). TRAIL-resistant MCF-7 cells served as a control cell line. Cells were pre-treated with 5 μM compound, followed by a subtoxic dose of rhTRAIL (25 ng/ml). After evaluation of the relative survival ratio, data were normalized using standard scores. A primary 'hit' was defined as a compound showing a standard score lower than -3σ, or a compound inducing more than 50% cell death when cells were cotreated with rhTRAIL, relative to control treatment. During the primary screening, we identified 30 candidate compounds, including daunorubicin

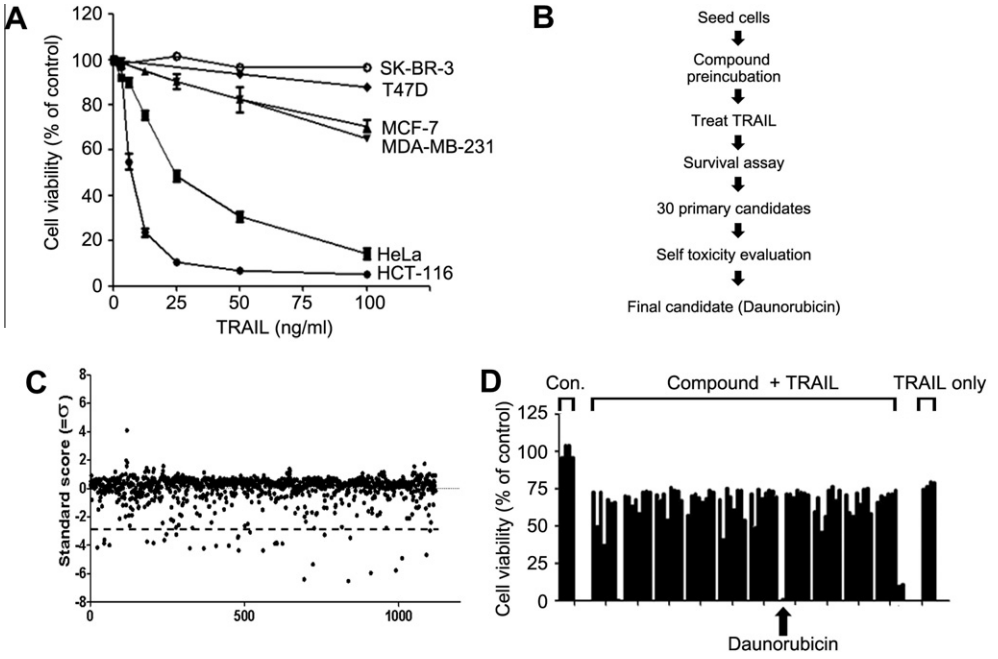

Fig. 1. Resistance of breast cancer cells to rhTRAIL and compound screening to isolate TRAIL-sensitizers. (A) Cell viability assay of TRAIL-induced cell death in various cell lines. The mean ± standard deviation (SD) ($n = 3$) values are shown. Cells were treated with rhTRAIL at the indicated doses for 20 h. Relative cell survival was evaluated by converting raw data from ATP content assays. (B) Flow chart of the compound screen used to identify sensitizers of TRAIL-induced cell death in MCF-7 cells. (C) Normalized standard score distribution for the 1200 small molecule screen. The -3σ cut-off used for hit definition is indicated as a dotted line. (D) Representative graph of an assay plate. Normalized relative survival (y axis) was plotted against well number (x axis). Wells A1 to H1 are controls (cells with DMSO only) and the wells in column 12 are experimental controls (DMSO with rhTRAIL). Sensitization by daunorubicin is indicated.

(Fig. 1C). We next evaluated the cytotoxicity of each compound, when cells were treated with compounds alone. We discarded 29 primary candidate compounds as they were toxic by themselves at 5 μM. Fig. 1D shows the result of an assay plate containing daunorubicin as the primary candidate compound. Cell viability was reduced to less than 10% when cells were treated with 5 μM daunorubicin and rhTRAIL (25 ng/ml). In contrast, treatment with rhTRAIL alone showed minimal effects in ATP assays (Fig. 1D).

3.2. Combination of daunorubicin and TRAIL induces apoptotic cell death

To verify the sensitizing effect of daunorubicin and TRAIL in MCF-7 cells, cells were treated with fixed concentrations of daunorubicin (2.5 μM) and increasing doses of rhTRAIL. When cells were incubated with subtoxic doses of TRAIL (12.5 ng/ml) and daunorubicin, MCF-7 cells underwent massive cell death with up to 80% of cells dying. In contrast, TRAIL-only treated cells showed 91% cell viability (Fig. 2A). Induction of massive cell death was confirmed by photography (Fig. 2B) and flow cytometric analysis of propidium iodide uptake (Fig. 2C). To determine if this cell death was apoptotic, we performed DNA fragmentation analysis and immunoblotting to detect caspase activation. As shown in Fig. 2D, the combination of daunorubicin and rhTRAIL induced DNA fragmentation, caspase activation, and cleavage of caspase substrates (Bid, Poly ADP ribosyl polymerase), indicating that this cell death was apoptotic (Fig. 2D).

3.3. Daunorubicin sensitized breast cancer cells to TRAIL

We next set investigated if the sensitizing effect of daunorubicin and TRAIL is cell-type specific (specific to MCF-7 cells) or potentially effective in other TRAIL-resistant breast cancer cells. MDA-MB-231, T47D, and SK-BR-3 cells were pre-incubated with daunorubicin and treated with rhTRAIL at various doses. As shown in Fig. 3, daunorubicin sensitized MDA-MB-231 and T47D cells to rhTRAIL. SK-BR-3 cells were already sensitive to daunorubicin, and we did not observe induction of additional cell death with rhTRAIL. These data suggest that the combination of daunorubicin and rhTRAIL was effective in inducing cell death in breast cancer cells. However, the mechanism involved in TRAIL-resistance in SK-BR-3 cells may be distinct to other breast cancer cells.

3.4. Daunorubicin induces loss of Mcl-1 protein in TRAIL-resistant breast cancer cells

To understand the mechanism of enhanced cell death initiated by daunorubicin, we aimed to identify cell death proteins that were modulated by daunorubicin incubation. Bcl-2 and Bcl-X_L expression levels were not altered by the daunorubicin and rhTRAIL (Fig. 4). However, protein expression of Mcl-1 and the phosphorylation level of p42/44 ERK were strongly reduced by combination treatment (Fig. 4A).

To determine if the sensitizing effect of daunorubicin was regulated similarly in different cancer cells, we examined various breast cancer cells including MDA-MB-231 and T47D cells, which were previously sensitized to rhTRAIL by daunorubicin pre-incubation. As shown in Fig. 4B, phosphorylation of ERK was regulated differently in various breast cancer cells. In MDA-MB-231 cells, phosphorylation of ERK was increased by daunorubicin, and reduced to normal levels by combination treatment. However, in T47D cells, daunorubicin treatment reduced phosphorylation of ERK, whereas combination treatment increased ERK phosphorylation to normal levels. These data suggest that ERK activity modulation is not a common mechanism for the sensitization of breast cancer cells to rhTRAIL treatment by daunorubicin. However, Mcl-1 protein expression level was similarly decreased by daunorubicin incubation in both cell lines, with comparable results to MCF-7 cells. In MCF-7 cells, the Mcl-1 expression level was de-

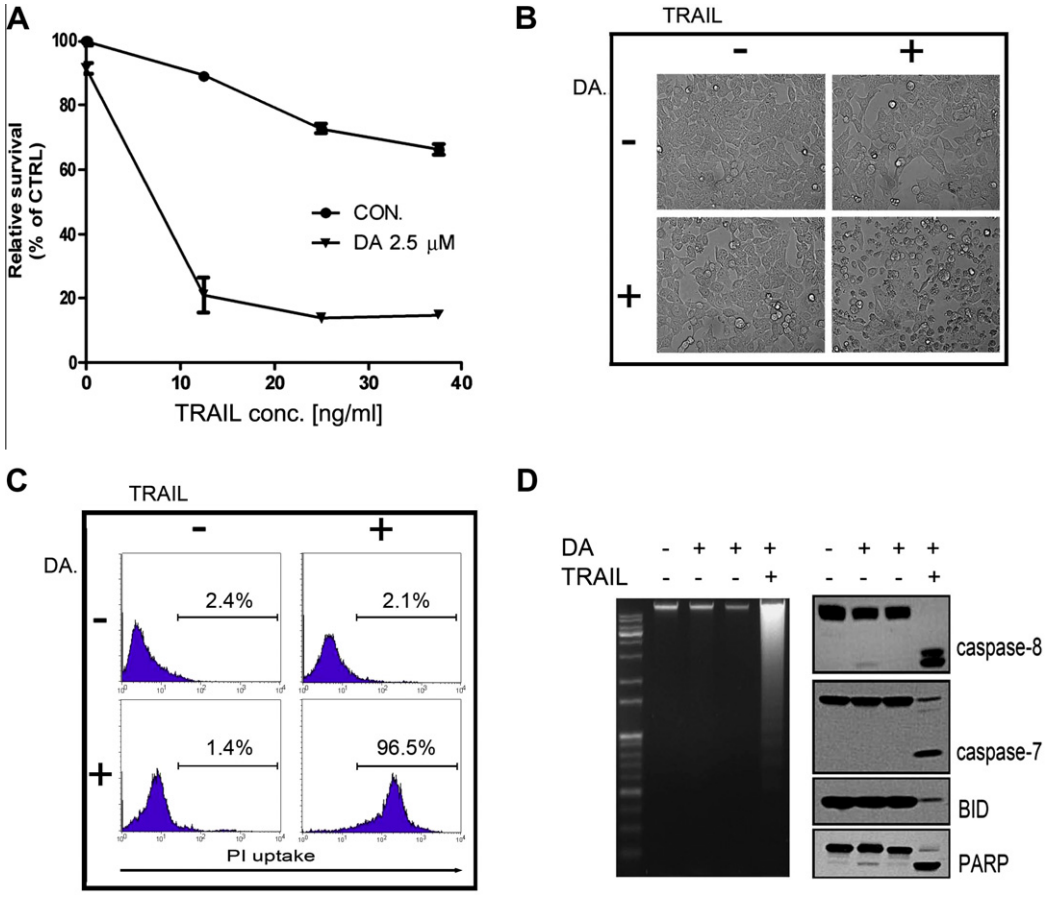

Fig. 2. Combination of daunorubicin and rhTRAIL induces synergistic cell death in MCF-7 cells. Cells were incubated with 2.5 μM daunorubicin, the indicated dose of rhTRAIL, or both. Cytotoxicity was measured using ATP content assays after 20 h of treatment. Representative experiment data are shown in (A) Similar results were obtained at least three times. (B) Photographs showing synergistic cell death resulting from combination treatment after 8 h of incubation. (C) Propidium iodide uptake assay using flow cytometry. (D) Combination treatment induced apoptotic cell death. Samples from C were subjected to DNA fragmentation and immunoblotting analysis as described in the Materials and Methods.

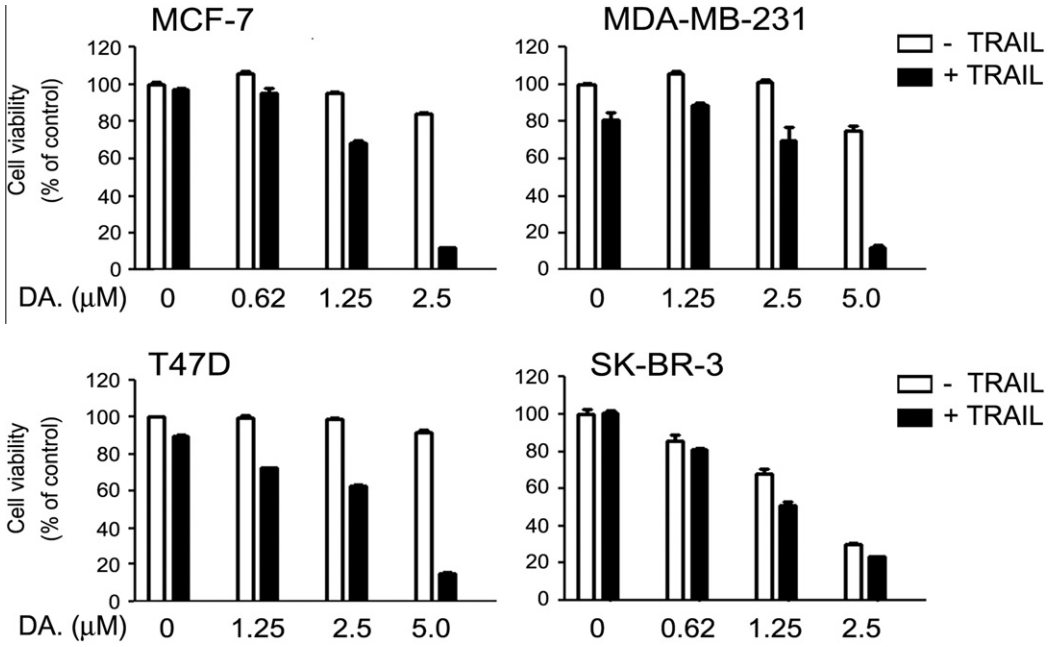

Fig. 3. Daunorubicin reverses resistance of breast cancer cells to TRAIL. The raw ATP content was determined in breast cancer cells treated with indicated concentrations of daunorubicin and rhTRAIL (25 ng/ml for MDA-MB231 and MCF-7, 100 ng/ml for T47D and SK-BR-3) for 20 h. Each concentration was tested in triplicate, and the SD is represented.

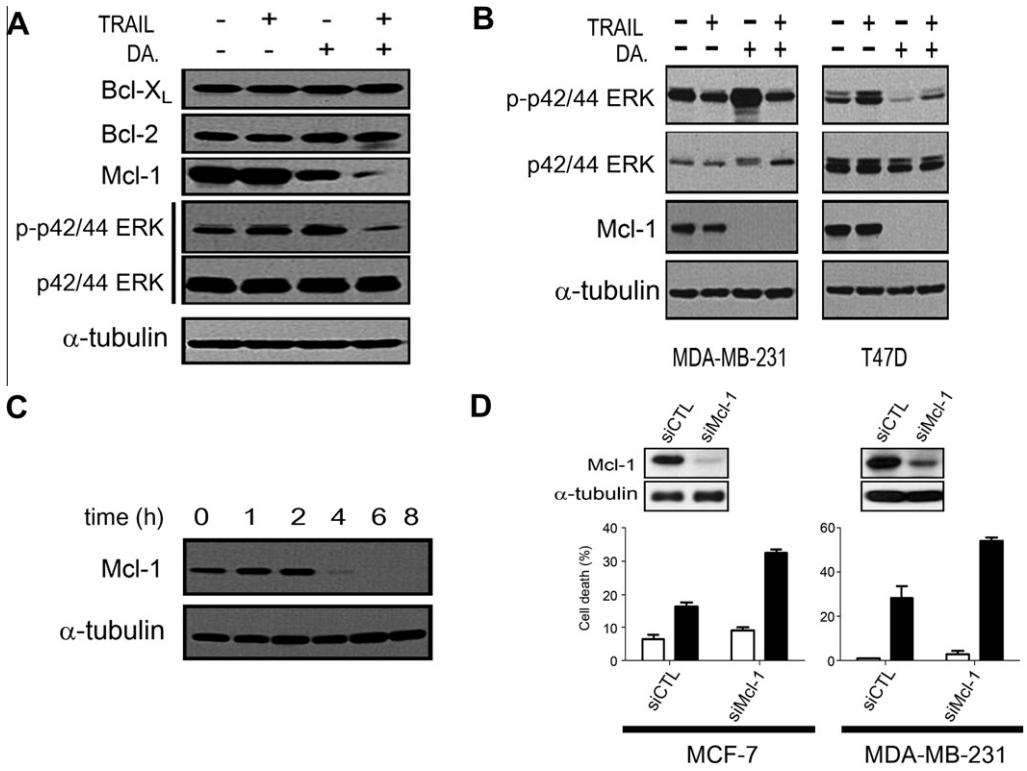

Fig. 4. Downregulation of Mcl-1 by daunorubicin sensitizes breast cancer cells to TRAIL. (A) Western blot analysis of apoptotic proteins in MCF-7 cells treated with rhTRAIL (25 ng/ml), daunorubicin (2.5 μM), or a combination of rhTRAIL and daunorubicin for 8 h. (B) Downregulation of Mcl-1 and p42/44 ERK activity detected by immunoblotting. Extracts from MDA-MB-231 and T47D cells treated with rhTRAIL and daunorubicin were analyzed by immunoblotting. (C) MCF-7 cells were incubated with 2.5 μM daunorubicin for the indicated times, and Mcl-1 expression levels were analyzed by western blotting. (D) Knockdown of Mcl-1 in breast cancer cells sensitized to rhTRAIL. MCF-7 and MDA-MB-231 cells were transfected with non-silencing siRNA (siCTL) or *Mcl-1* siRNA (siMCL-1). After 48 h of transfection, cells were treated with rhTRAIL (25 ng/ml) and cell death was evaluated by trypan blue staining. Data represent mean ± SD ($n = 3$). Mcl-1 knockdown efficiency was confirmed by immunoblotting.

creased by 2.5 μM daunorubicin in a time-dependent manner (Fig. 4C).

We then asked if the sensitization effect of daunorubicin on rhTRAIL treatment occurred through Mcl-1, by RNA interference of Mcl-1. As Fig. 4D shows, knockdown of Mcl-1 protein expression sensitized MCF-7 cells to TRAIL, suggesting that Mcl-1 is a critical inhibitor of TRAIL-induced cell death in MCF-7 breast cancer cells.

4. Discussion

The development of cancer therapy without side effects is the ultimate goal of clinical oncology. By this point, TRAIL has a wide therapeutic window and has attracted major interest as a cancer-specific agent with little or no side effects in normal cells [2]. Unfortunately, many primary tumors develop resistance to TRAIL [26]. Therefore, understanding the mechanism of TRAIL-resistance has been an important topic of research since its discovery.

Huge efforts have been made to identify resistance mechanisms in cancer cells to TRAIL. Some groups have identified the DNA damaging agent doxorubicin as a potent sensitizer of cancer cells to TRAIL [20–24]. In these reports, concentrations of doxorubicin and TRAIL used were very low, raising the possibility that low doses of combinations of anti-cancer drugs could be effective in killing cancer cells. Most of the mechanisms of sensitization identified to date have been cell-type specific. Such mechanisms include increased ceramide release by doxorubicin [22] and loss of Flip expression [23,24]. To date, there has been no report on the relationship between the sensitizing effects of DNA damaging agents on TRAIL and Bcl-2 family proteins, in particular, Mcl-1. In this study, we extended our investigation of the mechanism of resistance to TRAIL to Mcl-1 protein. This is the first report showing that DNA damaging agents induce loss of Mcl-1 protein in cancer cells, leading sensitization to TRAIL.

Mcl-1 is an anti-apoptotic Bcl-2 family protein that binds to pro-apoptotic Bcl-2 proteins, including Bax and Bik, and neutralizes their pro-apoptotic functions in cancer cells [27]. Cumulative data suggest that Mcl-1 is a critical mediator of cellular resistance to various anti-cancer therapies. The importance of Mcl-1 in suppressing TRAIL-induced cell death was identified in previous reports showing that reduced expression of Mcl-1 using RNA interference or microRNA-29b enhanced TRAIL-induced cell death in cancer cells [28,29]. A practical method of downregulating Mcl-1 expression is through use of the multiple kinase inhibitor sorafenib [30]. In hematopoietic malignant cells, sorafenib reduced the expression level of Mcl-1, consequently sensitizing cells to TRAIL. This effect was confirmed in patient-derived acute myelogenous leukemia cells [31], providing support for the use of Mcl-1 downregulation, and TRAIL to treat cancer cells. As in the case of sorafenib, the identification of therapeutic agents that preferentially downregulate Mcl-1 would be critical for the use of TRAIL in the clinical setting.

As shown by our data, the DNA-damaging agent daunorubicin, a structural analog of doxorubicin, could sensitize breast cancer cells to TRAIL by downregulating Mcl-1 protein. This effect was observed in all breast cancer cells tested (Fig. 4B), except SK-BR-3. Knockdown of Mcl-1 protein sensitized MCF-7 and MDA-MB-231 cells to rhTRAIL, in concordance with findings for daunorubicin (Fig. 4D). ERK phosphorylation was suppressed by combination treatment in MCF-7 cells. However, the phosphorylation status of ERK differed in other breast cancer cells, and inhibition of the ERK pathway did not sensitize cells to TRAIL (Fig. 4B, and data

not shown). Taken together, our data suggest that daunorubicin sensitizes breast cancer cells to TRAIL via downregulation of Mcl-1 protein.

Notably, the increased cell death rate of breast cancer cells by Mcl-1 knockdown still accounts for only a part of the sensitization effect. Mcl-1 knockdown sensitized cells to TRAIL with a 20% increase in cell death. As mentioned earlier, DNA damaging agents have been shown to upregulate the expression level of DR5 in leukemia cells. However, we could not detect upregulation of DR5 in any breast cancer cells, suggesting that the effect may be cell type-specific. Future studies to characterize the molecular mechanism behind sensitization of breast cancer cells to TRAIL by DNA damaging agents should focus on (a) membrane relocation of TRAIL receptors, (b) formation of the death-inducing signaling complex, and (c) modulation of stress signaling following use of DNA damaging agents.

It is possible that the sensitization of cancer cells to TRAIL by daunorubicin through Mcl-1 downregulation is a distinct mechanism occurring in breast cancer cells. This scenario warrants further study.

It is not clear whether downregulation of Mcl-1 by daunorubicin is an event at the transcriptional or post-translational level. Mcl-1 is a fragile protein that is degraded by the ubiquitin proteasome system [32]. It is possible that daunorubicin induces degradation of Mcl-1 through an unidentified signaling mechanism, to enhance ubiquitination of Mcl-1. However, the detailed mechanism of action needs to be characterized further.

In summary, we identified daunorubicin as a TRAIL-sensitizing compound of breast cancer cells, which were originally resistant to TRAIL. The identification of a DNA damaging agent as a sensitizer of TRAIL is in itself not a novel finding. However, we are the first to identify that reduced expression of Mcl-1 following daunorubicin treatment is one factor in sensitization of breast cancer cells to TRAIL. Collectively, these findings suggest that Mcl-1 downregulation is a promising strategy for cancer therapy when using TRAIL for the treatment of breast cancers.

Acknowledgments

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