Advances in Brief

Resistance to Tumor Necrosis Factor-related Apoptosis-inducing Ligand-induced Apoptosis in Neuroblastoma Cells Correlates with a Loss of Caspase-8 Expression[1]

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Abstract

Disruption of apoptotic pathways may be involved in tumor formation, regression, and treatment resistance of neuroblastoma (NB). Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a potent inducer of apoptosis in cancer cell lines, whereas normal cells are not sensitive to TRAIL-mediated apoptosis. In this study we analyzed the expression and function of TRAIL and its agonistic and antagonistic receptors as well as expression of cellular FLICE-like inhibitory protein and caspase-2, -3, -8, -9, and -10 in 18 NB cell lines. Semiquantitative RT-PCR revealed that TRAIL-R2 and TRAIL-R3 are the main TRAIL-receptors used by NB cells. Sensitivity to TRAIL-induced apoptosis did not correlate with mRNA expression of TRAIL receptors or cellular FLICE-like inhibitory protein. Surprisingly, caspase-8 and caspase-10 mRNA expression was detected in only 5 of 18 NB cell lines. Interestingly, only these five NB cell lines were susceptible to TRAIL-induced apoptosis in a time- and dose-dependent manner. Treatment with 5-aza-2′-deoxycytidine restored mRNA and protein expression of caspase-8 and TRAIL sensitivity of resistant cell lines, suggesting that gene methylation is involved in caspase inactivation. The TRAIL system seems to be functional in NB cells expressing caspase-8 and/or caspase-10. Because many cytotoxic drugs induce caspase-dependent apoptosis, failure to express caspase-8 and/or caspase-10 might be an important mechanism of resistance to chemotherapy in NB.

Introduction

Abnormal regulation of apoptosis has been implicated in tumor development and resistance to cancer therapy. Therefore, an understanding of the cellular mediators of apoptosis may lead to the development of new cancer therapies. Like TNF[3] and Fas ligand, TRAIL (Apo2L) has been shown to be a potent inducer of apoptosis in various cancer cell lines (1). In contrast to injection of TNF and Fas ligand, which are both lethal to mice (2), TRAIL exerts potent antitumor activity *in vivo* without exhibiting systemic toxicity (3, 4). Two agonistic receptors for TRAIL have been identified: TRAIL-R1 (DR4; Ref. 5) and TRAIL-R2 (DR5, KILLER; Ref. 6). Although TRAIL and TRAIL-R1 and -R2 are widely expressed in human tissues, normal cells are not sensitive to TRAIL-mediated killing (7).

Two antagonistic receptors are unable to transduce the death signal: TRAIL-R3 (DcR1, TRID; Refs. 5 and 6) is lacking the complete intracellular portion, and TRAIL-R4 (DcR2, TRUNDD; Ref. 8) contains a truncated, nonfunctional death domain. The preferential expression of these antagonistic receptors in normal tissues led to the hypothesis that both molecules protect normal tissues from TRAIL-induced apoptosis by competing with the agonistic receptors for limited amounts of ligand (5, 6, 8). Activation of death receptors leads to a signal transduction cascade initiated by death receptor-associated molecules such as FADD and caspase-8 (FLICE, MACH, respectively; Ref. 9). It has been suggested that FADD and caspase-8 also participate in TRAIL-induced cell death (10, 11), but other studies pointed instead to the involvement of caspase-10 (5). cFLIP is a recently identified protein homologue to caspase-8, that lacks catalytic activity and has been suggested to play a significant role in controlling the susceptibility of tumor cells to the apoptosis-inducing effects of TRAIL (12). NB is the most common extracranial malignant solid tumor of childhood and arises from the sympathetic nervous system. The more advanced stages of disease have a very poor prognosis, despite aggressive treatment regimens. Induction of cell death by direct activation of TRAIL receptors might be an attractive new treatment option in neuroblastoma. In this study, we analyzed expression and function of TRAIL, its receptors, and important intracellular signaling elements in 18 NB cell lines.

Materials and Methods

Tissue Culture. All NB cell lines were obtained from the Children's Hospital of Philadelphia cell line bank. Jurkat human T-cell lymphoma cells were purchased from American Type Culture Collection. Cells were grown at 5% CO_2 in RPMI 1640 supplemented with 10% fetal bovine serum, L-glutamine, penicillin, and streptomycin. Treatment of cells with 5-ADC at a concentration of 0.1, 1, 5, or 10 μM was performed for 1 week, with the medium and drug renewed twice weekly.

RNA Extraction and Semiquantitative RT-PCR. Total RNA was extracted from NB cell lines using the Qiagen RNeasy Kit (Valencia, CA). One μg of total RNA was reverse transcribed using the SuperScript Preamplification System (Life Technologies, Inc.). PCR was carried out according to a previously described protocol (13). PCR primers were designed to bracket cDNA sequences that cross an intron-exon boundary in genomic DNA. Primers for *TRAIL-R1, TRAIL-R2, TRAIL-R3,* and *TRAIL-R4* have been previously described, and their specificity has been confirmed (14). Primer sequences specific for *TRAIL*, human *c-FLIP*, *Caspase-2, -3, -8, -9,* and *-10,* and *GAPD* are available on request. All PCR primers were biotinylated at their 5′ ends. The housekeeping gene *GAPD* was coamplified as an internal standard control as previously described (13). Each PCR sample was analyzed on a nondenaturing 6% polyacrylamide gel. DNA was transferred to a nylon membrane (Hybond N+; Amersham) and immobilized by UV cross-linking. Detection of biotin-labeled DNA on X-ray films was performed according to the Southern-Light Protocol (Tropix, Bedford, MA; Ref. 13).

Assessment of Cell Viability and Apoptosis. NB cell lines were seeded into 96-well plates at a density of 5×10^3 cells per well and cultured in 10%

serum containing RPMI medium. After 24 h, cells were treated either with recombinant soluble human TRAIL (200 ng/ml; UBI, Lake Placid, NY) according to the manufacturer's instructions, with CHX (1 μg/ml; Sigma) alone, or simultaneously with TRAIL (200 ng/ml) + CHX (1 μg/ml). The caspase inhibitors zVAD-fmk (Enzyme Systems Products, Livermore, CA) and zIETD-fmk and zLEHD-fmk (Calbiochem) were dissolved in DMSO and used at a final concentration of 50 μM in culture medium where indicated. Each condition was performed in triplicate. Cell cultures were maintained for indicated treatment times. A colorimetric MTT assay was then performed as previously described (15). For study of dose dependency, cells were treated with increasing concentrations of TRAIL alone (0, 3, 6, 12.5, 25, 50, 100, and 200 ng/ml) or in the presence of CHX (1 μg/ml). Cell lines were considered sensitive to TRAIL-induced cell death if there was <70% viability after 24 h of TRAIL treatment at a concentration of 200 ng/ml. To assess specific apoptosis, the extent of DNA fragmentation in TRAIL-treated cells was detected by TUNEL with the Apoptosis Detection System (Promega, Madison, WI) and was also quantitated (optical density, 405 nm) using the Cell Death Detection ELISA (Roche Molecular Biochemicals, Indianapolis, IN), which detects cytoplasmic histone-associated DNA fragments. In addition, apoptotic cell death was confirmed by staining of TRAIL-treated cells with annexin V and propidium iodide (Roche Molecular Biochemicals) according to the manufacturer's instructions. Apoptosis was then monitored by flow cytometry analysis with a FACScan using CELLQuest software (Becton Dickinson, Mountain View, CA).

Western Blot Analysis. Cells were rapidly lysed in 800 μl/dish NP40 lysis buffer [1% NP40, 20 mM Tris (pH 8.0), 137 mM NaCl, 0.5 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 0.15 units/ml aprotinin, 20 μM leupeptin, 1 mM sodium vanadate]. Samples normalized for total protein content were separated by SDS-PAGE, electroblotted onto nitrocellulose, and immunostained. Anti-caspase-8 monoclonal antibody (UBI), anti-cFLIP (Alexis Biochemicals, San Diego, CA), and anti-TRAIL-R2 (Santa Cruz Biotechnology) were used according to the manufacturer's instructions. Detection of immunocomplexes was conducted using an ECL chemiluminescence system (Amersham Corp., Arlington Heights, IL).

Results

Expression of TRAIL and TRAIL Receptors in 18 Human NB Cell Lines. The mRNA expression of TRAIL and its agonistic (TRAIL-R1 and -R2) and antagonistic (TRAIL-R3 and -R4) receptors was assessed by semiquantitative RT-PCR analysis (Fig. 1A). TRAIL and TRAIL-R1 were not expressed in any of the NB cell lines. TRAIL-R4 was expressed at high levels in only one cell line and at lower levels in four additional cell lines. TRAIL-R2 and TRAIL-R3 were expressed at various levels in almost all of the cell lines, which suggests that they are the primary TRAIL receptors used by NB cells. Protein expression of TRAIL-R2 essentially correlated with mRNA expression levels (Fig. 1B).

TRAIL-induced Apoptosis in NB Cell Lines. The susceptibility of NB cells to TRAIL-induced apoptosis was tested by 24-h exposure of the cells to soluble TRAIL (200 ng/ml; Fig. 2). For comparison, Jurkat T cells were also exposed to TRAIL. Only 1 of 18 cell lines was susceptible to TRAIL-induced apoptosis (defined as a survival rate of <70% after 24 h of treatment with TRAIL). The same experiment was performed in the absence and presence of CHX, because inhibition of protein synthesis has previously been shown to sensitize cancer cell lines to CD95L/Fas-induced apoptosis (16). Coexposure to TRAIL+CHX enhanced TRAIL-induced cytotoxicity in four additional NB cell lines. However, 13 NB cell lines were still resistant to TRAIL-mediated apoptosis even in the presence of CHX. TRAIL-induced cell death in sensitive NB cells showed typical morphological signs of apoptosis, such as rounded cell bodies and nuclear condensation, as observed by phase contrast microscopy (data not shown). DNA strand breaks were confirmed by TUNEL labeling (data not shown), and DNA fragmentation was quantitated using a cell death ELISA (Fig. 3A). Exposure of phosphatidylserine on the outer leaflet
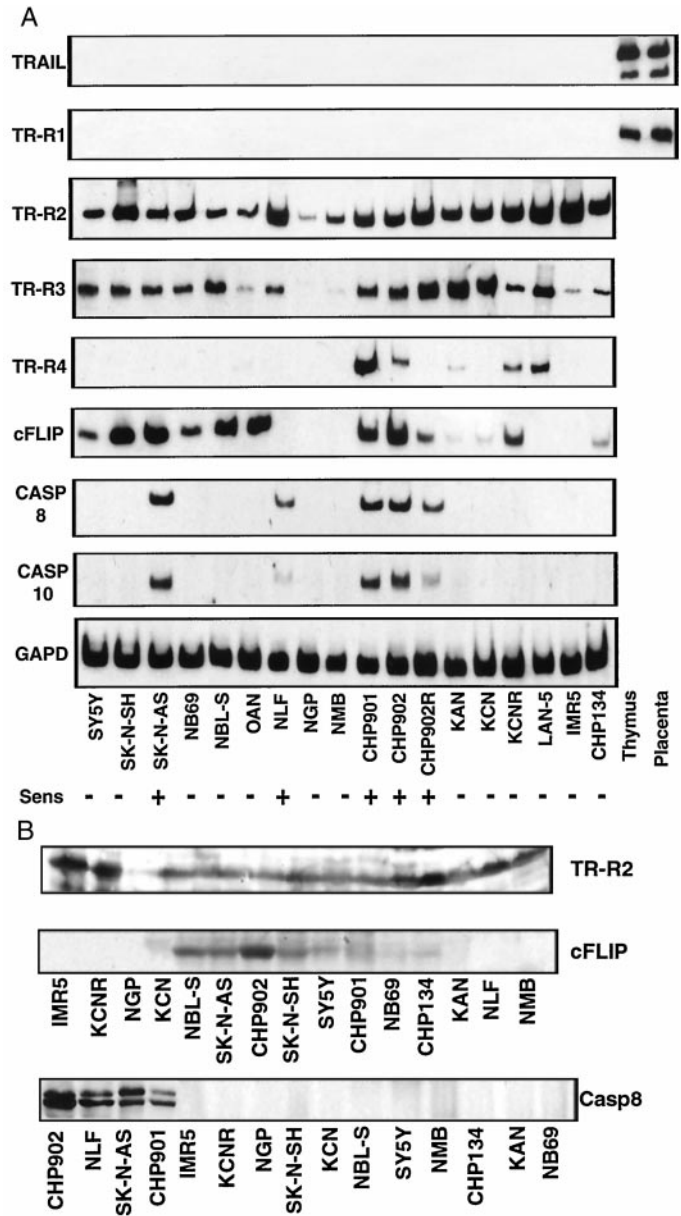

Fig. 1. A, RT-PCR of TRAIL system expression in 18 NB cell lines. Representative example of semiquantitative RT-PCR showing expression levels of TRAIL and TRAIL-R1, -R2, -R3, and -R4, cFLIP, caspase-8, and caspase-10 in the indicated NB cell lines. *Sens:* sensitivity (+) and resistance (−) to TRAIL-induced apoptosis. Expression of the housekeeping gene *GAPD* was used as internal control. B, protein expression of TRAIL-R2, cFLIP, and caspase-8 in 15 NB cell lines using Western blot analysis with specific polyclonal antibodies (see "Materials and Methods").

of the plasma membrane of apoptotic cells was demonstrated by annexin V-fluorescein isothiocyanate staining and flow cytometric analysis (data not shown).

Time and Dose Dependency of TRAIL-induced Apoptosis. The susceptibility of NB cells and the Jurkat T-cell line to TRAIL-induced apoptosis was tested over a range of concentrations of TRAIL. Shown as a representative example in Fig. 3B, apoptosis was maximal at 100 ng/ml TRAIL for CHP902 cells. Jurkat cells appeared more sensitive to apoptosis induced by TRAIL than CHP902 cells (IC_{50} = 6 ng/ml TRAIL *versus* IC_{50} = 50 ng/ml TRAIL). Studies on the kinetics of induction of apoptosis by TRAIL shown in Fig. 3C indicated that apoptosis was induced rapidly in Jurkat cells (maximum at 8 h), whereas 24 h was needed for maximal induction of apoptosis in CHP902 cells. Time and dose dependency of TRAIL-induced apoptosis was similar in the other four TRAIL-sensitive NB cell lines.
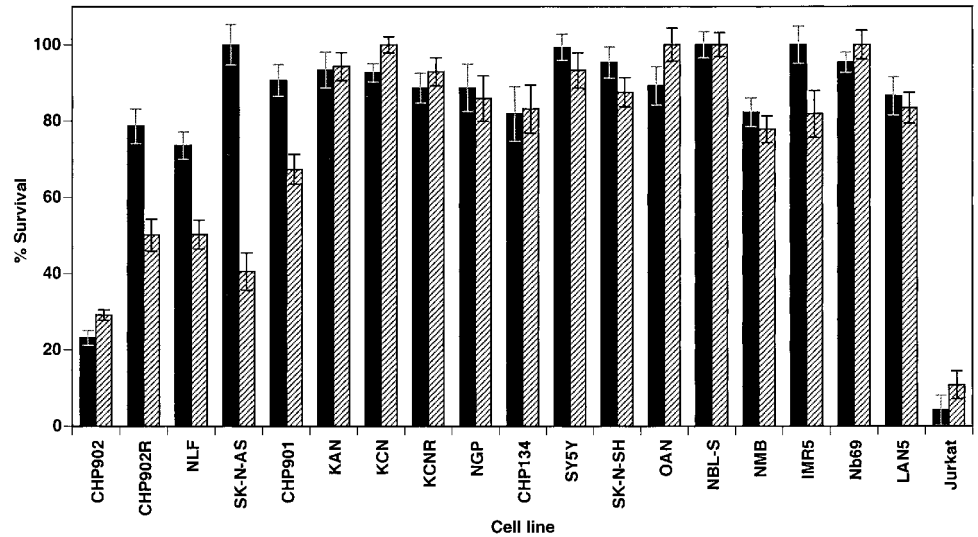
Fig. 2. Cytotoxicity of TRAIL to NB cells. NB cell lines were cultured in 10% serum medium in 96-well plates and treated with soluble human recombinant TRAIL (200 ng/ml) in the absence (■) or presence (▨) of CHX (1 μg/ml). Cell survival was determined after 24 h by MTT assay. Data points are the mean of triplicate samples (error bars, ± SD) and represent the percentage of survival compared with untreated control cells (CHX only treated cells). The representative results shown are from one of three different experiments.

Caspase Dependency of TRAIL-induced Apoptosis in NB. Because caspase activation is an important step in CD95/Fas-mediated apoptosis and has also been shown to play a role in TRAIL-induced apoptosis, we tested the susceptibility of TRAIL-sensitive NB cell lines to apoptosis in the presence of the broad spectrum caspase inhibitor zVADfmk and a specific caspase-8 and caspase-9 inhibitor (Fig. 3A). TRAIL-induced apoptosis in each of the TRAIL-sensitive NB cell lines tested was completely blocked in the presence of zVADfmk and almost completely inhibited in the presence of the specific caspase-8 inhibitor Z-IETD-FMK. In contrast, the caspase-9 inhibitor Z-LEHD-FMK had only a minor inhibitory effect on the cytotoxic effects of TRAIL. Thus, activation of caspase-8 seems to be essential for TRAIL-induced apoptosis in NB cells, whereas activation of caspase-9 appears to be rather redundant. Caspase-8 activation in response to TRAIL treatment in NB cell lines could directly be demonstrated by cleavage of the protein in a Western blot assay (data not shown).

Expression of cFLIP and Caspase-2, -3, -8, -9, and -10 in NB Cell Lines. Because critical involvement of caspases in TRAIL-induced apoptosis has been demonstrated before, we examined the expression profile of caspases and of the caspase-8 inhibitor cFLIP in our panel of NB cell lines. RT-PCR of 18 NB cell lines revealed high expression of cFLIP in 10 cell lines and various expression levels of caspase-2, -3, and -9 in all of the cell lines. Interestingly, caspase-8 and caspase-10 mRNA was detected in only 5 of 18 cell lines (Fig. 1A). The expression pattern of caspase-8 and cFLIP was confirmed on protein levels by Western blot analysis with specific antibodies (Fig. 1B).

Correlation of Caspase-8 and Caspase-10 Expression and TRAIL-induced Apoptosis. Most interestingly, a clear correlation of caspase-8 and caspase-10 expression and susceptibility to TRAIL-induced apoptosis became apparent: all of the 5 TRAIL-sensitive NB cell lines expressed caspase-8 and caspase-10 on mRNA levels, whereas all of the 13 resistant cell lines did not express any caspase-8 or caspase-10. On the other hand, no clear relation between TRAIL receptor expression, c-FLIP expression, or expression of any other caspase examined was evident.

Demethylation by 5-ADC Restores Caspase-8 and Caspase-10 Expression and TRAIL Sensitivity. Treatment of all of the TRAIL-resistant NB cell lines with the methyltransferase inhibitor 5-ADC resulted in dose-dependent restoration of caspase-8 and caspase-10 mRNA expression. Figure 4A shows SY5Y cells as a representative example. However, the induction of caspase-8 mRNA was weak in CHP134, NMB, OAN, and NGP cells. Treatment of TRAIL-sensitive SK-N-AS cells with 5-ADC further increased expression levels of caspase-8 and caspase-10 in these cells. Interestingly, sensitivity to TRAIL-induced apoptosis was also restored in SY5Y and IMR5 cells after treatment with 5-ADC (Fig. 4B). After cells were treated with 5-ADC, we demonstrated induction of caspase-8 protein and cleavage of caspase-8 in the presence of TRAIL+CHX. These data suggest that epigenetic changes such as methylation of the gene effect the expression of caspase-8 and caspase-10 and are a major determinant of TRAIL sensitivity in NB cells.

Discussion

NBs are rather resistant to current therapeutic approaches, including high-dose chemotherapy and stem cell transplantation. The search for novel therapies is therefore mandatory. It has been shown that NB cell lines are sensitive to CD95L-induced apoptosis, which seemed to be a promising experimental strategy to eliminate human NB cells *in vivo* (17). However, CD95 targeting *in vivo* has serious toxicity, which hampers its clinical use (2). On the other hand, TRAIL exerts potent antitumor activity *in vivo* without exhibiting systemic toxicity (3, 4). Thus, targeting the TRAIL system may be an equally effective but potentially safer approach of inducing apoptosis in human NB cells. To examine whether the TRAIL system might be suitable for experimental therapies of NB, we analyzed differential expression of agonistic and inhibitory receptors as well as important intracellular inhibitors and signaling elements to gain more insight into the complex regulation of the TRAIL receptor system in NB.

We show in our study that 5 of 18 NB cell lines are susceptible to TRAIL-induced apoptosis. Our results demonstrate that TRAIL sensitivity in NB is primarily regulated by expression of caspase-8 and/or caspase-10, rather than at the receptor level. Caspase-8 activation can be seen within minutes after the addition of TRAIL to sensitive cells, suggesting it may be one of the proximal compounds in the signaling pathway (14). Activation of caspase-10 and caspase-3 has also been reported in response to TRAIL treatment (5, 14). *Caspase-8* and *Caspase-10* are closely related genes that have both been mapped to 2q33-34, suggesting that the two genes have evolved by tandem duplication (18). Thus, it is possible that both genes are involved in the same gene rearrangement, mutational event, or epigenetic change. Our data suggest that the mechanism leading to the loss of caspase-8 and caspase-10 expression in NB is DNA methylation rather than deletion or recombination. Promoter hypermethylation is an important
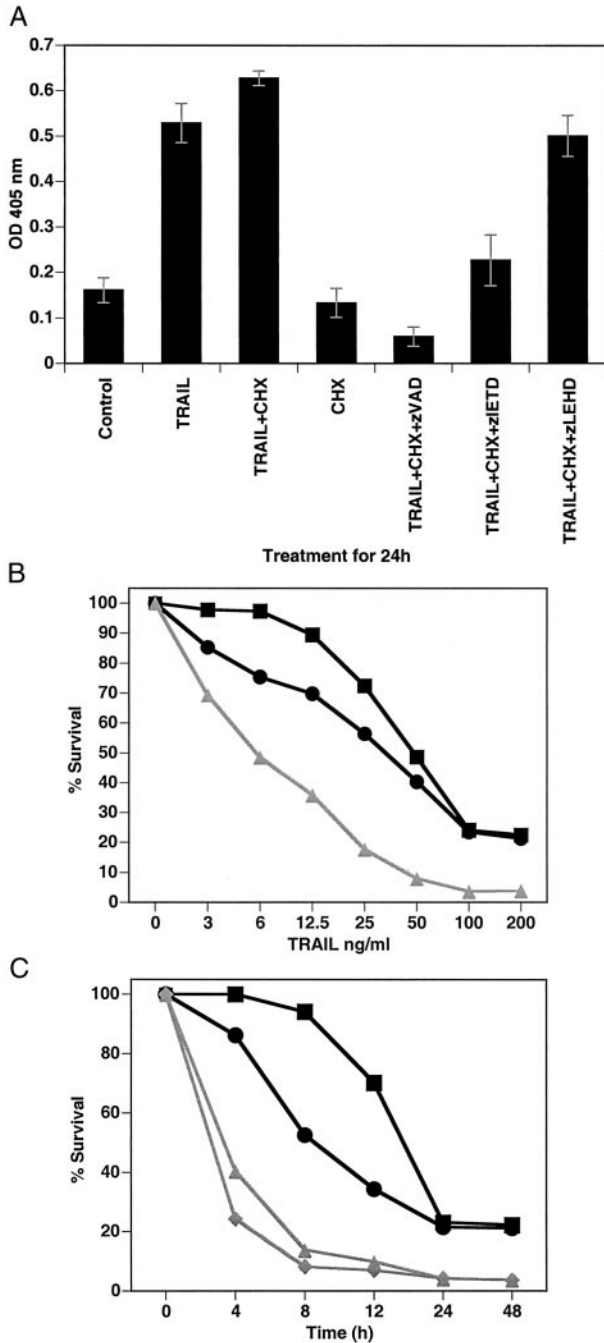
Fig. 3. Induction of apoptosis in Jurkat and neuroblastoma cells by TRAIL. *A,* cell death ELISA. Analysis of internucleosomal DNA cleavage by ELISA 24 h after stimulation. CHP902 cells treated with TRAIL or TRAIL+CHX show induction of internucleosomal DNA cleavage compared with untreated control cultures. Cultures were also treated with TRAIL in the presence of the caspase inhibitors zVAD, zIETD, and zLEHD. Results are the mean of triplicate samples; error bars, ± SD. Experiments are representative for all of the five sensitive NB cell lines and were repeated at least twice with similar results. *B,* dose dependency of TRAIL in Jurkat and in NB cells. Jurkat and CHP902 NB cells were incubated with recombinant TRAIL in the presence and absence of CHX for 24 h at the concentrations indicated. Cell survival of TRAIL-treated cells in the percentage of untreated control cells and of TRAIL+CHX-treated cells in the percentage of cells treated with CHX alone was measured by MTT assay. Data are the means of triplicate samples; SDs were <10%. Similar data were obtained in three independent experiments. ■, CHP902+TRAIL; ●, CHP902+TRAIL+CHX; ▲, Jurkat +TRAIL. *C,* kinetics of TRAIL-induced apoptosis in Jurkat and NB cells. Jurkat and CHP902 NB cells were incubated with recombinant TRAIL (200 ng/ml) in the presence and absence of CHX for the time periods indicated. Cell survival of TRAIL-treated cells in the percentage of untreated control cells and of TRAIL+CHX-treated cells in the percentage of cells treated with CHX alone was measured by MTT assay. Data are the means of triplicate samples; SDs were <10%. Similar data were obtained in three independent experiments. ■, CHP902+TRAIL; ●, CHP902+TRAIL+CHX; ▲, Jurkat+TRAIL; ◆, Jurkat+TRAIL+CHX.
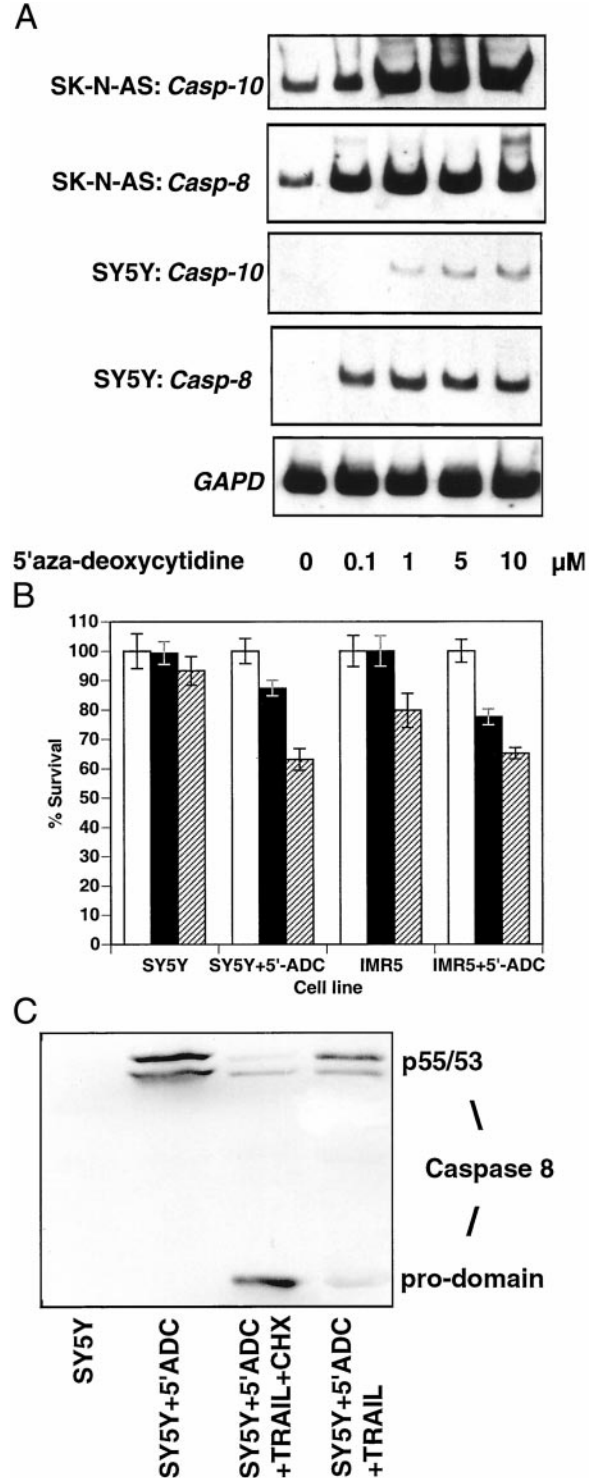

Fig. 4. Demethylation by 5-ADC restores caspase-8 and caspase-10 expression and TRAIL sensitivity. *A,* restoration of caspase mRNA expression. Representative example of semiquantitative RT-PCR showing expression levels of caspase-8 and caspase-10 in TRAIL-sensitive SK-N-AS cells and TRAIL-resistant SY5Y cells after treatment with the indicated concentrations of 5-ADC for 1 week. Expression of the housekeeping gene *GAPD* was used as internal control (as described in "Material and Methods"). *B,* restoration of TRAIL sensitivity. SY5Y and IMR5 cells lines were cultured in 10% serum medium ± 5-ADC in 96-well plates for 4 days. Cells were then treated with soluble human recombinant TRAIL (200 ng/ml) in the absence (■) or presence (▨) of CHX (1 μg/ml). Cell survival was determined after 24 h by MTT assay. Data points are the mean of triplicate samples and represent percentage of survival compared with untreated control cells (□; CHX-only treated cells). *C,* restoration of caspase-8 protein expression. Representative Western blot showing protein expression levels of caspase-8 in TRAIL-resistant SY5Y cells before (*Lane 1*) and after (*Lane 2*) treatment with 3 μM 5-ADC for 5 days. *Lanes 3* and *4:* cleavage of caspase-8 in 5-ADC-treated cells in response to 200 ng/ml TRAIL with or without 1 μg/ml CHX, respectively.

pathway for repression of gene transcription in cancer cells (19). Some tumor suppressor genes such as *p16*, *VHL*, and *MLH1* have been found to harbor promoter hypermethylation associated with loss of protein expression in cancer cells.

The fact that TRAIL-induced apoptosis in our study can be completely inhibited by the pancaspase inhibitor zVADfmk and largely by the specific caspase-8 inhibitor Z-IETD-FMK suggests an essential and nonredundant role of caspase-8 in TRAIL-mediated apoptosis of NB cells. Thus, the TRAIL receptor-signaling pathway appears to be similar to that identified for FAS and TNF receptors. The direct correlation of caspase-8 and caspase-10 expression with sensitivity of NB cells to TRAIL-mediated apoptosis is indicative of an early apical block of TRAIL-induced apoptosis attributable to a lack of caspase-8 and/or caspase-10. Caspase-8 knockout studies indicate that this enzyme is essential for apoptosis initiation by FAS, TNFR1, and DR3, but not for activation of the NFκB or JNK/AP-1 pathways by these receptors (20). Studies with a mutant human Jurkat T leukemia cell line that is deficient in caspase-8 confirm the requirement for this caspase in FAS-mediated apoptosis but show only partial attenuation of TNF-induced cell death (21). Thus, it appears more likely that the lack of caspase-8 expression is more important for the failure of NB cells to commit cell death than the lack of caspase-10 expression. However, the data obtained with the specific caspase-8 inhibitor cannot rule out the possibility that the downstream TRAIL signaling pathway can be triggered alternatively by activation of caspase-8 or caspase-10. Additional experiments with dominant negative forms of caspase-8 and caspase-10 are needed to elucidate the role of both caspases in TRAIL signaling.

In our study we found coexpression of TRAIL-R2 and TRAIL-R3 in the majority of cell lines. No expression of TRAIL-R1 and restricted expression of TRAIL-R4 transcripts does not suggest a significant regulatory role of these receptors in human NB. The lack of TRAIL-R1 might contribute to the TRAIL resistance of most NB cell lines, because it has been suggested for other cancer cell lines (22). However, the TRAIL-sensitive cell line CHP902 did also not express TRAIL-R1, which suggests rather that this receptor might not be needed for mediating the TRAIL signal in NB cells. The presence of mRNA encoding the "protective" TRAIL-R3 receptor did not correspond to resistance or sensitivity to TRAIL-induced apoptosis. In agreement with this, TRAIL-R3 and TRAIL-R4 have been detected in a number of other transformed cell lines that are sensitive to TRAIL-mediated apoptosis (23). Our data suggest that additional extracellular as well as intracellular factors such as caspase expression might modulate the previously proposed "decoy" model. However, the existence of additional TRAIL receptors as well as of soluble decoys has to be considered. As previously reported for other cell types, addition of the protein synthesis inhibitor CHX to four TRAIL-resistant NB cell lines rendered them sensitive to TRAIL, indicating that intracellular apoptosis inhibitors with a short half-life, such as cFLIP, may also be involved in the protection of NB cells from the cytotoxic effects of TRAIL. In our study 7 of 13 TRAIL-resistant cell lines demonstrated high expression of cFLIP on mRNA and protein levels. Although this correlation is not as convincing as the correlation of caspase-8 expression with TRAIL sensitivity, cFLIP might also contribute to the TRAIL resistance of NB cell lines. The role of cFLIP is controversial because in some cell types its overexpression can induce apoptosis (12), and resistance to apoptosis could also not be correlated with protein expression of the caspase inhibitor cFLIP in melanoma cell lines (24).

Induction of apoptosis via death receptors has been shown to represent one possible mechanism by which chemotherapy agents act on tumor cells (25). Deficient expression or activation of caspases may account in part for the failure of many current anticancer thera-

pies. NB cells may respond to cytotoxic agents only if they express a minimal level of caspase-8 and/or caspase-10. Our present results regarding the lack of caspase-8 and caspase-10 expression in a large subset of NB cell lines provides an explanation for the finding that these NB cell lines are resistant to TRAIL-mediated apoptosis. Our data may also explain the resistance of some NB cells to drug-induced apoptosis and thus have important consequences for additional attempts to manipulate tumor cell death. High levels of inactive, uncleaved caspase-2 and caspase-3 have recently been associated with poor survival in patients with acute myelogenous leukemia and acute lymphoblastic leukemia (26, 27). However, lack of caspase-8 and/or caspase-10 might not be the sole factor responsible for conferring resistance to TRAIL-induced apoptosis on NB cells. Regulation of the TRAIL system in NB is most likely also conferred by multiple agonistic and inhibitory receptors, a complex intracellular signaling mechanism involving a number of signaling adapters and activation of inhibitory molecules. On additional elucidation of the regulatory mechanisms of the TRAIL system, treatment with recombinant soluble TRAIL, possibly in combination with 5-ADC, may be an interesting, novel therapeutic approach for NB that deserves additional investigation.

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