Multiple mechanisms underlie resistance of leukemia cells to Apo2 Ligand/TRAIL

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Abstract
Targeting death receptors with tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) has the remarkable potential to selectively kill malignant cells whereas normal cells are largely unaffected by this treatment. However, some tumor cells, including leukemia cells, exhibit resistance to this molecule. To investigate the basis for resistance of leukemia cells to the zinc-bound form of Apo2 ligand (Apo2L/TRAIL), which is currently being evaluated in clinical trial, we isolated several resistant HL60 clones from parental HL60 cells by selection using the recombinant Apo2L/TRAIL. Differing resistance mechanisms were identified and characterized in these Apo2L/TRAIL-resistant clones. In one case, the level of the cell-surface death receptor DR4, but not DR5, was significantly decreased. However, these cells did undergo apoptosis in response to another form of recombinant TRAIL, histidine-tagged TRAIL, suggesting differing contributions of DR4 and DR5 in the response to these two forms of TRAIL. In the case of other clones, expression of procaspase-8 protein was lost and this was associated with a novel Leu22→Phe22 point mutation in CASP-8 gene. These results show that cells within a given tumor can have widely distinct mechanisms underlying resistance to Apo2L/TRAIL. [Mol Cancer Ther 2006;5(7):1844–53]

Introduction
Chemotherapy has provided significant survival benefit in the treatment of leukemia; however, it is associated with significant normal tissue toxicity, highlighting the need for therapeutic strategies that target tumor cells without compromising normal tissue function. Such a strategy was suggested by the identification of tumor-selective targeting molecules such as tumor necrosis factor–related apoptosis-inducing ligand (TRAIL; refs. 1, 2). Whereas early reports indicated that the histidine-tagged or FLAG-tagged version of TRAIL induced apoptosis in some normal human hepatocytes and astrocytes, respectively (3, 4), the non-tagged, zinc-bound recombinant Apo2 ligand (Apo2L)/TRAIL did not exert a similar toxic effect on these cells (5). In addition, no significant toxic effects of zinc-bound Apo2L/TRAIL have been observed in experimental animals (6, 7) or in primate toxicity studies (8), and this molecule was therefore selected for development for clinical applications (9). Importantly, the selectivity of recombinant TRAIL for tumor cells is consistent with the natural biological role of this molecule. It is expressed on the surface of activated immune cells such as natural killer cells, CD4+ T cells, macrophages, and dendritic cells, where it apparently functions as an immune effector molecule, mediating antitumor cytotoxicity and immune surveillance (10).

TRAIL-induced ligation and trimerization of the cell-surface death receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5), both of which contain a cytoplasmic region of 80 amino acids designated as the “death domain” (11), activates the extrinsic apoptosis pathway. DR4 and DR5 can recruit the initiator caspases caspase-8 and caspase-10 by a homotypic interaction between the death effector domains of the adaptor molecule Fas-associated death domain protein and the prodomain of the initiator caspase, thereby forming the death-inducing signaling complex (12). Within this death-inducing signaling complex, procaspase-8 (or procaspase-10) drives its autoactivation through oligomerization and subsequently activates the downstream effector caspases, such as caspase-3, caspase-6, and caspase-7, in type I cells (13, 14). In type II cells, the level of initiator caspase activation following death receptor ligation is insufficient to induce apoptosis and the intrinsic mitochondrial pathway is recruited to amplify the apoptotic signal through cleavage of the BH3 domain–containing proapoptotic molecule BH3 homologous domain BID by caspase-8 (15, 16).

In spite of the promising antitumor potential of recombinant TRAIL ligands, many tumor cell lines and some fresh tumor cells are unresponsive to TRAIL treatment (17, 18). Several different mechanisms have been identified by researchers that could block TRAIL-mediated apoptosis, such as (a) defects or mutations at the level of the TRAIL receptors (19, 20), including loss of DR4 expression through homozygous deletion or lack of expression of death receptor on the cell surface (21, 22), and competitive binding of TRAIL by the decoy receptors TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2) or the soluble receptor OPG (23, 24); (b) compromised function of the death-inducing signaling complex as a result of overexpression of cellular...
FLICE-inhibitory protein (25) or loss of caspase-8 expression by gene methylation (26); (c) lack of activation of the mitochondrial pathway by virtue of overexpression of anti-apoptotic (e.g., Bcl-2 and Bcl-XL; refs. 27, 28) or mutation of pro-apoptotic (e.g., Bax) Bcl-2 family members (29); and (d) caspase inhibition by up-regulation of the inhibitor of apoptosis protein family members (30, 31).

Primary leukemia cells and established leukemia cell lines studied to date have shown variable susceptibility to TRAIL (32–36), although the basis for susceptibility or resistance is not well understood. To characterize fully the possible resistance mechanisms of leukemia cells to Apo2L/TRAIL treatment, we examined the molecular basis for TRAIL resistance in HL60 human leukemia cell clones isolated by exposure to Apo2L/TRAIL in this project. The Apo2L/TRAIL-mediated apoptotic signaling pathway(s) in sensitive versus resistant HL60 cells was dissected by analyzing and comparing the sequential activation steps. Here, we report that distinct mechanisms are associated with resistance of HL60 cells to Apo2L/TRAIL, including decreased cell-surface expression of DR4 and loss of procaspase-8 protein, possibly due to a novel gene mutation identified in this study.

Materials and Methods

Cells and Reagents

The human acute myelogenous leukemia cell line HL60 was obtained from American Type Culture Collection (Rockville, MD). Apo2L/TRAIL-resistant HL60 variants were selected by exposure of HL60 cells to escalating doses of Apo2L/TRAIL (10 ng/mL, 20 ng/mL, 50 ng/mL, 100 ng/mL, 500 ng/mL, 1 μg/mL, 5 μg/mL, and 10 μg/mL) for 2 to 3 days. After each exposure, surviving cells were recovered and cultured in fresh medium for 3 days and then treated with the subsequent dose. Individual resistant clones were subcloned by limiting dilution and frozen down immediately after being developed. For experiments, the resistant clones were kept in continuous culture for only 3 to 4 weeks (<10 passages). All the cells were cultured in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum as well as 2 mmol/L L-glutamine (Life Technologies).

As noted above, we used the zinc-bound formulation of Apo2L/TRAIL provided by Genentech, Inc. (San Francisco, CA) for nearly all of the studies described here. In this article, we refer to this zinc-bound TRAIL as Apo2L/TRAIL. In certain experiments (as noted in Results), we also used His-tagged TRAIL, with the extracellular domain of human TRAIL (amino acids 95-281) fused at the NH2-terminal peptide (BD PharMingen, San Diego, CA), DcR1, DcR2, and caspase-8, recognizing the FLICE-inhibitory protein (25) or loss of caspase-8 expression by gene methylation (26); (c) lack of activation of the mitochondrial pathway by virtue of overexpression of anti-apoptotic (e.g., Bcl-2 and Bcl-XL; refs. 27, 28) or mutation of pro-apoptotic (e.g., Bax) Bcl-2 family members (29); and (d) caspase inhibition by up-regulation of the inhibitor of apoptosis protein family members (30, 31).

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Detection of Apoptosis

Apoptosis was quantified by Annexin V and propidium iodide staining (Caltag, Burlingame, CA) following the instructions of the manufacturer. Briefly, 106 cells in binding buffer were incubated with 3.5 μL Annexin V-FITC and 5 μL propidium iodide solution (50 μg/mL) for 15 minutes at room temperature. The cells were analyzed on a FACSscan (Becton Dickinson, San Diego, CA). The percentage of apoptotic cells was calculated as the percentage of cells being positive for Annexin V.

Blocking with Death Receptor – Specific Antibodies

Cells (106) in 100 μL PBS with 5% fetal bovine serum were incubated with 2 μg DR4 blocking antibody (4H6, Genentech), 3 μg DR5 blocking antibody (HS201, Apotech), both antibodies, or 3 μg mouse immunoglobulin G antibody (R&D Systems) as an isotype control or left untreated as a blank control for 30 minutes on ice. Consequently, the cells were treated with either Zinc-bound Apo2L/TRAIL (100 ng/mL) or His-tagged TRAIL (20 ng/mL for HL60 cells and 100 ng/mL for R1 cells) or left untreated for 20 hours followed by Annexin V apoptosis analysis.

Western Blot Analysis and Antibodies

Cells (106) were lysed on ice for 30 minutes in lysis buffer [0.5% NP40, 20 mmol/L Tris (pH 7.5), 120 mmol/L NaCl, 100 mmol/L NaF, 200 μmol/L Na3VO4, 50 mmol/L β-glycerophosphate, 10 mmol/L NaFPi, 4 mmol/L phenylmethylsulfonyl fluoride, 2 mmol/L benzamidine, 10 μg/mL leupeptin, 10 μg/mL aprotinin]. Forty micrograms of protein were separated on SDS-PAGE gel and immunoblottings analyses were done with antibodies to caspase-8, caspase-9, caspase-10, and Bid (Cell Signaling Technology, Beverly, MA), DcR1, DcR2, and caspase-8, recognizing the NH2-terminal peptide (BD Pharmingen, San Diego, CA), Fas-associated death domain and tumor necrosis factor receptor–associated death domain (BD Transduction Laboratories, San Diego, CA), Fas (CH11, Upstate), DR4 and DR5 (Oncogene, Cambridge, MA), and β-actin (Stressgen) using the recommended antibody concentrations by the manufacturer.

Flow Cytometric Analysis of Cell-Surface Receptor Expression

Cells (1 × 106) were incubated with 10 μg/mL antibody against DR4 (HS101, Apotech), DR5 (HS201, Apotech), DcR1 (eBioscience, San Diego, CA), DcR2 (eBioscience), Fas (CH11), mouse immunoglobulin G1 (DAKO, Denmark), or mouse immunoglobulin M (SouthernBioTech, Birmingham, AL) for 30 minutes at 4°C followed by 10 μg/mL phycoerythrin-conjugated goat anti-mouse immunoglobulin (BD Pharmingen) for 30 minutes at 4°C. The cells were analyzed on a FACSscan.

Reverse Transcription-PCR Analysis of Caspase-8 mRNA Isoforms

Total RNA from cell lines was isolated using the RNA-Easy mini kit (Qiagen, Valencia, CA) according to the instructions of the manufacturer. cDNA was synthesized from 5 μg total RNA using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) following the instructions of the manufacturer. Caspase-8 isoform–specific PCR
was done using Platinum Taq polymerase (Invitrogen) with the caspase-8/a,b,c primers 5'-ATGGACCTTCAGCAGAAAATCTT-3' (forward) and 5'-ATCAGAAGGGAAGACAAAGTTT-3' (reverse) under the following cycling conditions: initial activation at 94°C for 5 minutes, 35 cycles of denaturing at 94°C for 45 seconds, annealing at 52°C for 1 minute, and extension at 72°C for 1 minute, then final extension at 72°C for 10 minutes. Primers used to amplify other caspase-8 mRNA isoforms were as follows: caspase-8/b,d, 5'-CAGCCTTTGAGAAATCTTGAAG-3' (forward) and 5'-GTCTGCTAGTTGAGATT-3' (reverse); caspase-8/e, 5'-CTGGCTACAGGGTCATGCTCTAT-3' (forward); and caspase-8/f, 5'-CTGGCTACAGGGTCATGCTCTAT-3' (forward) and 5'-ATGGACTTCAGCAGAAAATCTCATCA-3' (reverse). The PCR reaction mixture was activated for 5 minutes at 94°C and incubated for 30 cycles (denaturing for 45 seconds at 94°C, annealing for 1 minute at 55°C, and extension for 1 minute at 72°C). Final extension was continued for 10 minutes at 72°C. Transcripts of glyceraldehyde-3-phosphate dehydrogenase as an internal control were amplified with the forward primer, 5'-CCCTCTAAAATCGTGG-3', and reverse primer, 5'-CCATCCACGCTCTTCTGG-3'. The PCR reaction mixture was activated at 95°C for 3 minutes, followed by 23 cycles of amplification (denaturing for 30 seconds at 94°C, annealing and extension for 1 minute at 60°C). Final extension was continued for 10 minutes at 60°C. PCR products were resolved by electrophoresis on 2% agarose gels or 4% to 12% acrylamide gel and visualized with ethidium bromide under UV light.

**DR4, DR5, and CASP-8 Gene Sequencing Analysis**

Total RNA was isolated as described above and mRNA was enriched on Oligotex mRNA spin-columns (Qiagen). Reverse transcription-PCR (RT-PCR) of the DR4 and DR5 genes was carried out using a One-Step RT-PCR Kit (Qiagen) under the following conditions: reverse transcription for 30 minutes at 50°C; initial PCR activation for 15 minutes at 95°C; three-step cycling of denaturation for 1 minute at 94°C, annealing for 1 minute at 60°C, and extension for 1 minute at 72°C for a total of 30 cycles; and final extension for 10 minutes at 72°C. The primers were designed as follows: DR4, 5'-ATGGACCTTCAGCAGAAAATCTT-3' (forward) and 5'-TTGCCATCTCAGGACAC-3' (reverse); DR5, 5'-ATGGACCTTCAGCAGAAAATCTT-3' (forward) and 5'-TTGAGACATGCCAGATCT-3' (reverse). The CASP-8 gene was amplified using High-Fidelity One-Step RT-PCR Kit (Invitrogen) with the primer pair 5'-CAGCATGACCTTCAGCAGAAAATCTT-3' (forward) and 5'-ATCAGAAGGGAAGACAAAGTT-3' (reverse) under the following cycling conditions: reverse transcription for 30 minutes at 50°C; initial PCR activation for 2 minutes at 94°C; three-step cycling of denaturation for 15 seconds at 94°C, annealing for 30 seconds at 50°C, and extension for 1 minute at 68°C for a total of 40 cycles; and final extension for 5 minutes at 68°C. The amplified PCR products were subcloned into a TOPO TA cloning vector (Invitrogen) and sequenced with an Applied Biosystems 3100 automated sequencer (Foster City, CA).

**Results**

**Characterization of the Apoptotic Response of Apo2L/TRAIL-Resistant HL60 Derivatives**

Human HL60 leukemia cells were treated with escalating doses of Apo2L/TRAIL as described in Materials and Methods and several resistant clones were initially obtained. To investigate whether different clones shared one common mechanism of resistance or whether more than one mechanism occurred, five clones were selected at random for analysis. Further characterization of these five clones revealed that three clones (R2, R3, and R5) were completely resistant to Apo2L/TRAIL whereas two (R1 and R4) exhibited incomplete resistance (Fig. 1A). Therefore, a detailed investigation of the resistance mechanisms in two representative clones (R1 and R2) was carried out and the mechanisms identified in these two clones were then validated in the others.

The dose-response of clones R1 and R2 to Apo2L/TRAIL is shown in Fig. 1B. Parental HL60 cells were sensitive to Apo2L/TRAIL treatment (~55% apoptotic cells seen after 24 hours) whereas Apo2L/TRAIL-induced apoptosis was significantly lower in both R1 and R2 cells. It should be noted that the observed resistance of R1 cells to Apo2L/TRAIL-induced apoptosis was not stable, in that when these cells were cultured in the absence of Apo2L/TRAIL for 3 to 4 weeks, their resistance was partially reversed (Fig. 1B, R1*). In contrast, this was not the case with R2 cells, which maintained resistance.

**Kinetics of Activation of Initiator Caspases and Relevant Molecules in the TRAIL-Mediated Apoptosis Pathway**

We first investigated the possibility that defective activation of the initiator caspase-8/caspase-10/caspase-9 was involved in resistance of R1 and R2 cells by analyzing the time course of activation of these molecules and of proteolysis of Bid in response to Apo2L/TRAIL treatment using Western blot analysis (Fig. 1C-E). Activation (cleavage) of the initiator caspases and cleavage of Bid were detected within 3 hours of exposure to 1 μg/mL Apo2L/TRAIL in parental HL60 cells. In contrast, the activation of initiator caspases and Bid cleavage in R1 cells was substantially inhibited. In R2 cells, which are completely resistant to Apo2L/TRAIL, procaspase-8 protein was undetectable (using the caspase-8 antibody against the COOH-terminal p18 subunit) and no cleavage of procaspase-10 was found. These results suggest that defects in the Apo2L/TRAIL-mediated apoptotic signaling pathways occur upstream of the initiator caspases caspase-8/caspase-10 in R1 cells whereas resistance in R2 cells could be due to loss of expression of procaspase-8, a critical mediator of Apo2L/TRAIL-induced apoptosis.

**Analysis of Adaptor Proteins in the TRAIL-Mediated Apoptosis Pathway**

Because TRAIL and Fas death receptors share common adaptor proteins and activate the same downstream apoptotic signaling pathway(s), comparing the response to Apo2L/TRAIL and Fas agonistic antibody allowed us to distinguish whether the defects were present in the
receptors themselves or somewhere along their shared apoptotic signaling pathway. As shown in Fig. 2A, R1 cells were sensitive to Fas agonistic antibody (CH11), suggesting that resistance of R1 cells to Apo2L/TRAIL occurs at the level of the TRAIL receptors themselves. Consistent with the observation that R2 cells lack procaspase-8, R2 cells were resistant to both Fas agonistic antibody and Apo2L/TRAIL. As shown in Fig. 2B, the expression levels of relevant adaptor molecules, such as Fas-associated death domain and tumor necrosis factor–associated death domain, were comparable in these cells. Additionally, the expression level of cellular FLICE-inhibitory protein was equal in these cells (data not shown). Furthermore, we observed that the expression levels of total and cell-surface Fas, as measured by Western blot analysis (Fig. 2B) and flow cytometry (Fig. 2C), respectively, were comparable in parental HL60, R1, and R2 cells, ruling out the possibility that resistance to Fas antibody treatment arises as a result of diminished expression of Fas receptors.

Analysis of TRAIL Receptors

We next evaluated total protein expression of DR4, DR5, DcR1, and DcR2 in HL60, R1, and R2 cells by using Western blotting. Because only cell-surface DR4 and DR5 are able to bind Apo2L/TRAIL and mediate apoptosis, we also examined the cell-surface expression of TRAIL receptors by flow cytometry. As shown in Fig. 3A, total expression of DR4 and DR5, measured by Western blot analysis, was similar in all of the cells. When cell-surface levels of DR4 and DR5 were analyzed, DR5 was found to be similarly expressed in all of the cells but the cell-surface expression of DR4 was found to be significantly reduced on R1 cells (Fig. 3B). The cell-surface expression of DR4 on three other resistant clones (R3, R4, and R5) was similarly decreased (data not shown). Interestingly, this was reversible as cell-surface DR4 expression on R1 cells increased after culture in the absence of Apo2L/TRAIL for 3 to 4 weeks (Fig. 3B), correlating well with the observed reversal of Apo2L/TRAIL resistance in R1* cells (Fig. 1B). No differences in decoy receptor expression (neither in total amount nor cell-surface expression) were observed between the parental and the resistant clones (Fig. 3A and B), suggesting that the pattern of decoy receptor expression does not seem to play a role in the resistance mechanisms involved here. Therefore, the results led us to hypothesize that decreased cell-surface DR4 distribution could be responsible for the resistance to Apo2L/TRAIL treatment.

Figure 1. Apoptotic response of HL60 wild-type cells and Apo2L/TRAIL-resistant clones to Apo2L/TRAIL. A, five Apo2L/TRAIL-resistant HL60 clones (R1, R2, R3, R4, and R5) exhibited variable resistance to Apo2L/TRAIL treatment. Cells (10^6) were treated with 1 μg/mL Apo2L/TRAIL for 24 h and apoptosis was quantitated by flow cytometric analysis of Annexin V-propidium iodide staining. Columns, mean of results obtained from three independent experiments; bars, SD. B, dose–response curves for HL60, R1, and R2 cells treated with Apo2L/TRAIL. Cells (10^6) were treated with Apo2L/TRAIL (0.01, 0.1, 1, or 10 μg/mL; M, media control) for 24 h and apoptosis was quantitated by Annexin V assay. Points, mean of results obtained from three independent experiments; bars, SD. C to E, immunoblot analysis of caspases and relevant molecules in response to Apo2L/TRAIL treatment. Aliquots of 1 × 10^6 HL60 (C), R1 (D), and R2 (E) cells treated with 1 μg/mL Apo2L/TRAIL were harvested following exposure to Apo2L/TRAIL (0, 1, 3, 6, 12, and 24 h) and assayed by immunoblotting for caspase-8, caspase-10, caspase-9, and Bid cleavage with specific antibodies. Procaspase-8 protein is expressed at similar levels in HL60 and R1 although cleavage was inhibited in R1. Procaspase-8 protein was not detected in R2 cells. Activation and cleavage of procaspase-10 and procaspase-9 and cleavage of Bid were inhibited in both R1 and R2 compared with HL60.
Because the level of total DR4 expression in R1 cells was comparable to that in parental HL60 (Fig. 3A), this suggested that the reduced cell-surface expression of DR4 might be due to defective targeting of intracellular DR4 molecule to the cell surface and/or increased turnover at the cell surface. We first sequenced the DR4 gene and found no mutation (data not shown), ruling out the possibility that decreased targeting of DR4 to the cell surface was due to the defects in the DR4 gene per se.

Somewhat surprisingly, given the previous results of others (37), DR5 alone did not mediate substantial Apo2L/TRAIL-induced apoptosis although it was predominantly expressed on the R1 cell surface. Thus, we hypothesized that DR5 on R1 cells might be nonfunctional. To investigate this possibility, we sequenced the DR5 gene in R1 cells, but no mutation was found (data not shown). Additionally, R1 cells underwent apoptosis when treated with anti-DR5 agonistic antibody (Fig. 3C). Because this antibody selectively targets the DR5 molecule without involving DR4, it is apparent that the DR5 receptor alone can transduce apoptotic signaling. In addition, these data suggested that DR5 agonistic antibody could overcome Apo2L/TRAIL resistance conferred by decreased cell-surface DR4 expression. In contrast, R2 cells were not responsive to DR5 agonistic antibody treatment, likely due to the loss of procaspase-8 protein expression (Fig. 3C).

To further confirm our hypothesis that decreased cell-surface DR4 on R1 cells was responsible for resistance to Apo2L/TRAIL-induced apoptosis, we carried out cell-surface DR4 and/or DR5 blocking assays with specific neutralizing antibodies (Fig. 4). In an initial study, we did a titration experiment that showed that the blocking effects of DR4 and DR5 antibodies were dose dependent and picked the lowest doses that could exert the highest blocking effects (data not shown). In HL60 cells, we recapitulated the cell-surface TRAIL receptor expression pattern in R1 cells by blocking cell-surface DR4 with a specific neutralizing antibody and found that Apo2L/TRAIL-triggered apoptosis was almost completely abrogated. This decreased level of apoptosis was the same as that seen by incubation with DR4 antibody itself, which has weak agonistic ability (38). This result strongly supported our hypothesis that decreased cell-surface DR4 distribution was responsible for resistance to Apo2L/TRAIL-induced apoptosis in R1 cells.

To determine whether the DR4 dependency was specific for zinc-bound Apo2L/TRAIL, we did the blocking assay using another form of TRAIL ligand, His-tagged TRAIL. As with zinc-bound Apo2L/TRAIL, blocking DR4 thoroughly abrogated His-tagged TRAIL–triggered apoptosis (Fig. 4A). We next asked what role DR5 plays in mediating apoptosis by Apo2L/TRAIL and His-tagged TRAIL. We found that blocking DR5 only marginally inhibited the response to Apo2L/TRAIL but greatly inhibited the ability of His-tagged TRAIL to induce apoptosis (Fig. 4A). Interestingly, we also found that R1 cells, which were resistant to Apo2L/TRAIL, were moderately susceptible to apoptosis induction by His-tagged TRAIL (Fig. 4B). Based on these results, it seems that DR5 mediates a greater apoptosis signal in response to His-tagged TRAIL than to Apo2L/TRAIL.

Identification of a Possible Mechanism for Loss of Caspase-8 Expression in R2 Cells

To identify the mechanism(s) underlying the loss of procaspase-8 protein in R2 cells, we first examined procaspase-8 expression at the protein level using an immunoblotting assay (Fig. 5A). No band was detected in the R2 lysate with the antibody directed against the COOH-terminal peptide of caspase-8 whereas very faint bands corresponding in size to isoforms caspase-8/a and caspase-8/b were seen with the antibody recognizing the NH2-terminal peptide.
Alternative splicing of the caspase-8 RNA precursor results in five transcript variants, caspase-8/a (55 kDa), caspase-8/b (54 kDa), caspase-8/c (51 kDa), caspase-8/d (32 kDa), and caspase-8/e (26 kDa), with a common NH2-terminal death effector domain (39). To explore the possibilities that the greatly reduced procaspase-8 protein expression is due to gene methylation or a shift in expression pattern from functional isoforms a/b to non-functional isoform d by alternative splicing, we assessed caspase-8 expression at the mRNA level using caspase-8 isoform–specific RT-PCR analysis (Fig. 5B). The expression levels of all five caspase-8 isoforms were comparable, indicating that all cells have the same transcriptional regulation pattern. We also found that treatment of R2 cells with the DNA demethylating agent 5-aza-2'-deoxy-cytidine at 5 μmol/L daily for 5 days did not restore the expression of procaspase-8 protein (data not shown). Thus, the above possibilities were ruled out.

Based on the discrepancy in caspase-8 expression at the translational (protein) and posttranscriptional (mRNA) levels in R2 cells, we speculated that there could be a missense or nonsense point mutation(s) in CASP-8 gene, generating a mutated protein that was subjected to rapid turnover, resulting in the reduced amount of procaspase-8 protein detected by Western blot analysis. To address this possibility, we sequenced the coding region of CASP-8 gene and identified a single C-to-T point mutation at position +64 that caused a Leu<sup>22</sup>→Phe<sup>22</sup> amino acid substitution in the first death effector domain of CASP-8 gene in R2 cells (Fig. 5C). Interestingly, we found that the other two completely resistant clones (R3 and R5) had the identical point mutation in CASP-8 gene in association with strikingly reduced procaspase-8 protein expression, indicating that the completely resistant cells shared a common resistance mechanism (data not shown). To test the possibility that inhibition of proteasome-mediated degradation would increase the level of mutant protein, we treated the cells with the proteasome inhibitor PS34. However, we found that PS34 treatment did not restore procaspase-8 protein in R2 cells to the level found in HL60 cells (Fig. 5D).

Finally, to determine whether the mitochondrial apoptosis pathway was also affected in R2 cells, we evaluated their response to Adriamycin treatment and found an identical response to that seen in parental HL60 cells (data not shown), indicating that the intrinsic mitochondrial apoptotic pathway used by Adriamycin was intact and also providing a rationale for combining Apo2L/TRAIL with therapies that induce apoptosis through a caspase-8-independent pathway, such as chemotherapy, to ensure killing of a heterogeneous tumor population.

Figure 3. Analysis of TRAIL receptors in HL60, R1, and R2 cells. A, the total cellular expression levels of TRAIL death and decoy receptors are similar in HL60, R1, and R2 cells as assayed by Western blot. B, cell-surface expression levels of TRAIL death and decoy receptors. As assessed by flow cytometric analysis (solid line, TRAIL receptor; filled peaks, mouse immunoglobulin G1 isotype control), R1 cells have significantly lower cell-surface expression of DR4 than HL60 cells (mean fluorescence of DR4 in HL60 cells versus R1 cells: 14.20 ± 2.55 versus 5.78 ± 0.54; P < 0.01), and cell-surface DR4 expression on R1* cells increases as compared with that on R1 cells (mean fluorescence of DR4 in R1* cells versus R1 cells: 7.27 ± 0.71 versus 5.78 ± 0.54; P < 0.05). The surface expression of the other receptors is similar in all three cell lines. Representative of three independent experiments.

C, apoptosis induced by 1 μg/mL anti-DR5 agonistic antibody for 24 h was measured by Annexin V apoptosis assay. A similar degree of apoptosis was induced in HL60 and R1 cells whereas R2 cells were resistant to this anti-DR5 antibody. Columns, mean of three independent experiments; bars, SD.
Discussion
In this study, we have carried out a detailed examination of the causes of resistance to Apo2L/TRAIL in HL60 leukemia cells. We showed that two distinct defects occur in several resistant clones isolated from a population of HL60 cells by exposure to escalating doses of Apo2L/TRAIL. One defect consisted of loss of procaspase-8 protein expression likely due to a point mutation in CASP-8 gene whereas the other consisted of decreased cell-surface DR4 expression. These results suggest that more than one resistance mechanism could also arise in patients’ tumors, especially in the setting of Apo2L/TRAIL treatment, which would select for resistant cells, similar to the process studied here. Screening additional TRAIL-resistant clones, as well as validating these observations in patient specimens, is warranted because more resistance mechanisms may be identified. The information gathered from this work may contribute to the design of strategies to avert the development of resistance or to overcome resistance mechanisms if they are encountered in the clinic. Furthermore, the results of this study could help to understand the molecular basis of mechanisms of tumor evasion that may occur during development of natural resistance to TRAIL-mediated immune surveillance.

Caspase-8 is shown to be a critical mediator of apoptosis triggered by both TRAIL and Fas ligand (13, 40, 41). Caspase-10, another initiator caspase in the death receptor pathway, cannot functionally substitute for caspase-8 (14, 42). On the other hand, there are also reports that endogenous caspase-10 is recruited to the TRAIL death-inducing signaling complex and is capable of signaling apoptosis in the absence of caspase-8 (43). Our results indicate that caspase-8 plays a pivotal role in mediating Apo2L/TRAIL-induced apoptosis in HL60 leukemia cells. With regard to reasons for the loss of procaspase-8 protein, there are at least two mechanisms that have been elucidated thus far. It has been widely reported that gene methylation can cause silencing of CASP-8 gene in some cancers such as childhood neuroblastoma, Ewing sarcoma, malignant brain tumors, and small-cell lung cancer (44–47). However, this was not found in the case of R2 cells in our study. Second, it has also been found that point mutations in CASP-8 gene are associated with reduction or loss of procaspase-8 protein in several types of cancers (48, 49). Our research indicates that a novel Leu22 → Phe22 point mutation in CASP-8 gene could result in a substantially reduced protein level and consequent resistance to Apo2L/TRAIL-induced apoptosis. We postulate that this point mutation in CASP-8 gene could preexist in a few cells that therefore gain a survival advantage in the HL60 cell population exposed to Apo2L/TRAIL. However, it would be difficult to identify the point mutation as well as the corresponding loss of procaspase-8 protein before treatment due to the low numbers of cells with the point mutation in the CASP-8 gene. This defect becomes obvious only after the tumor cells undergo selection by Apo2L/TRAIL exposure and subsequent subcloning. Furthermore, the CASP-8 point mutation which we showed in an established cell line could be an important but subtle cause for the recurrence of disease in the clinic and deserves more attention.

One intriguing aspect of TRAIL signaling is the existence of multiple receptors and, as yet, there is not a comprehensive understanding of their differential roles. Previous studies have revealed a complex situation that is still under intensive investigation. Whereas DR4 and DR5 have been shown to have similar ability to recruit Fas-associated death domain and caspase-8 in multiple cell lines (13), our results show that cell-surface DR4, but not DR5, is indispensable for significant apoptotic signaling by Apo2L/TRAIL in these HL60 cells. In addition, DR4 is of similar importance in mediating responses to both Apo2L/TRAIL and His-tagged TRAIL.

Figure 4. Comparison of the contributions of cell-surface DR4 and DR5 to induction of apoptosis by either Apo2L/TRAIL or His-tagged TRAIL. HL60 cells (A) and R1 cells (B) were treated with specific neutralizing antibodies to DR4, DR5, or both and then exposed to either Apo2L/TRAIL or His-tagged TRAIL. *, significant blocking effects by the indicated neutralizing antibodies as compared with the mouse immunoglobulin G antibody control. Columns, mean of results obtained from three independent experiments; bars, SD. In HL60 cells, blocking DR4 almost abrogated the apoptotic response to both forms of TRAIL. However, blocking DR5 inhibited apoptosis in response to His-tagged TRAIL to a greater extent than in response to Apo2L/TRAIL. R1 cells were modestly responsive to His-tagged TRAIL and blocking either DR4 or DR5 reduced this response.
TRAIL and DR5 alone cannot substitute for DR4. However, the role of cell-surface DR5 in inducing apoptosis in response to Apo2L/TRAIL and His-tagged TRAIL differs in that neutralizing cell-surface DR5 significantly decreases the apoptotic response to His-tagged TRAIL but not to Apo2L/TRAIL. As with our results, Jin et al. (22) showed that DR4 plays a more important role than DR5 in His-tagged TRAIL–induced apoptosis in the colon cancer study. In contrast, a very recent report, using either DR4- or DR5-selective Flag-tagged TRAIL variants in the presence or absence of cross-linking anti-FLAG antibody, indicated that DR5 may contribute more than DR4 to TRAIL-induced apoptosis in cancer cells that express both death receptors (37). Collectively, these results of others and the data presented in this article suggest that the differential roles of DR4 and DR5 in TRAIL-induced apoptosis are affected by the type of TRAIL ligand used.

The above findings lead us to speculate that DR5 responds to a higher-order multimerization signal from TRAIL than DR4. In this hypothetical model, cross-linked Flag-tagged TRAIL can result in super-multimerization of death receptors and thereby effectuate an apoptosis signal via DR5. This hypothesis is supported by the report that DR4 signals apoptosis on triggering by cross-linked and non-cross-linked TRAIL whereas DR5 signals only in response to cross-linked TRAIL (50). Recently, it was suggested that the His-tagged preparation has a low solubility and tends to aggregate, and it is therefore likely to over-multimerize death receptors (18). However, zinc-bound Apo2L/TRAIL does not possess this aggregation ability and therefore may not be able to signal considerable apoptosis via cell-surface DR5 alone. This could be the basis for our observations that R1 cells expressing substantial cell-surface DR5 but minimal cell-surface DR4 were modestly susceptible to His-tagged TRAIL–induced apoptosis but resistant to Apo2L/TRAIL. It has been reported that Apo2L/TRAIL can induce at least some formation of heterotrimeric receptor complexes (13). Therefore, the issues with regard to how these multiple TRAIL receptors interact in a heterotrimer to regulate the apoptotic signal await further investigation.

Notably, in view of our results about the lack of direct correlation between the level of cell-surface and total cellular DR4, it is clear that the use of total receptor levels evaluated by Western blot analysis, RT-PCR, or Northern blot analysis can be misleading, and this should be taken into account when interpreting the relationships between the expression levels of TRAIL receptors and the sensitivity of tumor cells to Apo2L/TRAIL. This discrepancy implies that there may be a defect in trafficking of DR4 to the cell surface. Other studies have previously raised this possibility. For example, Jin et al. have suggested that defective transport of DR4 protein to the cell surface may involve alterations in the pathways of glycosylation or trafficking. Using TRAIL-resistant clones of SW 480 colon adenocarcinoma, these investigators found that the total protein level of DR4 was similar between the parental cells and the TRAIL-resistant clones but that cell-surface expression of DR4 was reduced in the TRAIL-resistant cells. They also found that treatment with the glycosylation inhibitor tunicamycin could reverse the phenotype of TRAIL resistance by increasing the cell-surface expression of death receptors.

Figure 5. Analysis of caspase-8 in HL60, R1, and R2 cells. A, evaluation of pro-caspase-8 protein expression with NH2 terminus– and COOH terminus–specific antibodies. Pro-caspase-8 protein expression was significantly decreased in R2 cells. B, each of these cell lines had similar expression levels of mRNA for each of the five caspase-8 isoforms as determined by RT-PCR (C, negative control). C, sequencing of the coding region of CASP-8 genes. R2 cells harbored a C-to-T point mutation at position +64 that caused a Leu22–Phe22 amino acid change in the first death effector domain (DED). D, expression of pro-caspase-8 protein was not restored by pretreatment with the proteasome inhibitor PS341. Cells were pretreated with 10 nmol/L PS341 for 24 h and pro-caspase-8 expression was then measured by Western blot analysis; 10 nmol/L PS341 was the highest possible concentration at which no significant apoptosis was induced (data not shown).
Receptors (22). Further, Ren et al. (51) found that down-regulation of signal recognition particle subunits resulted in a dramatic decrease in cell-surface DR4 receptors but not DR5. Their results also suggest that defects in the pathways of protein sorting are involved in the decreased cell-surface expression of DR4. It will be important to further investigate the expression of signal recognition particle and other relevant molecules that affect DR4 cell-surface sorting in Apo2L/TRAIL-selected leukemia clones as well as primary patient samples.

In summary, our research has revealed that leukemia cells can employ different resistance mechanisms to escape apoptosis mediated through TRAIL receptors. These data may help to predict the type of resistance mechanisms likely to be found in patient samples and contribute to the design of Apo2L/TRAIL-based therapeutic strategies.

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