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Original Article

Selective TRAIL-triggered apoptosis due to overexpression of TRAIL death receptor 5 (DR5) in P-glycoprotein-bearing multidrug resistant CEM/VBL1000 human leukemia cells

Soo-Jung Park[1, 2,*], Khadijeh Bijangi-Vishehsaraei[1,2], Ahmad R. Safa[1,2]

[1]*Department of Pharmacology and Toxicology,* [2]*Indiana University Simon Cancer Center, Indianapolis, IN, USA.*
**Present address: Clinical Research Center, National Masan Hospital, Masan 631-710, South Korea.*

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Abstract: The death-inducing cytokine, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), holds enormous promise as a cancer therapeutic due to its highly selective apoptosis-inducing action on neoplastic versus normal cells. Our results revealed that TRAIL selectively triggered apoptosis in the P-glycoprotein (P-gp, ABCB1) and DR5 overexpressing CEM/VBL1000 multidrug resistant leukemia cell line, but not in the parental CEM cells. Moreover, TRAIL treatment reduced P-gp expression in these cells. Mechanistic analysis of TRAIL-induced apoptosis revealed that TRAIL hypersensitivity is due to robust upregulation of the TRAIL receptor DR5 at the protein and mRNA levels during development of MDR in the CEM/VBL1000 variant. DR5 upregulation was independent of the level of expression of endoplasmic reticulum stress regulator C/EBP homologous transcription factor (CHOP/GADD153). TRAIL-triggered apoptosis was associated with increased expression of FADD; activation of caspases-3, -8, -9, and -10; and cytochrome *c* release from mitochondria. Therefore, both the extrinsic and intrinsic apoptosis pathways are involved in this process. These findings for the first time reveal that TRAIL treatment selectively causes apoptosis in P-gp-overexpressing CEM/VBL1000 cells through strong upregulation of DR5. Moreover, this hypersensitivity to TRAIL and its effect on reducing P-gp expression in these cells hold significant clinical implications for using TRAIL to eradicate MDR malignant cells.

Keywords: TRAIL, P-glycoprotein, TRAIL death receptor 5 (DR5), apoptosis, caspases, death receptors

Introduction

Drug resistance is a major clinical problem and an important cause of treatment failure in cancer patients. Numerous mechanisms have been found to cause resistance to chemotherapeutic agents in cancer cells *in vitro* [1-8]. Many types of cancer display intrinsic resistance to multiple chemotherapeutic agents. Other cancers acquire multidrug resistance (MDR) during chemotherapy. MDR is frequently associated with the overexpression of P-gp (ABCB1), a 170 kDa ATP-dependent transmembrane protein encoded by the *MDR*1 gene. P-gp is capable of pumping a number of structurally unrelated chemotherapy drugs and other compounds out of the cell by utilizing the energy of ATP hydrolysis [8], resulting in decreased intracellular accumulation of the compounds, and hence resistance to drug cytotoxicity.

Defects in apoptotic signaling pathways in malignant cells contribute to the drug resistance in various cancer types [9-12]. Therefore, strategies to lower the thresholds for triggering apoptosis in various cancers may lead to new and more effective therapeutic regimens. The death-inducing cytokine, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), is a death cytokine that induces cancer cell apoptosis without harming normal tissues [13, 14] by activating two apoptosis pathways: (a) the extrinsic death receptor pathway, and (b) the intrinsic mitochondrial pathway [6, 14, 15]. It does so by binding to its cell surface receptors, DR4 and DR5, which trigger receptor recruitment of the death-adaptor protein FADD through homotypic binding interactions between death domains

(DD) in DR4/DR5 and the adaptor protein Fas-associated death domain (FADD) [16-18]. FADD in turn recruits apical procaspases-8 and/or -10 to the receptor complex through homotypic interactions between death effector domains (DED) in FADD and procaspase-8/10, thus forming the death-inducing signaling complex (DISC) [6, 16-19]. Once activated in the DISC, caspases-8/10 cleave the pro-apoptotic molecule Bid, which then translocates to the mitochondrial membrane, leads to release of mitochondrial cytochrome *c* into the cytosol, activates both caspases-9 and -3, and finally cause apoptosis [14-19]. Often, caspases-8/10 executioner caspase activation is sufficiently robust that the apoptosis response is caspase-9-independent [6, 18]. In contrast, neither decoy receptor 1 (DcR1) nor decoy receptor 2 (DcR2), which contain a truncated cytoplasmic death domain, mediates apoptosis after binding to TRAIL; DcR1 and DcR2 thus serve as decoy receptors that sequester TRAIL at the cell surface [6, 20, 21].

We were the first to show, in a number of cell lines from various tumor types displaying acquired MDR and expressing various degrees of resistance to several chemotherapeutic agents, that overexpression of P-gp enhances apoptosis triggered by TRAIL [22] or TRAIL recombinant adenovirus [23]. In this study, for the first time we reveal that DR5 is robustly overexpressed in P-gp-overexpressing CEM/VBL1000 cells, an MDR variant of human CCRF-CEM acute T-lymphoblastic lymphoblastic leukemia (ALL), and that TRAIL treatment selectively causes apoptosis in these cells through binding to DR5. To our knowledge, increased expression of DR5 in cancer cells overexpressing other ABC transporters has not been reported. Our data on this hypersensitivity to TRAIL and its effect on reducing P-gp expression in the CEM/VBL1000 MDR variant hold significant clinical implications for using TRAIL to eradicate MDR malignant cells.

Materials and methods

Cell lines and culture conditions

The human T-lymphoblastic leukemia CCRF-CEM (CEM/WT) and the P-glycoprotein-expressing, vinblastine (VBL)-resistant cell line, CEM/VBL1000, were obtained from Dr. Victor Ling (Department of Cancer Genetics, British Columbia Cancer Agency, University of British Columbia, Vancouver, British Columbia, Canada) and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and penicillin-streptomycin (50U/ml) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Five μg/ml vinblastine are added regularly to the CEM/VBL1000 culture medium to maintain drug resistance. CEM/VBL1000 cells are incubated in vinblastine-free medium for over 1 week prior to use in experiments.

Annexin V analysis for apoptosis measurement

For determination of cell death, cells were treated for 24 h with or without the indicated concentrations (0.1-5 ng/ml) of TRAIL. The cells were resuspended in 100 μl of staining solution containing annexin V-fluorescein and propidium iodide in a HEPES buffer (BD Pharmingen). After incubation at room temperature for 20 min, cells were analyzed by FACSCalibur flow cytometer. Annexin V binds to those cells that express phosphotidylserine on the outer layer of the cell membrane, and propidium iodide stains the cellular DNA of those cells with a compromised cell membrane. This allows for the discrimination of live cells (unstained with either fluorochrome) from apoptotic cells (stained only with annexin V and propidium iodide).

Treatment of cells with chemotherapeutic agents

CEM/WT and CEM/VBL1000 cells were treated with or without vinblastine (5 ng/ml), vincristine (5 ng/ml), or paclitaxel (0.1 μM) for 24 h, and the percentage of apoptotic cell was evaluated by the annexin V binding assay

Measurement of cytotoxicity by MTT assay

Cell survival was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. MTT is cleaved by mitochondrial enzymes, mainly by succinate dehydrogenase, to form a dark blue crystalline product, formazan. Reduced formation of formazan is caused by decreased mitochondrial dehydrogenase activity, inhibited cell proliferation, or cell death. Cells were seeded in 96-well plates at a concentration of 1 x 10^3 cells/well and treated with increasing concentrations of TRAIL (0.1-5 ng/ml) for 24 h. After treatment, the medium was incubated with 0.5 mg/ml MTT dye (Sigma-Aldrich, St Louis, MO) for 3 h, the dark blue crystals formed were dissolved at 0.1N HCl in isopropyl alcohol, and absorbance was measured at 570 nm using a spectrophotometer. Results are presented as percentage of survival, using controls as 100%.

TRAIL binding assay

CEM/WT and CEM/VBL1000 cells (2×10^5) were incubated with 0.1-5 ng/ml TRAIL, respectively, for 24 h. After treatment, the cells were incubated with anti-TRAIL antibody for 1 h and washed 3 times with PBS, then incubated with 5 μg/ml of FITC-conjugated anti-rabbit antibody (Sigma-Aldrich, St Louis, MO) at room temperature for 1 h, followed by rinsing with PBS. The fluorescence intensities of the samples were then measured by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). The data were analyzed using the CellQuest program.

Confocal microscopic analysis

CEM/WT and CEM/VBL1000 cells (1×10^6) were cultured in media containing 10% fetal bovine serum in the absence or presence of 1 and 5 ng/ml TRAIL. After 24 h incubation, the cells were attached to poly-L-Lysine coverslips (BD BioCoat), then fixed in 4% PBS buffered paraformaldehyde for 30 min on the ice. The fixed cells were washed in PBS, incubated in 2% bovine serum albumin (BSA) in PBS for 20 min, then primary antibody (UIC2, DR4, DR5, IgG_{2a}) was added at room temperature. After 30 min, the samples were washed in PBS/0.02 % Tween, then Texas Red goat anti-mouse IgG (Molecular Probes, Eugene OR) or FITC-labeled secondary antibody, plus DAPI, were added for 30 min. These samples were washed in cold PBS, mounted onto slides, then examined by confocal microscopy.

Western blot analysis

Cells were washed in ice cold PBS and extracted for 30 min with a buffer containing 50 mM Tris-HCl, pH 7.5, 140 mM NaCl, 5 mM EDTA, 5 mM NaN_3, 1% Triton X-100, 1% NP-40, 1 mM EGTA, and protease inhibitor cocktail. Lysates were cleared by centrifugation at 13,000 rpm for 30 min, and protein concentrations were determined using the Bradford protein assay. Proteins were denatured in 2% sodium dodecyl sulfate containing sample buffer, and same total protein amount was transferred onto PVDF membranes. The membranes were probed with specific antibodies. The following primary antibodies were used: rabbit anti-caspase-3 polyclonal antibody (1:1,000 v/v), rabbit anti-PARP polyclonal antibody (1:1,000 v/v), mouse anti-cytochrome *c* monoclonal antibody (1:1,000 v/v), and mouse anti-CADD153 monoclonal antibody (1:1,000 v/v) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The mouse anti-caspase-8 monoclonal antibody (1:1,000 v/v) and the anti-TRAIL-R2 (DR5) rabbit polyclonal antibody (1:1,000) were purchased from Cell Signaling Technology, Inc. (Beverly, MA). The rabbit anti-caspase-9 polyclonal antibody (1:1,000 v/v) was provided by Chemicon International (Temecula, CA). Immunocomplexes were detected using horseradish peroxidase conjugated either with anti-mouse, anti-rabbit, or anti-goat IgG followed by chemiluminescence detection (ECL, Thermo Scientific, Rockford, IL).

Treatment of cells with neutralizing antibodies to death receptors and apoptosis detection

To determine whether TRAIL-induced apoptosis occurs through the death receptors, cells were pretreated with the TRAIL-R1 (anti-DR4) or TRAIL-R2 (anti-DR5) antibody (10 μg/ml, R & D Systems, Minneapolis, MN) 3 h before treatment with 1 and 5 ng/ml TRAIL for 24 h. In control experiments, cells were treated normal IgG_{2a} before TRAIL treatment. Apoptosis was the measured by annexin V assay as described above.

RNA isolation and RT-PCR analysis

Total RNA from CEM/WT and CEM/VBL1000 cells was isolated by Tri Reagent TR-118 (Molecular Research Center, Cincinnati, OH) as described by the manufacturer. One microgram of total RNA was used in reverse transcription reactions with M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA) with dNTPs and an oligo dT primer (Promega, Madison, WI) as described by the manufacturer. One μg of the resulting total cDNA was then used as the template in PCR to measure the mRNA level of interest by using following primers: DR4 (forward) 5′-CTGAGCAACGCAGACTCGCTGTCCAC-3′ and (reverse) 5′-AAGGACACGGCAGAGCCTGTGCCAT-3′; DR5 (forward) 5′-CTGAAAGGCATCTGCTCAGG TG-3′ and (reverse) 5′-CAGAGTCTGCATTACCTTCT AG-3′. For *β*-actin, we used the following prim-

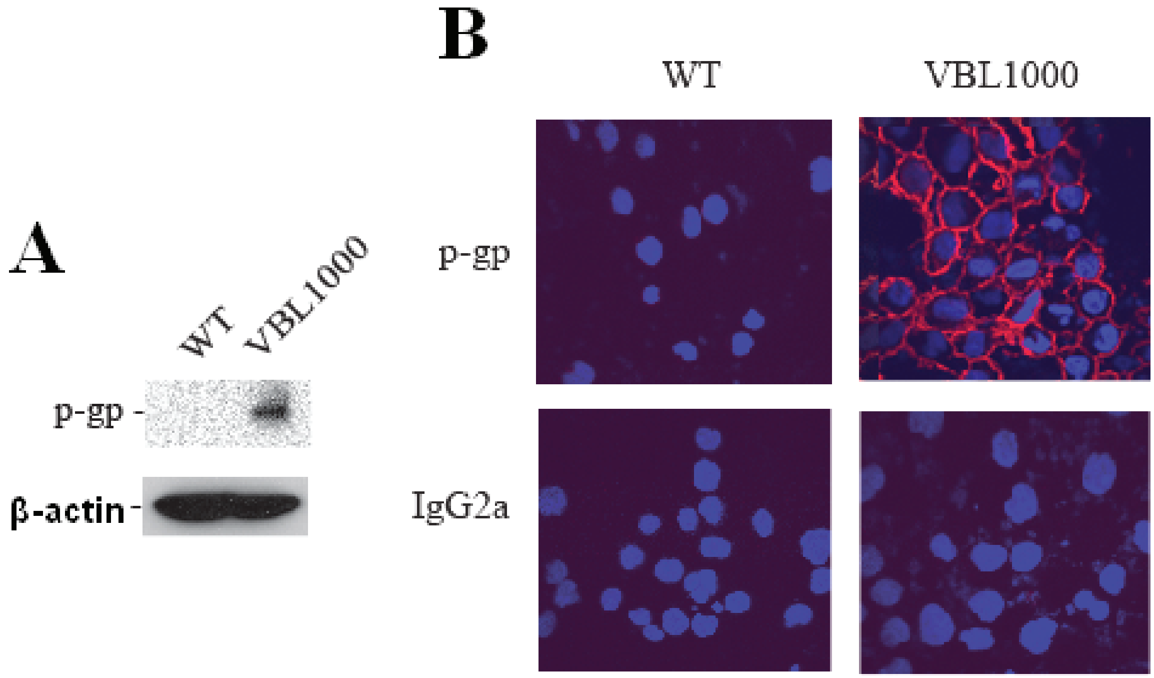

Figure 1. Detection P-gp in CEM/WT and CEM/VBL1000 cells. (A) Western blot analysis. P-gp was detected using 1 μg/ml anti-P-gp monoclonal C219 (1:1,000), and the blot was developed as described in the Materials and Methods. (B) Confocal microscopic analysis. Cells were incubated with P-gp monoclonal antibody UIC2 (1:500), and subsequently labeled with Texas Red-conjugated secondary antibodies (1:1,000).

ers: forward 5′-CAGAGCAAGAGAGGCATCCT-3′; and reverse 5′-TTGAAGGTCTCAAACATGAT-3′ [23]. The reactions were performed at 94°C for denaturation, 58°C for annealing, and 72°C for extension for 30 cycles. *β*-Actin mRNA levels were used as internal controls. Amplified fragments were separated on 1.5% agarose gels and visualized by ethidium bromide staining.

Results

We first examined P-gp expression in CEM/WT and CEM/VBL1000 cells. Western blot analysis with the C219 antibody showed that CEM/VBL1000 expressed a high level of P-gp, while parental CEM/WT cells did not. To investigate the surface expression of P-gp, both CEM/WT and CEM/VBL1000 cells were fixed and incubated with UIC2 antibody, which recognizes an extracellular epitope of P-gp. Confocal microscopic analysis determined that CEM/VBL1000 overexpressed P-gp on the cell surface, while no P-gp was detected on the surface of CEM/WT cells. These results confirm that while parental CEM/WT is P-gp deficient, CEM/VBL1000 cells overexpress P-gp (**Figure 1**).

TRAIL induces apoptosis and inhibits proliferation of CEM/VBL1000 cells

Both cell lines were then tested for their sensitivity to chemotherapeutic agents and TRAIL. Vinblastine, vincristine, and paclitaxel are known P-gp substrates. P-gp-expressing CEM/VBL1000 cells were almost completely resistant to these chemotherapeutic agents, while CEM/WT were very sensitive (**Figure 2**). In contrast, while the CEM/WT cells were resistant to TRAIL, CEM/VBL1000 exhibited a marked sensitivity to TRAIL-induced apoptosis in a dose-dependent manner.

To determine whether TRAIL induced cell growth inhibition, CEM/WT and CEM/VBL1000 cells were treated with TRAIL and the MTT assay was performed. As shown in **Figure 2**, the growth of CEM/VBL1000 cells treated with 0.1-5 ng/ml of TRAIL was inhibited in a dose-dependent manner. However, TRAIL did not inhibit the growth of CEM/WT cells (**Figure 2**).

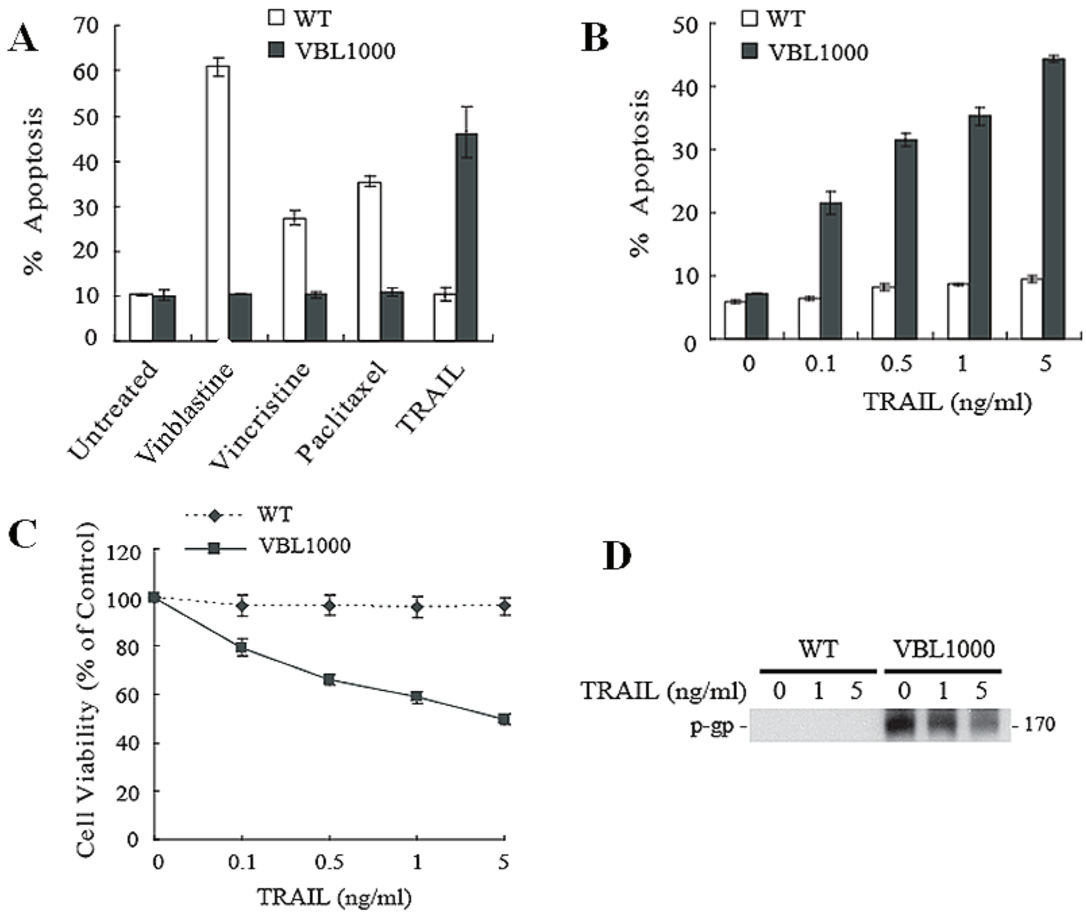

Figure 2. Analysis of TRAIL sensitivity in CEM/WT and CEM/VBL1000 cells. (A-B) Analysis of chemotherapeutic agents and TRAIL sensitivity. Cells were exposed to the indicated concentrations of vinblastine (5 ng/ml), vincristine (5 ng/ml), paclitaxel (0.1 μM), and TRAIL (5 ng/ml, or 0.1-5 ng/ml) for 24 h. After these treatments, the percentage of apoptotic cells was determined by annexin V staining followed by flow cytometry. (C) Effect of TRAIL on the viability of CEM/WT and CEM/VBL1000 cells. Cells were treated with 0.1-5 ng/ml TRAIL for 24 h, and viability was assessed by MTT assay. Viability results are given as a percentage of control cells. The results represent the mean ± SD of three independent experiments. (D) Western blot analysis of P-gp showing that TRAIL downregulates P-gp expression.

Western blot analysis of P-gp after treating CEM/VBL1000 cells with 1 and 5 ng/ml TRAIL for 24 h showed that TRAIL significantly downregulates P-gp expression (Figure 2).

TRAIL-induced apoptosis is mediated via DR5

We next investigated whether TRAIL-induced apoptosis in P-gp expressing CEM/VBL1000 cells is mediated by TRAIL receptors. First, the binding of TRAIL to its receptors was examined by flow cytometry after treating CEM/WT and CEM/VBL1000 cells with 0.1-5 ng/ml TRAIL for 24 h. As shown in Figure 3, TRAIL binding was increased in a dose-dependent manner in CEM/VBL1000 but not CEM/WT cells. Next, we examined the expression of the TRAIL receptors on the surface of CEM/VBL1000 and CEM/WT cells. As shown in Figure 4, CEM/WT cells expressed very little DR4 and DR5 compared to CEM/VBL1000 cells. TRAIL interacts with five receptors: death receptor 4 (DR4) and DR5 mediate apoptosis activation, while decoy receptor 1 (DcR1), DcR2, and osteoprotegerin counteract this function [21, 22, 24]. Only two of the TRAIL receptors, DR4 and DR5, contain functional death domains and are capable of inducing apoptosis [16-18]. Therefore we investigated the expression of DR4 and DR5 by using confocal microscopic analysis and flow cytometry with

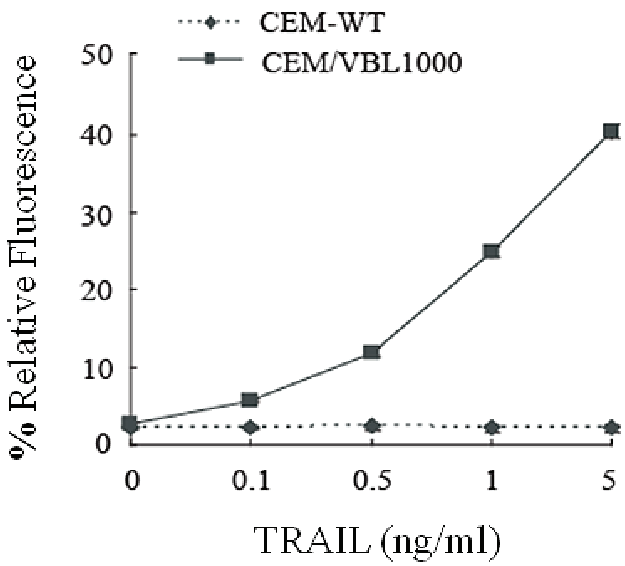

Figure 3. Flow cytometric analysis of TRAIL binding in TRAIL-treated CEM/WT and CEM/VBL1000 cells. Cells were treated with TRAIL for 24 h. Cells were then incubated with anti-TRAIL antibody (1:100 v/v) for 1 h and subsequently labeled with 5 µg/ml FITC conjugated secondary antibody for 1 h. Dotted line: CEM/WT. Solid line: CEM/VBL1000.

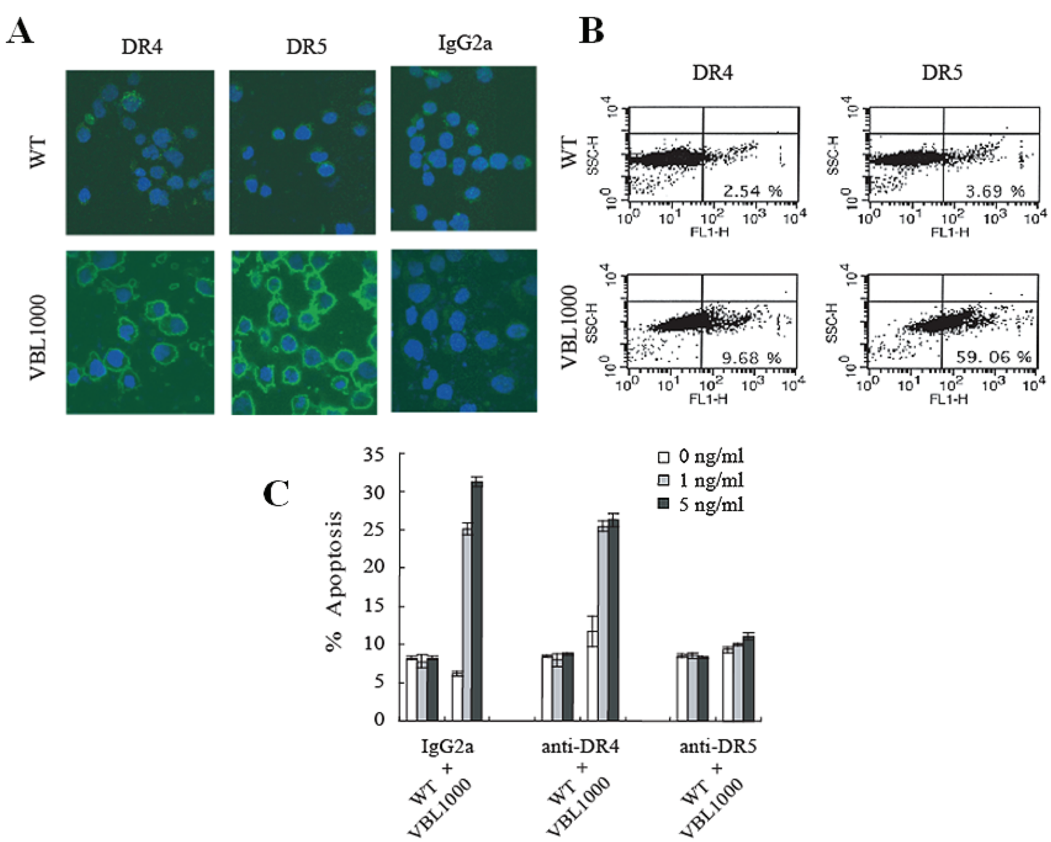

Figure 4. Evaluation of surface expression of TRAIL receptors and the effect of neutalizing anti-TRAIL receptors antibodies on TRAIL-induced apoptosis. (A) Confocal microscopic analysis and (B) flow cytometry. Cells were incubated with anti-TRAIL-R1 (DR4) or anti-TRAIL-R2 (DR5) antibody (1:500), and subsequently labeled with FITC-conjugated secondary antibodies (1:1,000) (C) Effect of neutralizing antibodies to DR4 and DR5 on TRAIL-induced apoptosis. Cells were pretreated with the anti-DR4 or anti-DR5 antibodies 3 h before treatment with 1 and 5 ng/ml TRAIL for 24 h. Mouse IgG_{2a} was used as the control isotype antibody. Apoptosis was detected by annexin V binding assay, as described in the Materials and Methods.

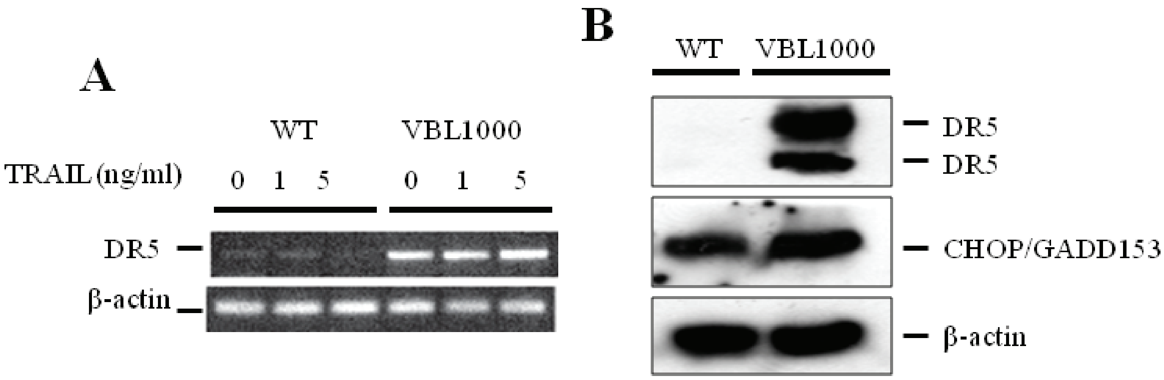

Figure 5. Expression of DR5 at mRNA and protein levels. (A) The mRNA levels of *DR5* and β-actin in the CEM/WT cell line and its MDR variant CEM/VLB10000 were determined by RT–PCR as described in the Materials and Methods. (B) Western blot analysis of DR5 using 1 μg/ml anti-DR5 polyclonal antibody (1:1,000) or mouse anti-CADD153 monoclonal antibody (1:1,000) as described in the Materials and Methods.

anti-DR4 and -DR5 antibodies. Figure 5 reveals that DR4 and DR5 were constitutively expressed on CEM/VBL1000 cells. In contrast, CEM/WT cells were deficient in DR4 and DR5 expression. Moreover, flow cytometry analysis revealed that CEM/VBL1000 expressed DR5 more than DR4, and the expression of both receptors on the surface of CEM/VBL1000 was upregulated following TRAIL treatment (Figure 4).

To investigate the relative importance of DR4 and DR5 for apoptosis induction by TRAIL, we examined the effect of neutralizing antibodies against DR4 and DR5, each at a concentration of 10 μg/ml. The neutralizing anti-DR4 antibody had no inhibitory effect on TRAIL-induced apoptosis in CEM/VBL1000 cells, while neutralizing anti-DR5 antibody showed a significant reduction in apoptosis. These results reveal that DR4 is not involved in TRAIL-induced apoptosis, but TRAIL triggers apoptosis via a DR5 signaling pathway in CEM/VBL1000 cells (Figure 4).

DR5 mRNA and protein expression in CEM/WT and CEM/VBL1000 cells

To determine whether DR5 expression is regulated at the protein and/or mRNA levels, we performed RT-PCR and Western blot analysis. The data in Figure 5 clearly show that the expression of DR5 mRNA is strongly upregulated in CEM/VBL1000 cells compared to CEM/WT cells. Western blot analysis also showed that DR5 is robustly increased at the protein level. Therefore, the results in Figure 5 show that upregulation of DR5 in CEM/VBL1000 cells occurs at both the mRNA and protein levels. Interestingly, in parallel with overexpression of the DR5 receptor, FADD is also upregulated in CEM/VBL1000 cells (data not shown).

TRAIL-induced apoptosis is mediated through mitochondria

To further determine the mechanism of the TRAIL-induced apoptosis, we investigated caspase activation, cytochrome *c* release from mitochondria, and PARP degradation, by treating CEM/WT and CEM/VBL1000 cells with 1 and 5 ng/ml TRAIL for 24 h. The regulation and execution of apoptotic cell death is carried out by a family of cysteine proteases with aspartic acid specificity known as caspases. Figure 6 shows that TRAIL induced the proteolytic cleavage of inactive procaspases-8 and -10, which are involved in death receptor-induced apoptosis, into active caspases-8 and -10 only in CEM/VBL1000 cells. As shown in Figure 6, exposure to TRAIL also resulted in processing of procaspases-9 and -3, as well as the release of cytochrome *c*, only in CEM/VBL1000 cells. Moreover, one of the substrates for caspases during apoptosis is poly(ADP-ribose) polymerase (PARP), an enzyme that appears to be involved in DNA repair and genome surveillance and integrity in response to environmental stress. Therefore, the cleavage of PARP was used as an indicator of caspase activation, and it was seen only in CEM/VBL1000 cells (Figure 6). These results reveal that TRAIL-induced apoptosis in P-

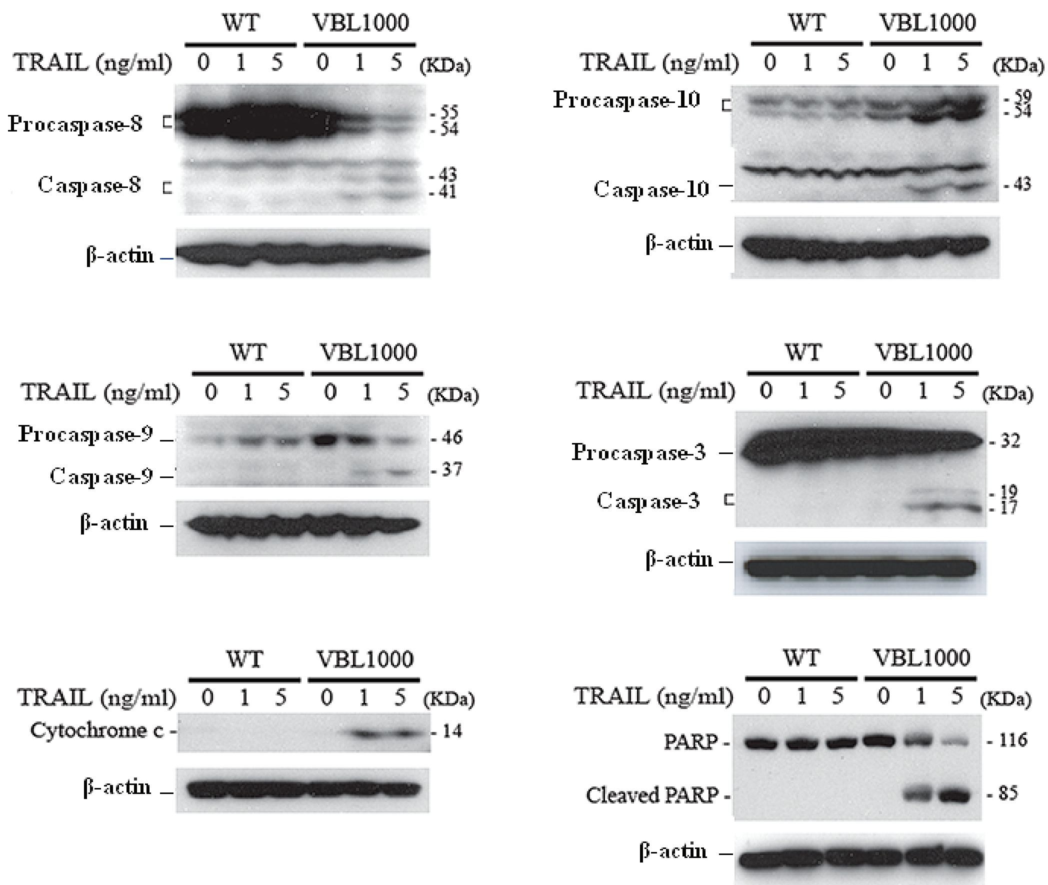

Figure 6. Effect of TRAIL on activation of caspases, cytosolic accumulation of cytochrome *c*, and cleavage of PARP. Cells were exposed to 1 and 5 ng/ml TRAIL for 24 h. Subsequently, cells were lysed and proteins used for Western blot analysis of the indicated proteins using specific antibodies to caspases-8, -9, -3, and PARP. To detect cytochrome *c* released from the mitochondria, the cytosolic fractions were prepared and used as described in the Materials and Methods.

gp-expressing CEM/VBL1000 cells is mediated via the mitochondrial apoptosis cascade.

Discussion

In this study, we found for the first time that TRAIL triggers selective apoptosis in the P-gp- and DR5-overexpressing CEM/VBL1000 MDR variant compared to its parental drug-sensitive CEM/WT leukemia cell line. Our results clearly show that the development of MDR in these cells is associated with upregulation of the TRAIL receptors DR4 and DR5, and that overexpression of DR5 is stronger than DR4. Moreover, pre-treating these cells with a neutralizing antibody against the DR5 receptor strongly reduced the degree of apoptosis induced by TRAIL. However, DR4 neutralizing antibody did not significantly affect TRAIL-triggered apoptosis in these cells, revealing that TRAIL induced apoptosis occurs preferentially via DR5. We [22, 23] and others [25, 26] have shown that in malignant cell lines from various tumor types, TRAIL triggers more efficient apoptosis through DR5 than DR4 in the apoptosis signaling cascade. It appears that the role of P-gp in TRAIL-triggered apoptosis in various MDR cells is complex. In *MDR*1 transfected MCF-7 breast cancer cells, we previously showed that the interaction of P-gp with DR5 in plasma membranes enhanced TRAIL binding to DR5, robustly stimulating P-gp ATPase activity and inducing mitochondrial depolarization [22].

We have previously shown that P-gp interacts with and forms a complex with DR5 in plasma membranes [22]. Our results in this report show that TRAIL treatment decreases the expression of P-gp. Therefore, it is tempting to speculate that in the absence of TRAIL, P-gp interacts with DR5 to prevent DR5 from initiating DISC formation. The TRAIL-induced decrease in P-gp expression makes DR5 available to recruit FADD and procaspases-8 and -10, allows subsequent activation of these caspases, DISC formation, and induction of apoptosis. It is well documented that DR5 is upregulated at the transcription level via the transcription factor CHOP/GADD153 [28, 29]. However, our results showed that DR5 upregulation is independent of increased CHOP expression in CEM/VBL1000 cells. Interestingly, many chemotherapeutic drugs that upregulate the expression of P-gp also increase the expression of DR5 [30]. It would be interesting to investigate whether specific transcription factors upregulate both P-gp and DR5.

In addition to upregulating DR5 and FADD, we found that the death receptor initiator caspases -8 and -10 are activated during the selective TRAIL-induced apoptosis in CEM/VBL1000 MDR cells, but not in CEM/WT cells, indicating involvement of the death receptor pathway in TRAIL-induced apoptosis in the MDR variant cell line. TRAIL-induced apoptosis also resulted in the release of cytochrome *c* and activation of caspases-9 and -3 only in CEM/VBL1000 cells. These results reveal that TRAIL-induced apoptosis in CEM/VBL1000 cells is mediated via both the DR5 and mitochondrial apoptosis cascades.

On the basis of the pattern of caspase cascade activation, two types of cells have been characterized. First, in type I cells, the caspase cascade is triggered upon oligomerization of cell surface death receptors and undergoes a sequential activation of caspase-8 to the principal mediator of apoptosis, caspase-3 [31-33]. Second, an alternative apoptotic pathway is seen in type II cells and involves mitochondrial damage and caspase-9 activation [31-33]. Upon receiving apoptotic stimuli, cytochrome *c* is release from the mitochondrial inner membrane and leads to the formation of the apoptosome, which in turn cleaves and activates caspase-9. Active caspase-9 then causes activation of caspase-3, which in turn cleaves many cellular substrates resulting in the biochemical and morphological features characteristic of apoptosis [6, 34, 35]. Our results show that CEM/VBL1000 cells have adopted a combination of apoptosis mechanisms operative in both type I and II cells.

Our study for the first time shows that TRAIL-induced apoptosis in P-gp-overexpressing CEM/VBL1000 cells is mediated via DR5 and the mitochondrial apoptosis cascade. Furthermore, our data on the selective sensitivity of the CEM/VBL1000 variant and other MDR cells [22] to TRAIL and the effect of this cytokine on reducing P-gp expression in these MDR cells may have significant clinical implications for using TRAIL to eliminate MDR cancer cells. Significantly, the recently published first Phase I clinical trial revealed that recombinant TRAIL administration is safe and well tolerated, and that dose escalation achieved peak TRAIL serum concentrations equivalent to those associated with preclinical antitumor efficacy [36].

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Please address correspondence to: Ahmad R. Safa, Department of Pharmacology and Toxicology and Indiana University Simon Cancer Center, 980 W. Walnut Street, R3-C524, Indianapolis, IN 46202; Tel: (317) 278-4952; Fax: (317) 274-8046; E-mail: asafa@iupui.edu

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