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Adriamycin Sensitizes Adriamycin-Resistant HL-60/ADR Cells to TRAIL-Mediated Apoptosis

Hong Ke[1]
Yiyu Wang[2]
Dongming Ren[2]
Lei Wang[2]

[1] Key Lab of Immunology, Medical College, China Three Gorges University, Yichang 443002, Hubei Province, China.
[2] Department of Oncology, Gezhouba Central Hospital, Third Clinical College of China Three Gorges University, Yichang 443002, Hubei Province, China.

Correspondence to: Lei Wang
Tel: 86-717-6397 438
E-mail: wl196565@163.com

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CJCO http://www.cjco.cn
E-mail: 2008cocr@gmail.com
Tel (Fax): 86-22-2352 2919

OBJECTIVE To study whether an adriamycin-resistant cell line (HL-60/ADR) can be sensitized by adriamycin (ADR) to TRAIL-mediated apoptosis.
METHODS The mRNA levels of the TRAIL receptor and apoptosis-related signaling molecules involved in the TRAIL-mediated apoptotic pathway were measured by RT-PCR. The protein levels of apoptotic-related signaling molecules involved in the TRAIL-mediated apoptotic pathway and processed caspase-3, caspase-9, and caspase-8 were measured by Western blots. Apoptosis was assessed by flow cytometry. Mitochondrial membrane potential was analyzed by DiOC6(3) staining. Cytotoxicity was determined by the colorimetric MTT viability/proliferation assay.
RESULTS Treatment with a combination of TRAIL and subtoxic concentrations of ADR resulted in synergistic cytotoxicity and apoptosis for both the parental HL-60 and the HL-60/ADR cells. For HL-60, there was a 5-fold potentiation and synergy in cytotoxicity for TRAIL and for HL-60/ADR, cytotoxicity to TRAIL was potentiated 6-fold with ADR. Adriamycin treatment modestly up-regulated TRAIL-R2 (DR5), but had no effect on the expression of Fas-associated death domain, c-FLIP, Bcl-2, Bcl-xL, Bax, and IAP family members (cIAP-1, cIAP-2, XIAP, and survivin). The protein levels of pro-caspase-8 and pro-caspase-3 were not affected by ADR, whereas pro-caspase-9 and Apaf-1 were up-regulated. Combined treatment with TRAIL and ADR resulted in activation of caspase-9 and caspase-3, but there was no detectable processing of caspase-8 beyond the background levels. There was significant depolarization of the mitochondrial membrane by the combined treatment of both cell lines and it was more pronounced in the parental HL-60 cell line. The combined treatment with TRAIL and ADR resulted in 42.6% of the HL-60/ADR cells undergoing DNA fragmentation, whereas treatment with either ADR or TRAIL alone resulted in 5.46% and 21.3% DNA fragmented cells, respectively. Similar results were obtained with the HL-60 cells.
CONCLUSION These findings demonstrate that ADR can still signal ADR-resistant tumor cells, resulting in the modification of the TRAIL-mediated signaling pathway and apoptosis.

KEY WORDS: adriamycin, TRAIL, apoptosis, HL-60 cells, multidrug resistance.

Introduction

Leukemia, which is a major cause of cancer deaths has an incidence that continues to rise. The anthracycline antibiotic adriamycin (ADR) has been widely used in the treatment of leukemia for more than 30 years. However, its utilization is hampered by dose-limiting cardiotoxicity and its propensity to develop resistance to a wide range of

functionally unrelated anticancer agents[1].

The development of drug resistance in leukemia patients has led to the exploration of alternative therapeutic strategies such as antitumor cytokines/antibodies, tumor-directed gene therapy, high-dose combination chemotherapy, total body irradiation in combination with bone marrow transplantation, and utilization of chemosensitizers. Despite these enormous efforts, currently these strategies have not been successful in treating leukemia. Systemic antitumor immunity is crucial for long-term protection against tumor relapse and metastasis[2]. Thus, immune-based therapeutics are particularly attractive for the development of effective treatments against leukemia.

TRAIL is a new member of the tumor necrosis factor (TNF) superfamily that has been shown to kill tumor cells selectively, and it is one of the antitumor cytotoxic factors used by activated lymphocytes[3]. In vitro studies have demonstrated that TRAIL exerts cytotoxic effects against various tumor cells but not against the majority of normal cells. The in vivo antitumor activities of TRAIL in mice and nonhuman primates showed no toxic side effects[4,5]. As opposed to Fas (CD95/Apo-1) and TNF, which cause severe toxicity upon in vivo administration, TRAIL can be well tolerated and is a potential candidate for immune-based antitumor therapeutics[6]. However, it has been demonstrated that applications of TRAIL alone or in combination with chemotherapeutic drugs induces apoptosis in normal human hepatocytes[7], but this controversy was resolved by Koschny et al.[8]

It has been demonstrated that human leukemia cell lines are sensitive to TRAIL-mediated apoptosis[9]. However, there is no evidence concerning the sensitivity of drug-resistant leukemia cells to TRAIL. Also, drug-resistant tumor cells often develop cross-resistance to many apoptotic-inducing stimuli, including those mediated by cytotoxic cytokines/lymphocytes[10]. Previous findings revealed that immune resistance can be reversed by sensitizing agents such as chemotherapeutic drugs. However, it is not clear whether a drug can be used as a sensitizing agent in tumors that are resistant to the same drug. The present study uses two representative human leukemia cell lines, HL-60, an ADR-sensitive leukemia cell line and HL-60/ADR, an ADR-resistant cell line derived from HL-60. The objectives of this study were to determine: *i*) whether the leukemia cell lines are sensitive to TRAIL-mediated apoptosis; *ii*) whether the HL-60/ADR cells can be sensitized by ADR to TRAIL-mediated apoptosis, although they are resistant to ADR; and *iii*) to explore possible mechanisms of ADR-mediated sensitization.

Materials and Methods

Drugs and reagents

ADR and soluble recombinant human TRAIL were purchased from the Sigma Chemical Co, USA. Stock solutions of ADR were prepared in PBS. Fetal calf serum and RPMI-1640 were purchased from Gibco, USA.

Cell culture

The human leukemia multidrug resistant cell line HL-60/ADR was maintained in RPMI-1640 medium supplemented with 10% fetal calf serum, glutamine 2 mmol/L, benzylpenicillin 100 kU/L, streptomycin 100 mg/L, and ADR 0.10 μmol/L at 37ºC in a 5% CO_2 atmosphere. Prior to use in experiments, HL-60/ADR cells were cultured in drug-free medium for 2 weeks.

Reverse transcription reaction

Total cellular RNA was isolated from cells using the TRIzol reagent (Sigma) in accordance with the manufacturer's instructions. The reverse transcription reaction was performed using Promega products. The cDNA was stored at -20ºC until required for analysis.

PCR

PCR amplification of 2.5 μl of the cDNAs was performed using the following gene-specific primers: 506 bp of DR4, primer sequences: 5'-CTG AGC AAC GCA GAC TCG CTG TCC AC-3', 5'-TCC AAG GAC ACG GCA GAG CCT GTG CCA T-3'; 502 bp of DR5, primer sequences: 5'-GCC TCA TGG ACA ATG AGA TAA AGG TGG CT-3', 5'-CCA AAT CTC AAA GTA CGC ACA AAC GG-3'; 612 bp of DcR1, primer sequences: 5'-GAA GAA TTT GGT GCC AAT GCC ACT G-3' , 5'-CTC TTG GAC TTG GCT GGG AGA TGT G-3'; 453 bp of DcR2, primer sequences: 5'-CTT TTC CGG CGG CGT TCA TGT CCT TC-3', 5'-GTT TCT TCC AGG CTG CTT CCC TTT GTA G-3'. Internal control for equal cDNA loading in each reaction was assessed using the following gene-specific GAPDH primers: GAPDH forward, 5'-GAA CAT CAT CCC TGC CTC TAC TG-3' and GAPDH reverse, 5'-GTT GCT GTA GCC AAA TTC GTT G-3' (355-bp expected product). PCR amplifications were carried out using the following temperature cycling parameters: for DR4, DcR2, and GAPDH, 95ºC; 1 min, 60ºC; 1 min; 35 cycles; and for DR5 and DcR1, 94ºC; 1 min, 65ºC; 1 min, 40 cycles. The amplified products were resolved by 2% agarose gel electrophoresis and the bands were visualized by ethidium bromide staining.

Cytotoxicity assay

Cytotoxicity was determined by the colorimetric MTT viability/proliferation assay. Cells were pretreated with 0.50 μmol/L of ADR in 50 μl/well for 18 h. Various concentrations (0.04, 0.1, and 0.20 μmol/L) of soluble TRAIL, 50 μl /well, were then added to the tumor cells, and the plates were incubated at 37ºC and 5% CO_2 for another 6 h for maximal killing. Subsequently, 10 μl of MTT reagent (5 g/L) was added to each well. The reaction was stopped 4 h after incubation and then the absorbance values at 570 nm was measured by a microELISA

reader (Sigma). Positive controls were left untreated and substituted with 50 µl of medium. For negative control, 100 µl of medium alone without cells were used. The percentage cytotoxicity was calculated using the background-corrected reading as follows: Cytotoxicity = [1-(absorbance of experimental well/absorbance of positive control)] × 100%

Flow cytometry analysis of apoptosis

Cells were treated with ADR (0.50 µmol/L, 18 h), TRAIL (0.20 µmol/L,6 h), and a combination of TRAIL and ADR (18 h pretreatment with drug and 6 h with TRAIL). Then, cells were harvested by centrifugation and washed twice with PBS, and resuspended in a final volume of 300 µl PBS. One ml of ethanol was added to the resuspended cells with vigorous mixing. Fixed cells were stained for 5 min with propidium iodide, incubated in the dark for 30 min, and then the fluorescent intensity was analyzed by flow cytometry (FACScan, Beton Dickinson, USA). Data were analyzed with CellQuestTM software (Beton Dickinson, USA).

Western blot analysis

The leukemia cells were incubated for 18 h in the presence or the absence of ADR (0.50 µmol/L). The cells were then broken in lysis buffer at 4ºC with sonication. The lysates were centrifuged at 15,000 × *g* for 15 min and the concentration of the protein in each lysate was determined with Coomassie brilliant blue G-250. Loading buffer was then added to each lysate, which was subsequently boiled for 3 min and then resolved by 12% SDS/PAGE and transferred to a nitrocellulose membrane. The membrane was blocked for 30 min in blocking buffer, and then incubated with the respective antibody for 1 h at room temperature. After washing with Tris-buffered saline containing 0.2% Tween 20 twice, the membranes were incubated for 60 min with alkaline phosphatase-conjugated antimouse or antirabbit IgG antibody and developed with tetrazolium salts. As a control, tubulin protein was blotted concurrently. All antibodies were from Santa Cruz Biotechnology.

Analysis of mitochondrial membrane potential by DiOC6(3) staining

The leukemia cell lines were either left untreated or treated with 0.50 µmol/L ADR (18 h), 0.20 µmol/L TRAIL (6 h), or ADR+TRAIL (18 h pretreatment with 0.50 µmol/L ADR, followed by 6 h treatment with 0.20 µmol/L TRAIL). After incubation, the leukemia cells were then stained with DiOC6(3) to quantify the mitochondrial membrane potential[11], by adding to each sample 50 µl of 40 µM DiOC6(3) (Molecular Probes, Inc.), a mitochondria-specific dye used to detect membrane depolarization. Then the cells were allowed to incubate for 30 min at 37ºC, followed by washing twice in PBS/0.1% BSA. After washing, 500 µl of PBS/0.1% BSA was added to all samples which were then analyzed by flow cytometry.

Statistical analysis

All data were expressed as the mean ± SD and statistically analyzed by the *t*-test. $P < 0.05$ was considered significant.

Results

TRAIL receptor expression in human leukemia cell lines

We first examined whether the HL-60 and HL-60/ADR leukemia cell lines expressed TRAIL receptors that are needed for signaling of TRAIL-mediated cytotoxicity. Oligonucleotide primers derived from unique regions of each of the two death-signaling receptors (DR4 and DR5), and two decoy receptor (DcR1 and DcR2) sequences were designed and used in RT-PCR analyses. The TRAIL-sensitive cell line, CEM, was used as a positive control for TRAIL receptor expression. The amplified products were resolved by 2% agarose gel electrophoresis and were of the expected product size. Both HL-60 and HL-60/ADR cell lines express the mRNAs for all four receptors (Fig.1).

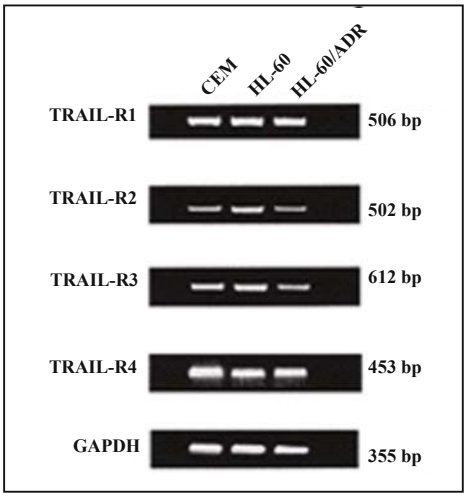

Fig.1. RT-PCR analysis of TRAIL receptor expression in human leukemia cell lines.

Sensitivity of human leukemia cell lines to soluble TRAIL and ADR

We assessed the cytotoxic effect of soluble TRAIL and ADR on the human leukemia cell lines. As shown in Fig.2A, both HL-60 and HL-60/ADR cell lines exhibited sensitivity to TRAIL-mediated cytotoxicity in a concentration-dependent manner as measured by the MTT assay. The parental cell line, HL-60, showed higher sensitivity to TRAIL-induced cytotoxicity when compared with the ADR-resistant variant HL-60/ADR. The maximal cytotoxicity was observed at 4 µmol/L of TRAIL with 95.6% for HL-60 and at 83.3% for HL-60/ADR cells. No further cytotoxicity beyond these levels

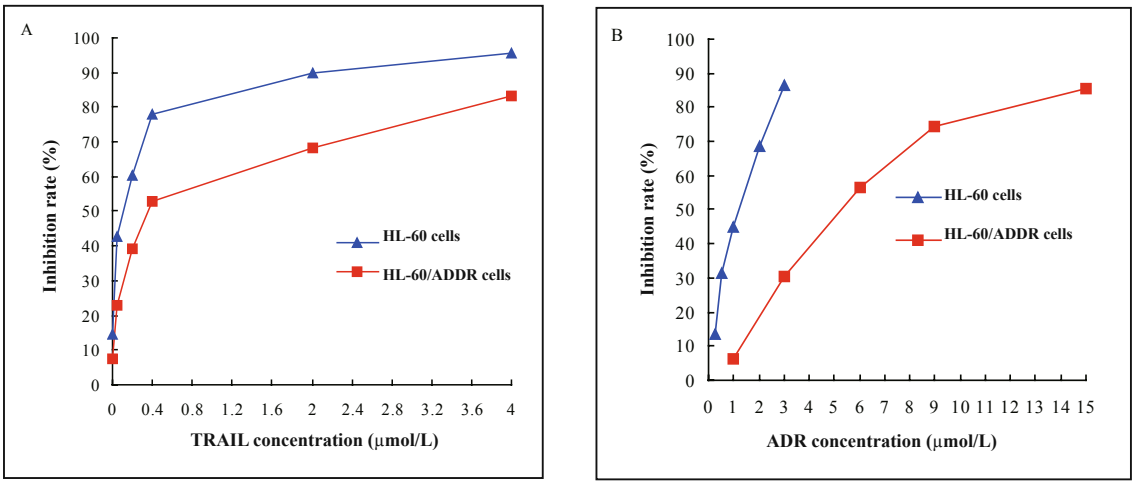

Fig.2. Sensitivity of leukemia cell lines to TRAIL (A) and ADR (B) mediated cytotoxicity (n = 4, mean ± SD).

was observed by increasing the concentration of TRAIL (data not shown). The sensitivity of leukemia cell lines to various concentrations of ADR (0.25~15 μmol/L; Fig.2B) was also determined. The parental HL-60 cell line was sensitive to ADR and exhibited 86.6% cytotoxicity at concentrations of 3 μmol/L ADR. The HL-60/ADR cell line was resistant to ADR as expected. In HL-60/ADR cells, ADR killed only 30.6% of the cells at concentrations of 3 μmol/L ADR. These findings demonstrate that the HL-60/ADR cell line is more resistant to TRAIL and ADR than the HL-60 cell line.

A combination of TRAIL with ADR induces synergistic cytotoxicity in leukemia cells

We examined whether the sensitivity of the leukemia cells to low noncytotoxic concentrations of TRAIL could be enhanced by ADR. HL-60 and HL-60/ADR cell lines were either left untreated or pretreated with subtoxic (0.50 μmol/L) concentrations of ADR for 18 h. Thereafter, the cells were incubated with low concentrations (0.004~4 μmol/L) of TRAIL for another 6 h for maximal killing, and the percentage cytotoxicity was determined by the MTT assay. Kinetics of the effect indicated that plateau cytotoxicity was achieved with TRAIL at 6 h (data not shown). Significant synergy was achieved in both cell lines by the combined treatment with subtoxic concentrations of TRAIL and with subtoxic concentrations of ADR. For HL-60, there was a 5-fold potentiation and synergy in cytotoxicity for TRAIL (Table 1), and for HL-60/ADR, cytotoxicity to TRAIL was potentiated 6-fold with ADR (Table 2). These findings demonstrate that subtoxic concentrations of antitumor drugs can override the resistance to low concentrations of TRAIL, and that ADR can sensitize both the ADR-sensitive (HL-60) and ADR-resistant (HL-60/ADR) leukemia cells to TRAIL. These findings also demonstrate that ADR-mediated sensitization can take place in an ADR-resistant line.

Table 1. Synergistic cytotoxicity of TRAIL in combination with ADR on the HL-60 cell line.

Group	IC_{50}/μmol/L	Reversal fold
TRAIL	1.50 ± 0.17	-
ADR + TRAIL	0.300 ± 0.012*	5.00

n = 4; mean ± SD; *, P < 0.01 *vs.* TRAIL group.

Table 2. Synergistic cytotoxicity of TRAIL in combination with ADR on the HL-60/ADR cell line.

Group	IC_{50}/μmol/L	Reversal fold
TRAIL	1.50 ± 0.17	-
ADR + TRAIL	0.251 ± 0.012*	5.98

n = 4; mean ± SD; *, P < 0.01 *vs.* TRAIL group.

Table 3. Decrease in the mitochondrial transmembrane potential (⊿ψm) of human leukemia cell lines upon treatment with a combination of ADR and TRAIL.

	Control	Mean fluorescence intensity		
		ADR	TRAIL	ADR+TRAIL
HL-60	-	21.3 ± 3.1	31.7 ± 3.1	67.5 ± 7.3*
HL-60/ADR	-	14.6 ± 0.5	25.1 ± 5.7	51.1 ± 1.7*

n = 3; mean ± SD; *, P < 0.01 *vs.* TRAIL group.

Synergy with combined TRAIL and ADR measured via apoptosis

Morphological changes of the leukemia cells after TRAIL and ADR treatment alone or in combination were evaluated by the acridine orange staining procedure. Combined treatment of the HL-60 or HL-60/ADR cells with TRAIL (0.20 μmol/L) and ADR (0.50 μmol/L) resulted in extensive membrane blebbing and bright orange areas of condensed chromatin, hallmarks of apoptosis (data not shown). Apoptosis was also evaluated by measuring the DNA content of the cells by the DNA fragmentation assay using the PI staining procedure. The combination treatment of TRAIL and ADR

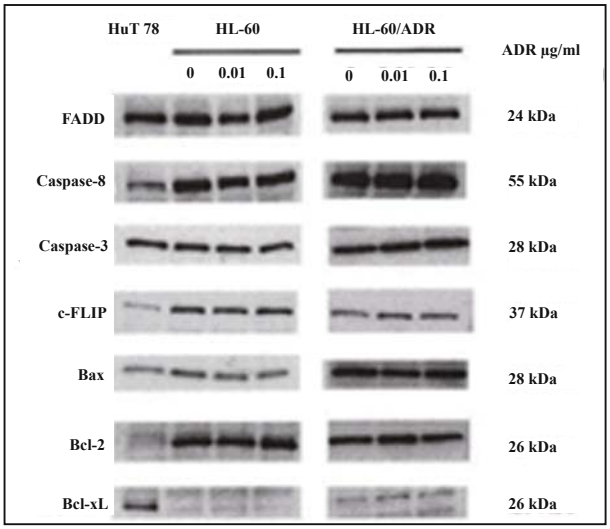

Fig.3. Effects of ADR on the expression of gene products by Western blot analyses. The human T-cell leukemia cell line, HuT 78, was used as a positive control.

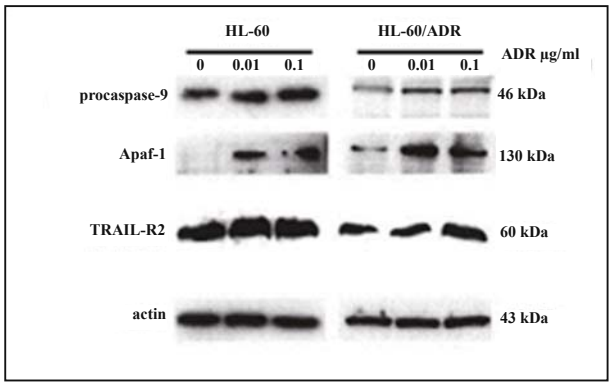

Fig.4. Up-regulation of pro-caspase-9, Apaf-1, and TRAIL-R2 upon ADR treatment of HL-60 and HL-60/ADR cells as detected by Western blot analyses.

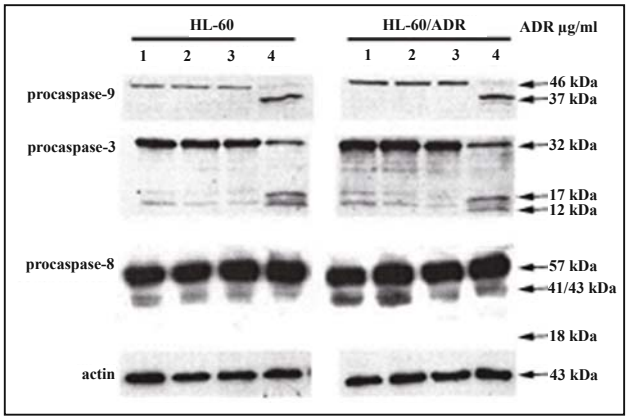

Fig.5. Western blot analysis of processed caspase-3, caspase-9, and caspase-8. The HL-60 and HL-60/ADR cells were either left untreated (Lane 1) or treated with ADR (0.50 μmol/L for 18 h; Lane 2), TRAIL (0.20 μmol/L for 6 h; Lane 3), TRAIL/ADR (18 h pretreatment with ADR, followed by 6 h treatment with TRAIL; Lane 4).

resulted in 42.6% of the HL-60/ADR cells undergoing DNA fragmentation, whereas treatment with ADR or TRAIL alone resulted in 5.46% and 21.3% DNA fragmented cells, respectively. Similar results were obtained with the HL-60 cells. Altogether, these findings demonstrate that the combined treatment with low concentrations of TRAIL and ADR results in synergistic cytotoxicity by apoptosis of both the ADR-sensitive and ADR-resistant leukemia cells.

Mechanism of TRAIL-mediated apoptosis by ADR

We investigated the potential molecular mechanism of sensitization of leukemia cells to TRAIL by ADR, by examining possible alterations in the expression levels of proapoptotic and antiapoptotic signaling molecules involved in the TRAIL-mediated apoptotic pathway. We chose to examine the levels of FADD, c-FLIP, procaspase-8, and procaspase-3, which are involved in the "Type I" death receptor-mediated apoptosis pathway. We also examined Bcl-2 family members (Bax, Bcl-2, and Bcl-xL), which are apoptotic regulators for the mitochondrial-death pathway. IAP family proteins, inhibitors of caspase-3, caspase-7, and caspase-9, were also examined. Leukemia cells were either left untreated or treated with 0.50 μmol/L of ADR for 18 h.

There were no detectable modulations of FADD, c-FLIP, procaspase 8, procaspase 3, or the Bcl-2 family members, Bax, Bcl-2, and Bcl-xL at the transcriptional regulation (mRNA) level (data not shown). These findings were confirmed by Western blots (Fig.3). Also, there was no detectable change in the expression level of the IAP family members (cIAP-1, cIAP-2, and XIAP) at either the transcriptional or protein levels as detected by RT-PCR and Western blot analysis (data not shown). However, there was a slight up-regulation of TRAIL-R2 (DR5) by ADR treatment as detected by Western blot analysis (Fig.4). Also, the protein levels of pro-caspase 9 and Apaf-1, the constituents of the apoptosome, were up-regulated in both the HL-60 and HL-60/ADR cells upon treatment with 0.50 μmol/L ADR for 18 h (Fig.4).

There was significant depolarization of the mitochondrial membrane by the combined treatment in both cell lines, but was more pronounced in the parental HL-60 cell line (Table 3). These results suggest that ADR pretreatment activates the mitochondrial (type II) apoptotic pathway (Apaf-1 and pro-caspase-9, decrease in $\Delta\psi m$) and facilitates TRAIL-mediated signaling for apoptosis. The above findings demonstrate that ADR significantly up-regulates some components of the apoptosome. Therefore, we determined whether the caspase cascade is also activated by the combined treatment of leukemia cells with TRAIL and ADR. The HL-60 and HL-60/ADR cells were either left untreated or treated with ADR (0.50 μmol/L for 18 h), TRAIL (0.20 μmol/L for 6 h), or TRAIL plus ADR (18 h), followed by 6 h of treatment with TRAIL, and total protein lysates were

subjected to Western blot analysis (Fig.5). The results showed that combined treatment of leukemia cells with TRAIL plus ADR results in the processing and activation of caspase-9 as well as the downstream effector, caspase-3. There was no detectable processing of caspase-8 beyond the background levels. These findings support the concept that apoptosis resulting from the combined treatment is mediated via the type II pathway involving mitochondria.

Discussion

Leukemia is best characterized by an early development of resistance to chemotherapy. The development of drug-resistant variants with the MDR phenotype remains as a major obstacle in the successful treatment of the disease, and has led to the search for alternative therapeutic strategies, including gene/immunotherapy. In this study, we explored the use of recombinant human TRAIL against the ADR-resistant HL-60/ADR leukemia cells. Our results showed that the HL-60/ADR cells were more resistant to TRAIL compared to parental HL-60 cells. We provided evidence for the first time that ADR sensitizes, through intracellular signaling, ADR-resistant leukemia cells to TRAIL-mediated apoptosis. Thus, the sensitization mechanism is likely to be distinct from the direct cytotoxic mechanism exerted by ADR. Furthermore, ADR-mediated sensitization is independent of the acquired MDR phenotype of HL-60/ADR cells. Our findings also suggest that ADR-mediated sensitization might be a result of selective up-regulation of apoptotic genes such as pro-caspase-9 and Apaf-1 and depolarization of the membrane potential of the mitochondria.

We first characterized the TRAIL receptor expression and the sensitivity of the HL-60 and HL-60/ADR cells to TRAIL-mediated apoptosis. HL-60/ADR cells express DR4, DR5, DcR1, and DcR2 as detected by RT-PCR analysis (Fig.1), although they are more resistant to TRAIL than the HL-60 parental cells. These results suggest that there is not a strict correlation between the expression of mRNA encoding for DcR1 and DcR2 and sensitivity to TRAIL. Our findings corroborate findings by others[12]. The results also suggest that the lower sensitivity of the HL-60/ADR cells to TRAIL is not at the receptor level, and is likely attributable to other intracellular regulatory mechanisms involved in the TRAIL-signaling pathway, such as the high expression of the antiapoptotic proteins Bcl-2 or Bcl-xL[13].

The drug-resistant HL-60/ADR cells showed lower sensitivity to TRAIL as compared with the ADR-sensitive cells. This suggests that the selective pressure applied by prolonged ADR treatment may co-select for tumor cells that have lost the capacity to undergo apoptosis in response to other unrelated apoptotic stimuli. Indeed, a previous report documented that when the HL-60 cells were selected for resistance to ADR, these cells also became resistant to Fas-mediated apoptosis[14]. Therefore, as leukemia cells develop resistance to drugs, they may also develop cross-resistance to TRAIL. The development of cross-resistance suggests that drugs and death receptors may use a common apoptotic pathway, and such a cross-resistance phenotype cannot be explained by the MDR mechanism alone.

Because the sensitivity of HL-60/ADR cells to TRAIL required high concentrations of TRAIL, we were able to enhance the TRAIL-mediated killing by low concentration of TRAIL by pretreatment with subtoxic concentrations of ADR followed by lower concentrations of TRAIL. Our results corroborate previous studies that demonstrated that drugs can synergize with TRAIL in killing resistant tumor cell lines in other tumor systems[15]. However, different from those studies, we showed that although the HL-60/ADR cells were resistant to ADR, they were still sensitized by subtoxic concentrations of ADR to TRAIL-mediated killing. Thus, MDR resistance to drugs does not prevent the sensitization process by the same drugs.

The sensitizing mechanism may involve changes in activities of proapoptotic and/or antiapoptotic molecules, or direct interaction with DNA that may alter the expression of apoptotic signaling molecules. In an attempt to study underlying molecular mechanisms of ADR-mediated sensitization, the expression levels of a number of molecules involved in the TRAIL-mediated apoptosis signal-transduction pathways were evaluated upon ADR pretreatment.

The regulation and execution of apoptotic cell death is carried out by a family of cysteine proteases with aspartic acid specificity known as caspases. Caspases are present in living cells as inactive zymogens; their activation is through autocatalytic processing by caspase cascades which are divided into initiators (e.g., caspase-8, caspase-9, and caspase-10) and effectors/executioners (e.g., caspase-3, caspase-6, and caspase-7). On the basis of the pattern of caspase cascade activation, two types of apoptosis have been characterized thus far[16]. In type I apoptosis, the caspase cascade is triggered upon the oligomerization of cell surface death receptors and undergoes a sequential activation of the initiator caspase, caspase-8, to the principal mediator of apoptosis, caspase-3. An alternative apoptotic pathway is seen in type II apoptosis, and involves mitochondrial damage and caspase-9 activation. Upon apoptotic stimuli, cytochrome c is released from the mitochondrial inner membrane and binds to the adaptor molecule Apaf-1, which recruits pro-caspase-9 and forms the apoptosome complex (cytochrome c/Apaf-1/caspase-9) that results in the activation of caspase-9. Active caspase-9 then causes the activation of caspase-3 and caspase-6. The activation of the effector caspase, caspase-3, is the merging point of the two caspase cascade pathways. Activated (pro-

cessed) caspase-3 cleaves death substrates and leads to apoptotic cell death.

No consistent changes, in the expression level of pro-caspase-8 and pro-caspase-3, FADD, or Bax were identified at the transcriptional regulation (mRNA) level that could explain the increased sensitivity of leukemia cells to TRAIL-mediated apoptosis caused by ADR (data not shown). However, ADR pretreatment modestly up-regulated DR5 in both cell lines at the protein level (Fig.4). This corroborates findings by others. Others have suggested that Bcl-2 and Bcl-xL expression in HL-60 and HL-60/ADR might contribute to their resistance to some apoptotic stimuli, including the Fas ligand. HL-60/ADR cells express higher levels of Bcl-xL mRNA compared with HL-60 cells, which could explain their lower sensitivity to apoptotic-inducing stimuli. However, ADR did not modulate Bcl-2 and Bcl-xL levels in both cell lines. Interestingly, the expression of the inhibitory c-FLIP, pro-caspase-3, and pro-caspase-8 and the adaptor FADD were unaltered upon 18 h of ADR pretreatment at the protein level as determined by Western blot analysis (Fig.3), and did not correlate with the enhancement of sensitivity to TRAIL-mediated apoptosis. The expression level of the IAP family members (cIAP-1, cIAP-2, survivin, and XIAP) were unaltered at the transcriptional or translational levels upon ADR treatment in both cell lines (data not shown). However, the results in Fig 4 show clearly that ADR pretreatment significantly up-regulated the expression of pro-caspase-9 and Apaf-1, components of the apoptosome, at the protein level. Also, combined treatment of leukemia cells with ADR and TRAIL resulted in significant depolarization of the membrane potential of mitochondria ($\downarrow \Delta\psi m$; Table 3). The findings demonstrate that ADR sensitizes the HL-60/ADR cells to TRAIL-mediated apoptosis via the type II mitochondrial pathway.

In summary, the present findings demonstrate that chemotherapeutic drugs such as ADR can significantly enhance the sensitivity of leukemia cells to TRAIL-induced apoptosis by mechanisms that modulate intracellular signaling pathways to potentiate apoptosis. Furthermore, our studies demonstrate that a cytotoxic drug can exert a sensitizing effect through intracellular signaling and gene modulation, although the tumor is resistant to the same drug and expresses the MDR phenotype. Additionally, the concentrations of ADR that enhanced the sensitivity of cells to TRAIL in vitro are within a subtoxic range that maybe clinically useful in patients. Therefore, ADR and similar drugs are potentially of value for clinical therapy in combination with TRAIL in the treatment of drug-resistant leukemia cells.

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