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Induction of Caspase 8 by Interferon γ Renders Some Neuroblastoma (NB) Cells Sensitive to Tumor Necrosis Factor-related Apoptosis-inducing Ligand (TRAIL) but Reveals That a Lack of Membrane TR1/TR2 Also Contributes to TRAIL Resistance in NB

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ABSTRACT

The resistance of neuroblastoma (NB) cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis has been attributed to a lack of caspase 8 expression. Here we demonstrate a clinically applicable molecular targeting strategy that not only increases caspase 8 expression *ex vivo* in NB cell lines but also in the tumor tissues of NB patients receiving IFN-γ treatment. We identify the functional caspase 8 promoter, which is different from the methylated region reported previously, and show promoter activity is up-regulated by IFN-γ through a IFN-γ activation site-containing region. IFN-γ also induces TRAIL expression in NB cell lines. However, the IFN-γ restoration of caspase 8 in some NB cells revealed persistent TRAIL resistance in most NB cell lines examined. This additional lesion in the TRAIL path is because of a loss of cell membrane TRAIL receptors (TR1/TR2) not only in cell lines but in most of the NB tumor tissues evaluated. Restoration of TR2 expression by transfection enhances IFN-γ-induced TRAIL sensitivity. Furthermore, we have found that we can improve TRAIL sensitivity in NB by reconstituting caspase 8 with IFN-γ and TR2 with chemotherapeutic agents.

INTRODUCTION

TRAIL/Apo2L[2] is a member of the tumor necrosis factor ligand superfamily. TRAIL-induced apoptosis has been demonstrated in a wide variety of transformed cell lines of diverse tissue types, including Ewing's sarcoma, melanoma, colon cancer, lung cancer, breast cancer, kidney cancer, brain cancer, skin cancer, Burkitt lymphoma, and various leukemia cell lines (1–4). Although TRAIL mRNA is expressed in most normal tissues, particularly in spleen, prostate, lung, and intestine (1, 5), TRAIL-induced apoptosis is not readily detected in tests of normal mammary epithelial cells, human renal proximal tubule epithelial cells, human lung fibroblasts, skeletal muscle cells, and monocytic cells (1, 6). Caution has been raised by the findings that the *in vitro* culture of human liver cells and brain slices with certain TRAIL formulations causes limited apoptosis (7, 8), yet it is not clear whether this will occur *in vivo*, and different recombinant versions of TRAIL vary widely in hepatocyte toxicity (2, 9). With little toxicity in nonhuman primates and mice, the antitumor activity of TRAIL has been shown *in vivo* using human mammary adenocarcinoma, colon carcinoma, and glioma xenografts in mice (2). These features establish TRAIL as a promising cancer therapeutic agent.

NB is the second most common solid tumor in children, and progress in improving the outcome of advanced staged NB has been slow. Recent studies indicate that most NB cell lines are TRAIL resistant, especially those that have amplified N-*myc* and a poor prognosis (10–12). TRAIL resistance correlates with a lack of caspase 8 expression (10–13), and methylation of a region in the caspase 8 gene correlates with decreased caspase 8 expression in NB cell lines and tumor tissue (12). Treatment with 5-azacytidine, a DNA methylation inhibitor, has been shown to induce caspase 8 and cell death in selected NB cells, and subsequent treatment with TRAIL increases the NB cell death (10–12).

Induction of caspase 8 is indispensable for TRAIL-induced apoptosis. Whereas the mechanisms silencing caspase 8 expression are not precisely defined, therapeutic strategies for inducing its expression need to be explored. Aside from the preliminary strategies aimed at inhibition of DNA methyltransferase (14), it has been reported that IFN-γ induced TRAIL sensitivity in resistant Ewing's sarcoma cell lines (15), and induced caspase 8 in cell lines of breast cancer (16), colon cancer (17), erythroid progenitor cells (18), and recently in NB (19). To explore the potential therapeutic value of activation of the TRAIL pathway in NB, we examined the mechanism of IFN-γ induction of caspase 8 and whether restoration of caspase 8 would restore TRAIL sensitivity in NB.

MATERIALS AND METHODS

Cell Lines and Cytokines

NB cell lines (20) were cultured as described previously (21). Cells were treated with indicated concentrations of IFN-γ (Sigma), TRAIL (R&D, Minneapolis, MN), TRAIL neutralizing antibody (eBioscience, San Diego, CA), or diluent for the indicated times. When noted, cells were treated with 5 μg/ml cycloheximide (Sigma) to block protein synthesis or 10 mM Hoechst33342 for 5 min at 20°C to stain DNA. The human recombinant IFN-γ administered to patients was from Genentech (Thousand Oaks, CA).

Promoter Cloning and Promoter Activity Assay

The transcription start point was determined by the 5′ most sequence of all of the expressed sequence tags representing known caspase 8 sequence as documented in GenBank. Genomic sequence was based on GenBank sequence GI9958173. The selected region was amplified by PCR and cloned into the promoterless luciferase reporter vector pGL2-basic (Promega) followed by sequence confirmation. Each reporter plasmid and pCMVβgal were cotransfected into BE2, and the luciferase activity was normalized to β-galactosidase activity. The luciferase and β-galactosidase activity assay is as described previously (22).

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[2] The abbreviations used are: TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; NB, neuroblastoma; TR, tumor necrosis factor-related apoptosis-inducing ligand receptor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription-PCR; GFP, green fluorescent protein; FACS, fluorescence-activated cell sorter; nt, nucleotide; GAS, IFN-γ activation site.

MTT Assay

Cells grown in 96-well plate were treated with 100 μl of 0.5 mg/ml of MTT in phenol-free RPMI 1640 for 3 h at 37°C, followed by 100 μl of isopropanol for 20 min. Absorbance was measure at 570 nm and 690 nm, and the viability inhibition is represented as a percentage of control cells {percentage inhibition = [1 − (treated/control)] × 100; Ref. 23}.

RNA Analysis

Total RNA was isolated with RNeasy kit (Qiagen, Valencia, CA). Fifteen μg of total RNA were analyzed by Northern blot hybridization as described previously (21) and hybridized with ^{32}P-labeled insert DNA isolated from plasmids containing human caspase 8 (kindly provided by Michael J. Lenardo, National Institute of Allergy and Infectious Diseases, Bethesda, MD) or GAPDH. cDNA made from 0.1 μg of total RNA was used in each RT-PCR reaction. The primers for TR1 and TR2 are as described previously (4); β-actin is 5′-ctcttccagccttccttcct-3′ and 5′-caccttcaccgttccagttt-3′. Touchdown PCR was performed, and the annealing temperatures were from 65–57°C by 0.5°C decrease per gradient.

Western Blot Analysis

Cells were lysed, and Western blot analysis was performed as described previously (24). Forty μg of protein per sample was analyzed. The anticaspase 8 antibody (Cell Signaling, Beverly, MA) was diluted to 1:1000.

Transient Transfection Assay

Ten million cells were plated in a 100-mm dish with DMEM and cotransfected with 4 μg of pcDNA-GS-TR2 (CMV-TR2 expression plasmid; Invitrogen, Carlsbad, CA) or the empty vector pcDNA-GS and 1 μg of pEGFP-C1 plasmid (GFP; Clontech Laboratories, Inc., Palo Alto, CA), using 45 μl of LipofectAMINE 2000 (Life Technologies, Inc., Gaithersburg, MD) according to the manufacture's instruction. The GFP-positive cells were isolated through cell sorting at 16 h after transfection, and plated in a 96-well microtiter plate and incubated with reagents noted.

FACS Analysis

One million cells were stained with anti-TR1 (IgG2a, M271) or anti-TR2 (IgG1, M413) monoclonal antibodies (kindly provided by David Lynch, Immunex, Seattle, WA), or isotype control (anti-CD7, 3A1 mAb kindly supplied by Ron Gress, National Cancer Institute, Bethesda, MD). Cells were washed in FACS buffer (PBS +2% BSA +0.1% NaN3) and incubated for 30 min at 4°C with FITC-conjugated goat antimouse immunoglobulin (Caltag, Burlingame, CA). Live cells were discriminated by staining with propidium iodide. Immunofluorescence was detected on a FACScalibur (Becton Dickinson, Franklin Lakes, NJ). A minimum of 10,000 cells were acquired and analyzed using Cell Quest software.

Immunohistochemistry

Immunohistochemical Detection of TR1 and TR2. Frozen sections from 16 NB tumor tissues were fixed in acetone and 4% paraformaldehyde at room temperature for 10 min. A Ewing's sarcoma tumor tissue studied previously was also included as positive control for each antibody. Endogenous peroxidase activity was quenched in 1% hydrogen peroxide. The sections were incubated in 10% normal goat serum in PBS for 1 h, in the primary antibodies anti-TR1 (5 μg/ml) or anti-TR2 (5 μg/ml; Immunex) at 4°C overnight, and in peroxidase conjugated goat antimouse immunoglobulins (Dako Corporation, Carpinteria, CA) at room temperature for 30 min. The peroxidase reaction was developed with 3,3′-diaminobenzidine (Vector Laboratories, Burlingame, CA), and the slides were counterstained with hematoxylin.

Immunohistochemical Detection of Caspase 8. Paraffin sections of 5 μm were deparaffinized in xylene and rehydrated in alcohol. Endogenous peroxidase activity was quenched for 10 min at room temperature in methanol containing 1.5% hydrogen peroxide. After washing twice with water, sections were then subjected to antigen retrieval by incubation in Dako Antigen Retrieval solution (Dako; pH 6.2), for 15 min in a mW oven. The sections were washed in PBS and incubated for 1 h with a blocking solution consisting of 10% normal goat serum (Vector Laboratories) and 0.4% Tween 20 (Roche Diagnostics Corporation, Indianapolis, IN) in PBS at room temperature. The mouse monoclonal caspase 8 antibody (Upstate Biotechnology, Lake Placid, NY) was applied overnight at 4°C at a concentration of 1:75. Then, the sections were washed with PBS and incubated with goat antimouse immunoglobulins conjugated to peroxidase-labeled dextran polymer (Dako Envision+ Peroxidase) for 30 min at room temperature. The peroxidase reaction was developed with 3′3-diaminobenzidine (Dako), and the slides were counterstained with hematoxylin.

RESULTS

IFN-γ Transcriptionally Induces Caspase 8 in NB Cell Line. KCNR is an N-*myc* amplified NB cell line derived from a patient with recurrent NB tumor (21, 25), which we found resistant to TRAIL-induced cell death. To investigate whether IFN-γ could induce caspase 8 expression, we treated KCNR with IFN-γ (200 ng/ml) and isolated total RNA after various durations of treatment for Northern analysis. IFN-γ induced caspase 8 mRNA within 12 h with peak levels after 48 h of treatment (Fig. 1*a*). Caspase 8 mRNA was also induced by IFN-γ when the cells were treated with 5 μg/ml of cycloheximide (data not shown), indicating that new protein synthesis is not required for the induction.

The relatively early induction of caspase 8 by IFN-γ without the requirement for new protein synthesis suggested regulation at the transcriptional level. To study the mechanism of IFN-γ induction of caspase 8, we evaluated the caspase 8 promoter using a luciferase reporter system. Because a caspase 8 promoter region has not been identified, we cloned a region 5′ to exon 1 (−327 nt) encompassing the transcription initiation site, named C8P-327, into a reporter vector and found the region had high promoter activity (Fig. 1*b*). After 12 h of IFN-γ (200 ng/ml) treatment, the promoter activity of C8P-327 increased 2.5-fold (Fig. 1*b*). Deletion of 163 nt at the 5′ end of C8P-327, which contains a GAS element, dramatically diminished both basal and IFN-γ-induced luciferase activity (Fig. 1*b*).

IFN-γ Induces Caspase 8 in NB Tumor Tissue. It is necessary to study the IFN-γ induction of caspase 8 *in vivo* to determine its therapeutic relevance and value. We had the opportunity to evaluate the caspase 8 expression in NB tumor tissue after systemic IFN-γ treatment and compared that with the tumor of the same patient before IFN-γ therapy. NB tissue specimens were collected previously from patients that entered a clinical protocol (NCI 90-C-0210) involving the use of IFN-γ (26). The protocol was approved by the Institutional Review Board of the National Cancer Institute, and written informed consent was obtained from all of the patients, or their parent or legal guardian. Patients received daily IFN-γ treatment (0.1 mg/m^2 via s.c. injection) for 5 days continuously before resection of the tumor for isolation of tumor infiltrating lymphocytes. We evaluated caspase 8 expression in tumor specimens before and after IFN-γ treatment from 7 patients by immunohistochemistry. In the tumors from three of the seven patients, caspase 8 expression was increased after IFN-γ treatment reflecting both an increase in number of cells expressing caspase 8 and in the intensity of caspase 8 expression (Fig. 1*c*). There was no significant change in caspase 8 expression in the tumors from the other four patients.

IFN-γ Sensitizes NB Cells to TRAIL-induced Apoptosis. To determine whether IFN-γ-treated caspase 8-expressing cells were sensitive to TRAIL treatment, TRAIL (100 ng/ml) was added to KCNR cells that had been pretreated for 48 h with IFN-γ. Treatment of KCNR cells with TRAIL alone did not affect cell morphology (Fig. 1*d*, *top panel*) or cell number (<5% decrease; Fig. 1*e*). However, in IFN-γ-treated cells, TRAIL induced a >40% decrease in cell number within 24 h, and this was diminished if cells were incubated with the caspase 8 inhibitor z-IETD-fmk (100 nM; Fig. 1*e*). Hoechst staining of the cells revealed there was in the IFN-γ/TRAIL-treated cells an

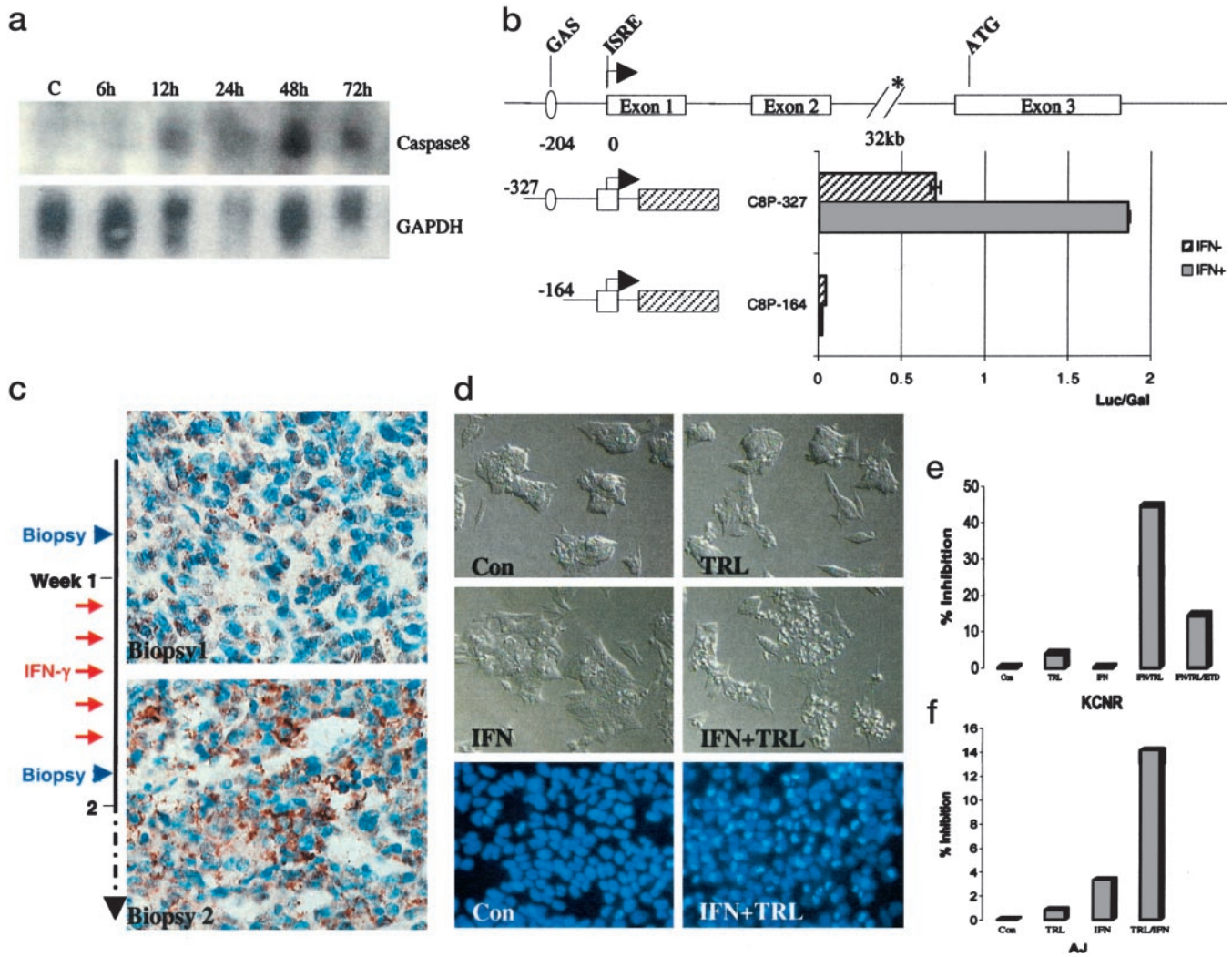

Fig. 1. IFN-γ sensitizes KCNR to TRAIL by inducing caspase 8. *a*, Northern analysis of caspase 8 expression in KCNR treated with IFN-γ for indicated periods of time. *b*, caspase 8 promoter is up-regulated by IFN-γ treatment. *Top*, the structure of the 5′ end of caspase 8 gene. The GenBank index number of the genomic sequence is GI9958173. The transcription start point (→) is determined as the most 5′ end of all expressed sequence tag sequences representing caspase 8. Region of hypermethylation in NB (∗; Ref. 12). *Bottom left*, constructs of luciferase (▨) driven by caspase 8 promoter or the 5′ end deletion of the region, which does not have GAS element (○). *Bottom right*, the promoter activity and the response to IFN-γ treatment of caspase 8 promoter with or without GAS. The promoter activity is assessed by luciferase activity (*Luc*) and normalized by β-galactosidase (*Gal*). ▨, no IFN-γ treatment; ▩, with IFN-γ treatment. *c*, IFN-γ induction of caspase 8 *in vivo*. Immunohistochemical staining for caspase 8 expression in a NB tumor before IFN-γ treatment (*Biopsy 1*) and after IFN-γ treatment (*Biopsy 2*). *d*, the IFN-γ and TRAIL treatment causes morphological changes and condensation of nuclei in KCNR. Cells are treated as indicated (*top* and *middle*, ×100 magnification) and stained with Hoechst 33342 (*bottom*, ×400 magnification). *Con*, control; *TRL*, TRAIL (100 ng/ml) treatment; *IFN*, IFN-γ (200 ng/ml) treatment; *IFN+TRL*, IFN-γ and TRAIL combined treatment. *e*, IFN-γ treatment sensitizes KCNR to TRAIL. Cell viability was measured by the MTT assay, and the data represent survival inhibition as a percentage of control cells {% inhibition = [1 − (treated/control)]×100}. This is a representative experiment of performed and the variation among experiments is within 5%. *IFN/TRL/IETD*, IFN-γ and TRAIL combined treatment in the presence of 100 nM z-IETD-fmk. *f*, primary NB culture AJ was treated with indicated conditions and assessed by MTT assay; *bars*, ±SD.

increase in the number of cells with condensed nuclei, a hallmark of apoptosis (Fig. 1*d*, *bottom panel*). Consistent with this finding, we observed that the sensitivity of a primary NB culture, AJ, to TRAIL could be enhanced by pretreatment with IFN-γ (Fig. 1*f*). FACS analysis showed that ~30% of AJ cells were TR2 positive.[3]

IFN-γ Induces Caspase 8 in Most TRAIL-resistant NB Cell Lines Tested but Confers Sensitivity in Only a Minority of Cell Lines. To determine whether the results obtained with the KCNR NB cell line were applicable to NB cell lines in general, we screened a panel of 15 NB cells for TRAIL sensitivity. Twenty-four h after plating, cells were treated with 100 ng/ml of TRAIL. We found that of the 15 NB cell lines tested, only 1, CHP212, was TRAIL sensitive (Fig. 2*a*). To evaluate whether IFN-γ induced caspase 8 in other cell lines, we evaluated the expression of caspase 8 mRNA by RT-PCR and Northern analysis in IFN-γ-treated cells for 48 h with a cell line known to express caspase 8, AS, as control. In the untreated cells, caspase 8 was constitutively expressed in CHP212 and AS, the two TRAIL-sensitive NB cell lines (Fig. 2*b*). After culture with IFN-γ, caspase 8 mRNA was detected at various levels in 8 of 14 caspase 8-negative NB cell lines (Fig. 2*b*), and Western analysis confirmed an increase in $p55/p57^{caspase\ 8}$ protein in the IFN-γ-treated cell lines (Fig. 2*c*).

To evaluate the effect of TRAIL on caspase 8-expressing NB cells, cells were treated with IFN-γ (200 ng/ml) for 48 h followed by TRAIL (100 ng/ml) for 24 h. However, 7 of 8 caspase 8-expressing cells were still resistant to TRAIL-induced apoptosis (Fig. 2*d*). The only TRAIL-sensitized cell line, KCNR, had a moderate level of caspase 8 expression compared with those that remained resistant to TRAIL-induced cell death (Fig. 2, *b* and *c*). This indicated that a mechanism(s) other than caspase 8 deficiency contributed to TRAIL

[3] X. Yang, M. Merchant, C. Mackall, C. Thiele, unpublished observations.

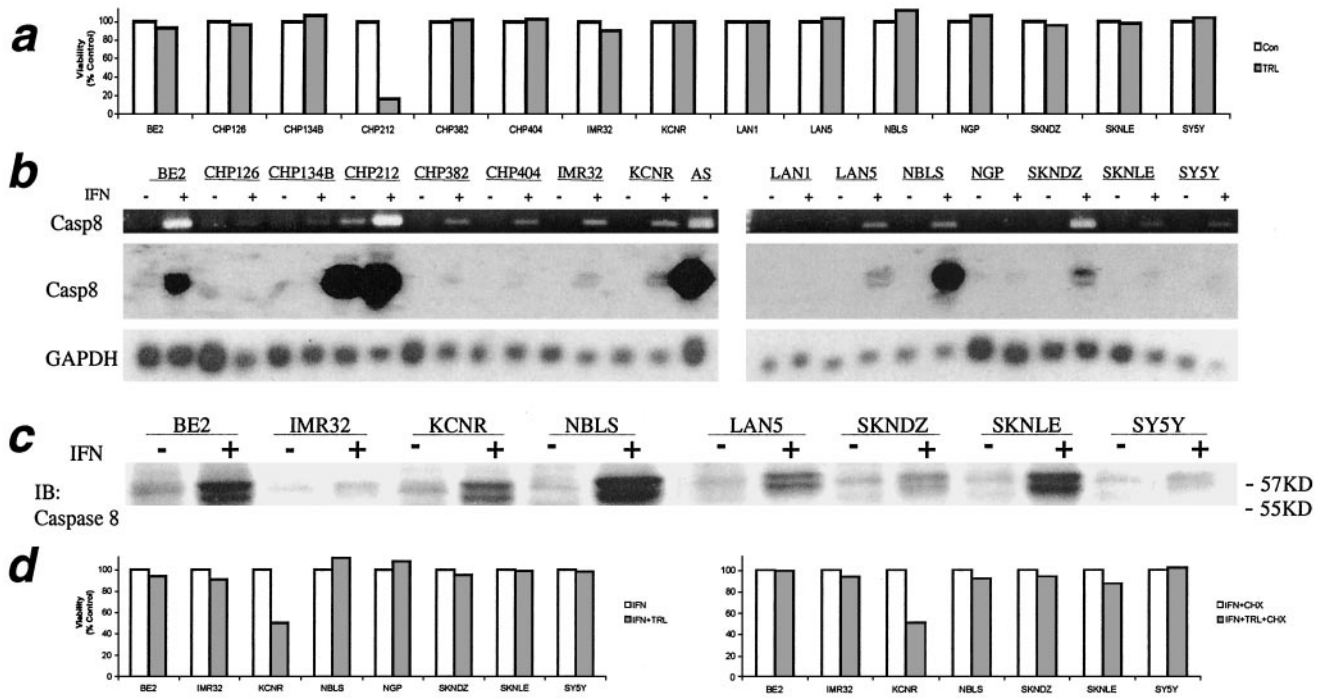

Fig. 2. IFN-γ induces caspase 8 in NB cell lines but often fails to restore TRAIL sensitivity. *a*, NB cell lines are treated with TRAIL, and viability is assessed by MTT assay. *b*, IFN-γ induces caspase 8 mRNA expression in 8 of 14 NB as assessed by RT-PCR (*top*), Northern analysis of caspase 8 (*middle*), and GAPDH (*bottom*), respectively. *c*, Western analysis of caspase 8 in selected NB cell lines in the absence or presence of IFN-γ. *d*, the viability of 7 of 8 caspase 8-expressing NB cell lines is not affected by TRAIL (*left*) and cycloheximide (*CHX*) treatment dose not alter TRAIL sensitivity (*right*) as assessed by MTT assay.

resistance in NB. Because the effect of some short-lived inhibitors of apoptosis, such as the caspase 8 inhibitor cFLIP, can be blocked by cycloheximide treatment (27), we incubated the IFN-γ pretreated cells with 5 μg/ml cycloheximide before adding TRAIL. Despite cycloheximide treatment, the cells remained resistant to TRAIL-induced apoptosis (Fig. 2*d*), although caspase 8 was expressed and potential short-lived inhibitors were blocked.

IFN-γ/TRAIL-resistant NB Lacks Surface TR. Our finding that the restoration of caspase 8 was not sufficient to induce TRAIL sensitivity in many NB cell lines indicated the presence of other deficiencies in the TRAIL pathway in NB. Fourteen TRAIL-resistant NB cells were cultured with or without IFN-γ (200 ng/ml) for 48 h, after which RNA was isolated, and a number of genes involved in death receptor signaling were evaluated by RT-PCR. We found expression of TR2 mRNA and variable expression of TR1 mRNA in NB cells, but neither TR2 nor TR1 mRNA expression correlated with TRAIL resistance (Fig. 3*a*). We also found that TRADD, FADD, TRAF2, RIP, and BID were widely expressed, and c-FLIP was variably expressed in NB cells (data not shown), yet none of these correlated with TRAIL resistance.

Functional TRs are distributed on the cell surface as monomers and trimerize on activation by TRAIL. Using FACS analysis, we found that none of the NB cell lines tested expressed surface TR1, and many NB cell lines lacked surface TR2 as well, despite expression of TR1/2 mRNA (Fig. 3*b;* Table 1). On the basis of the mean channel fluorescence intensity and positive staining percentage, we stratified the cell line panel into those cell lines with a relatively high level of surface TR2 expression and those cell lines with a relatively low level or no surface TR2 expression. Most of the TRAIL-resistant cell lines that express caspase 8, *e.g.*, those that remained resistant to TRAIL-induced apoptosis even after IFN-γ induction of caspase 8, had either no or very low levels of surface TR2 expression (Fig. 3*b;* Table 1). Nonsignaling TRs TR3 and TR4 were rarely expressed in the NB cells as assessed by FACS analysis, and were not correlated with TRAIL sensitivity (data not shown).

TR2 Deficiency in NB Tumor Tissue. The finding of low TR expression in NB is at variance with the interpretation of some reports (11, 19). To determine whether the loss of TR expression occurs in primary NB tumors and to rule out that it is an artifact of longer-term cell culture, we evaluated TR1 and TR2 expression in 16 NB tumor specimens using immunohistochemistry. Tumor tissues from all 16 of the primary NBs were negative for TR1 (Fig. 3*c, panel 1*), whereas Ewing's sarcoma stained using the same techniques showed significant expression (Fig. 3*c, panel 2*). Assessment of tumor tissue for TR2 expression revealed no expression in 14 cases (Fig. 3*c, panel 3*) and focal expression limited to small groups of tumor cells (<10%) in the remaining two samples examined (Fig. 3*c, panel 4*).

Ectopic TR2 Expression Sensitizes NB to IFN-γ/TRAIL Treatment. Our data indicate that a lack of TRs correlates with TRAIL resistance in NB cells expressing caspase 8 after IFN-γ treatment (Table 1). Both TR1 and TR2 are functional TRs that convey the death signal once activated by the ligand. To determine whether restoring one of the functional TRs would sensitize cells to TRAIL, we cotransfected two TRAIL-resistant NB cell lines, BE-2 and NBLS, with pcDNA-GS-TR2 and GFP, or pcDNA-GS and GFP. Twenty-four h after cotransfection, the GFP-positive cells were isolated and cultured with IFN-γ for 12 h followed by 48 h with TRAIL and IFN-γ. The TR2-transfected GFP-positive cells remained resistant to TRAIL-induced apoptosis (Fig. 4*a, top,* and *c*). However, after IFN-γ and TRAIL treatment cells underwent apoptosis as they became round, detached, and stained positive for annexin (Fig. 4*b, right*). There was a 5-fold decrease in the GFP-positive cells compared with the same cells treated with TRAIL alone (Fig. 4*a, top,* and *c*). In cells transfected with pcDNA-GS and GFP, IFN-γ and TRAIL treatment had no effect on the number of GFP-positive cells (Fig. 4*a, bottom,* and *b, left*).

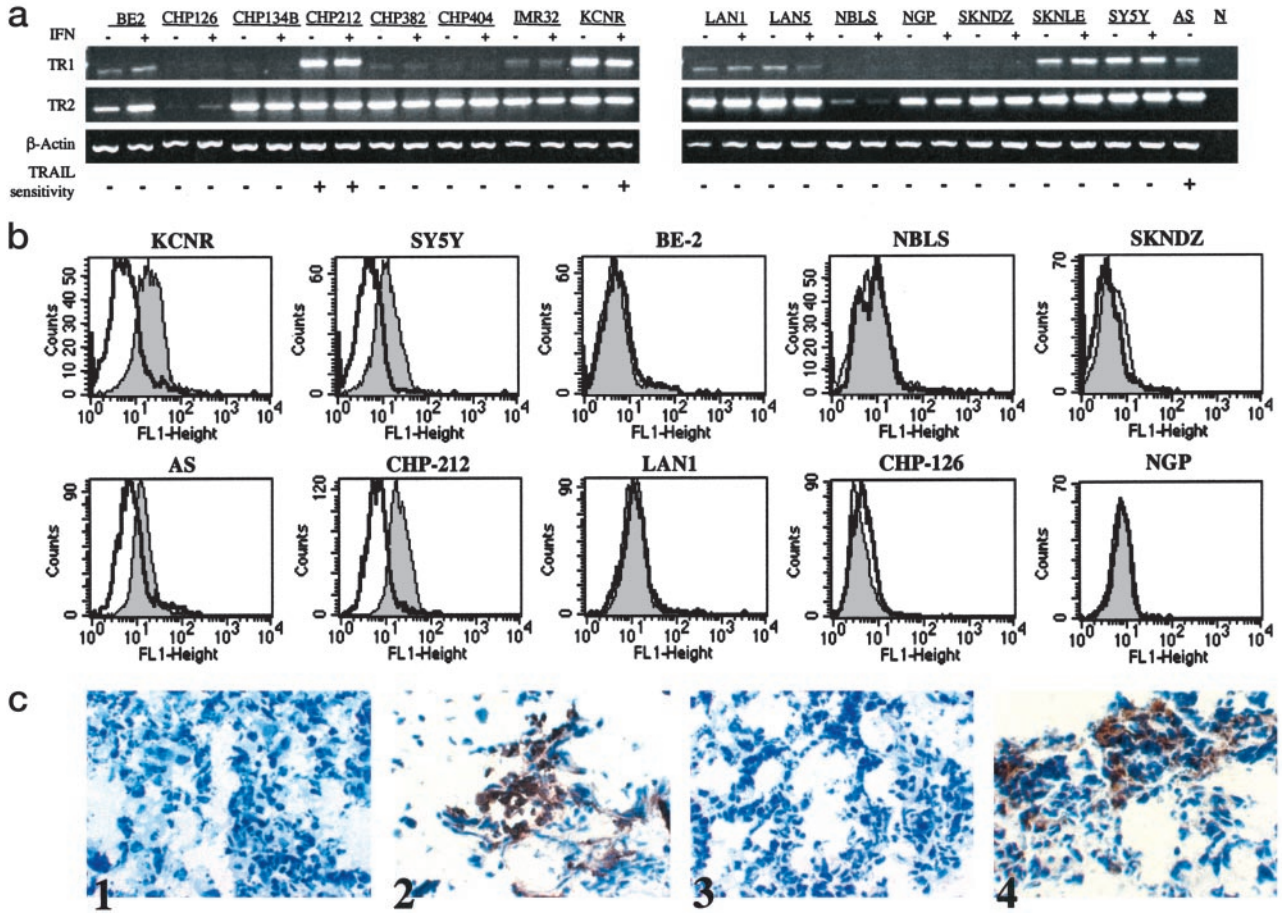

Fig. 3. TRAIL-resistant NB cells lack TR1 and TR2. *a*, assessment of TR1 and TR2 mRNA expression in NB cell lines by RT-PCR analysis. *N*, negative control. *b*, typical positive and negative TR2 surface expression in NB cell lines as assessed by FACS analysis. Cells were labeled with anti-TR2 monoclonal antibody M413 (*shaded*) or anti-CD7 monoclonal antibody as negative control (*open*). *c*, TR1 and TR2 expression *in vivo*. *Panel 1*, absent TR1 expression in a primary NB, which was representative of 16 tumors analyzed. *Panel 2*, extensive TR1 expression in a primary Ewing's sarcoma is shown as a positive control. *Panel 3*, absent TR2 expression in a primary NB, which was representative of 14 of 16 tumors analyzed. *Panel 4*, focal expression of TR2, which was observed in 2 of 16 tumors analyzed.

Treatment with IFN-γ alone induced cell death in TR2-transfected cells but not in control-transfected cells (Fig. 4, *a–c*). Because IFN-γ-induced cell death is TR2 dependent and TRAIL is the only known ligand inducing cell death via TR2, we hypothesized that IFN-γ may be inducing TRAIL expression in NB, and this leads to cell death of TR2 transfectants. To test this we investigated the expression of TRAIL before and after IFN-γ treatment. In both BE-2 and NBLS, TRAIL expression was induced by IFN-γ (Fig. 4*d*). Additional study showed that IFN-γ induced TRAIL in 10 of the 15 cells tested (data not shown). To test the role of TRAIL in IFN-γ-induced cell death we evaluated the IFN-γ killing effect of TR2 transfectants in the presence of 10 μg/ml anti-TRAIL antibody. IFN-γ treatment significantly decreased the number of BE2 cells with high TR2/GFP transfection as selected by GFP expression level, and the killing effect was partially blocked by anti-TRAIL treatment (Fig. 4*e*).

Table 1 Analysis of TRAIL resistance in human NB cells

Cell Line	TRAIL sensitivity[a]	Caspase8[b]	Surface TR2[c]	Surface TR1[c]	C-FLIP[d]
A4573[e]	+	+	+	+	/
SKNAS	+	+	+	−	+
CHP212	+	+	+	−	+
KCNR	+	+	+	−	+
BE2	−	+	−	−	+
NBLS	−	+	−	−	+
LAN5	−	+	−	−	−
SKNDZ	−	+	−	−	−
SKNLE	−	+	+	−	+
SY5Y	−	−	+	−	−
CHP382	−	−	+	−	−
CHP404	−	−	+	−	−
IMR32	−	−	+	−	−
LAN1	−	−	+	−	−
CHP126	−	−	−	−	−
CHP134B	−	−	−	−	−
NGP	−	−	−	−	−

[a] + indicates a decrease in cell viability ≥20% in TRAIL treatment compared to control as assessed by MTT assay.
[b] As detected by Western analysis after IFN-γ treatment.
[c] + represents a minimum shift of 5 units in mean channel fluorescence intensity above isotype control staining as evaluated by FACS analysis.
[d] As detected by RT-PCR.
[e] A known TRAIL sensitive Ewing's sarcoma cell line as positive control.

Chemotherapy Induces TR2 Expression and TRAIL Sensitivity. Whereas we have shown that TR2 transfection combined with IFN-γ treatment is effective at sensitizing NB to TRAIL-induced apoptosis, *in vivo* gene transference is still far from being clinically useful. The tumor suppressor protein p53 has been reported to induce TR2 expression in colon and lung cancer cells (28, 29). Because many antineoplastic agents now in use are DNA-damaging agents that are known to induce p53 (30), we reasoned that chemotherapy might increase expression of TR2 and sensitize NB to TRAIL when combined with IFN-γ. We pretreated SKNDZ, a relatively chemoresistant NB cell that is also TRAIL resistant, with Adriamycin (0.3 μg/ml) or etoposide (6 μg/ml) for 24 h, then the cells that survived chemotherapy were harvested and treated with IFN-γ and TRAIL for 48 h. The chemotherapy pretreated SKNDZ was found to be surface TR2 pos-

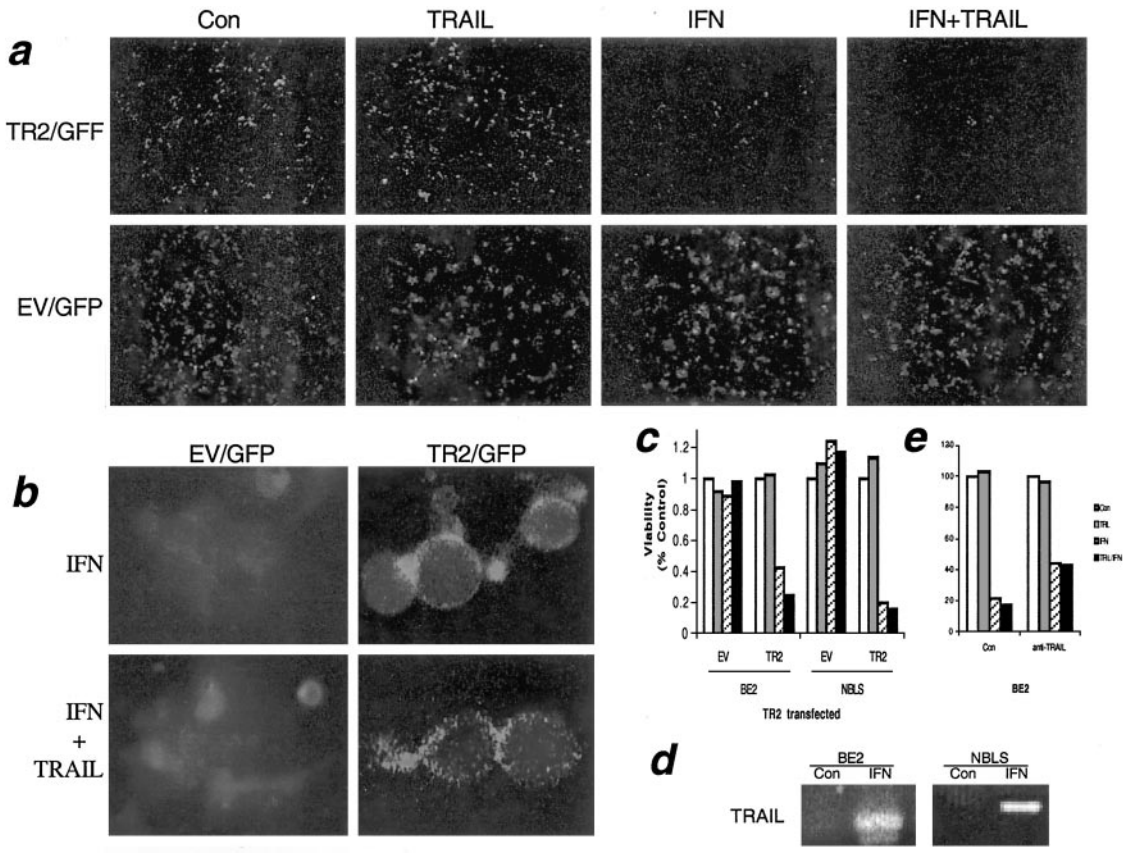

Fig. 4. Restoration of TR2 sensitizes resistant NB cells to IFN-γ/TRAIL treatment. *a*, ×100 magnification of BE2 cells treated as indicated. *TR2/GFP*, cotransfected with pcDNA-GS-TR2/pEGFP-C1; *EV/GFP*, cotransfected with pcDNA-GS/pEGFP-C1. *b*, ×400 magnification of TR2/GFP or EV/GFP cotransfected BE2 cells treated as indicated and stained with annexin V-phycoerythrin (*red*). *c*, the viability of TR2/GFP transfected cells after treatment with medium (*open*), TRAIL (*shaded*), IFN (*hatched*), and TRAIL/IFN (*black*). Four random fields were counted for GFP-positive cells for each condition and the mean value is used to indicate the GFP-positive cell viability. *d*, assessment of TRAIL expression by RT-PCR in BE2 and NBLS before and after IFN-γ treatment. *e*, the survival of TR2/GFP cotransfected BE2 after the indicated treatments in the presence of diluent (*Con*) or TRAIL-neutralizing antibody.

itive by FACS (Fig. 5*a*, *right*), and showed a 3–4-fold increase in cell death after IFN-γ and TRAIL treatment compared with cells not selected by chemotherapy (Fig. 5, *a* and *b*).

DISCUSSION

Considering the central role of caspase 8 in TRAIL-induced apoptosis (13, 31), the finding of *in vivo* caspase 8 induction is of therapeutic significance. As a Food and Drug Administration-approved cytokine used to treat chronic granulomatous disease and atopic dermatitis, IFN-γ can be a well-tolerated treatment (32–34). The administration of IFN-γ used in our clinical trial for NB was of much lower dosage and shorter course compared with the maximum tolerated dose that ranges from 3 to 10 mg/m^2 depending on the source of the IFN-γ and the clinical center (35–39), yet it induced caspase 8

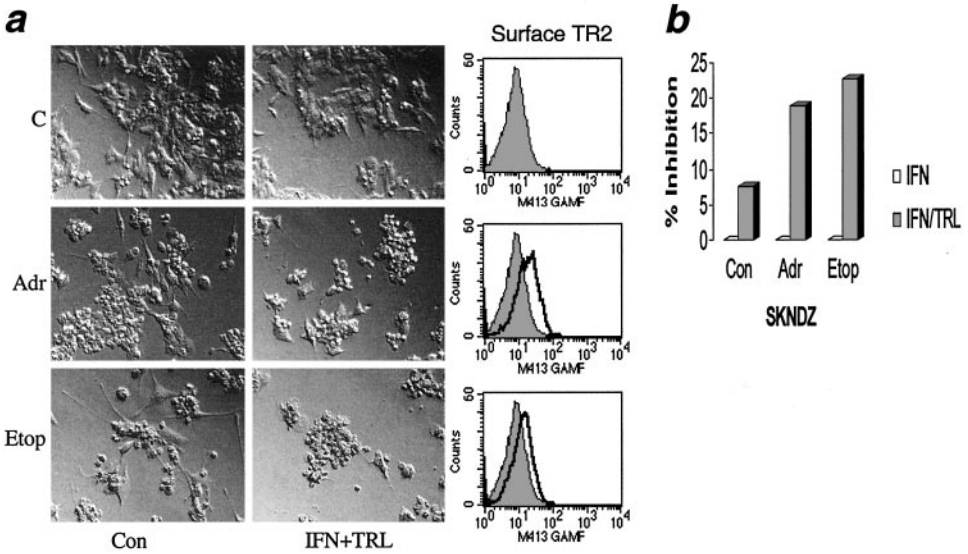

Fig. 5. Chemotherapy induces TR2 and increases TRAIL sensitivity. *a*, Adriamycin (0.3 μg/ml; *Adr*) or Etoposide (6 μg/ml; *Etop*) pretreated SKNDZ showed improved response to IFN-γ/TRAIL treatment (*left* and *middle*) as well as increased TR2 (*right*) as shown by FACS analysis. Cells in the absence (*shaded*) or the presence (*open*) of indicated drug were labeled with anti-TR2 monoclonal antibody M413 for FACS analysis. *b*, the viability inhibition of chemotherapy preselected SKNDZ cells (*Adr* and *Etop*) by IFN-γ/TRAIL was increased compared with that of nonchemotherapy-selected (*Con*) SKNDZ as measured by MTT.

expression in three of seven patients. Side effects were generally mild, and consisted mostly of fever and chills, and there were no intolerable toxicities that led to cessation of IFN-γ administration. In some preclinical settings, as we showed in the primary NB culture AJ and the cell line KCNR, IFN-γ treatment alone was able to increase TRAIL sensitivity in the resistant NB cells. On the other hand, most of the NB cell lines in our study remained TRAIL resistant even after restoration of caspase 8 expression to the same or even higher levels than that expressed in KCNR. A recent report (19) showed that IFN-γ plus TRAIL cooperated to trigger apoptosis in various resistant tumor cell lines. However, the report examined only 3 NB cell lines, and this limited study may not have detected the heterogeneity of response found in our panel of 15 cell lines. The induction of TRAIL sensitivity in this report is dependent on the dose of IFN-γ, and it is possible that IFN-γ induced apoptosis in these cells, which cannot be clarified without showing the effect of IFN-γ treatment alone.

After evaluating components of the death receptor pathway, we find that the TRAIL-resistant NB cells lack surface receptors in addition to their caspase 8 deficiency described previously. Whereas this finding is at variance with previous reports, the data presented by Hopkins-Donaldson *et al.* (11) in fact showed low surface TR expression in TRAIL-resistant NB cell lines, but its impact was underappreciated because of the caspase 8 deficiency. Our immunohistochemistry study of NB tumor specimens confirmed the lack of TRs in NB tissue. Our results indicate that TRAIL therapy in most NB would require restoration of both caspase 8 and surface receptor expression. Wu *et al.* (28) found that TR2 expression was DNA damage-inducible p53-regulated in human colon cancer cells. Many antineoplastic agents now in use are DNA-damaging agents that are known to activate the tumor suppressor protein p53, and one of the genes transcriptionally induced by p53 is *TR2* (28, 29). The administration of drugs that activate p53 in combination with TRAIL has been proposed to lead to a more efficient regression of certain cancers, because the p53-induced expression of TR2 on tumor cells may override the inhibitory effect of the antiapoptotic proteins of a tumor cell (40). In our chemoresistant NB cell SKNDZ, the survival of cells selected by chemotherapy pretreatment decreased after the combined IFN-γ/TRAIL treatment. The chemotherapy induction of TR2 was observed in 5 NB cell lines but not in BE2, in which p53 is not functional because of the loss of one allele on chromosome 17 and a missense mutation in exon 5 of the other allele (41). Because chemotherapy is the major treatment for NB and p53 is rarely mutated or silenced in primary NB, it is a relevant model for NB therapy. Although p53 mutation may occur in a relapsed NB after cytotoxic therapy (41), the combination of IFN-γ/TRAIL with cytotoxic drugs after the initial diagnosis may be preferred. Conversely, therapeutics that are not often used in NB treatment but are potentially able to induce p53 and TR2, such as irradiation, may also be used to increase TRAIL sensitivity.

Transcriptional regulation of caspase 8 has not been fully delineated. We have identified the caspase 8 promoter region and found that it responded to IFN-γ treatment *in vitro* using a functional promoter reporter assay. Furthermore, we focused on a 163-bp region that contains a GAS element, a known *cis*-element responding to IFN-γ signaling, and we found that this region is required for caspase 8 promoter activity and the response to IFN-γ. Consistent with this is the finding that IFN-γ induction of caspase 8 is blocked by overexpression of dominant-negative Stat-1 (19), because Stat-1 is known to stimulate transcription of IFN-γ-inducible genes through the GAS (42, 43). A detailed analysis of the caspase 8 promoter region is under way to identify additional factors required to activate or repress caspase 8 transcription. We find that IFN-γ does not induce caspase 8 in some NB cell lines, and this heterogeneous response to IFN-γ may not have been noted in more limited analysis (19). Consistent with our *in vitro* finding is the observation that caspase 8 was not induced in NB from some of the patients receiving IFN-γ. All of the patients in our protocol had significant increases in serum β_2-microglobulin levels after 5 days of IFN-γ treatment, indicating a general IFN-γ response, and suggesting distinct mechanisms for caspase 8 induction and MHC class I induction *in vivo*. Defects in the IFN-γ signaling pathway or repression of caspase 8 transcription may account for the failure to induce caspase 8 in some NB. Methylation of an intronic caspase 8 region in NB was found to correlate with caspase 8 silencing, and N-*myc* amplification and expression (12). Whereas we also find that the same region is methylated in most NB cell lines, the methylation status does not correlate with IFN-γ induction of caspase 8, and the IFN-γ treatment does not change the methylation status of this region.[4] 5-Aza-cytidine has been shown to induce caspase 8 in a few NB cell lines (11, 12), and Hopkins-Donaldson *et al.* (11) showed improved TRAIL sensitivity after caspase 8 was induced in NB cell line SH-SY5Y. TRs were found methylated in some NB cell lines (44), so it is possible that 5-aza-cytidine also contributes to TRAIL sensitivity induction through TR1/TR2 demethylation. The combination of IFN-γ and 5-aza-cytidine may cause a greater caspase 8 induction, and more NB cells may respond to this type of treatment, thus induction may be achieved at lower doses.

Our study indicates that there are additional lesions in the TRAIL/death receptor path aside from the silencing of caspase 8 expression and the deficiency of TRs. Cell lines such as SK-N-LE remain TRAIL resistant even after caspase 8 is induced and TR2 expressed. Other mechanisms of resistance may entail SMAC and XIAP, which were shown recently to be important in mediating TRAIL-induced apoptosis in colon cancer cells although the death receptor path was intact (45). A recent study used SMAC peptide to sensitize for TRAIL treatment in various tumor cell lines and glioma xenografts in a mouse model (46). The existence of multiple lesions in death receptor path in NB raises the possibility that this path plays an important role in tumorigenesis and/or evasion from therapies. It also indicates that the clinical application of TRAIL will require a multimodality approach.

We also found that IFN-γ induced TRAIL in NB, and this was sufficient to induce death in cells expressing ectopic TR2 via transfection. The recent finding that retinoic acid-induced TRAIL in APL accounted for the apoptosis-inducing effect of retinoic acid in the more differentiated APL cells (47) would support our model that the IFN-γ induction of TRAIL in NB cells would be an additional mechanism sensitizing the tumor to cell death. Furthermore, the usage of other IFN-γ-inducing agents should be evaluated. For example, anticancer cytokines such as interleukin 12 and interleukin 18, which induce IFN-γ in T cells (48–50), may be used to stimulate IFN-γ production by tumor-infiltrating T cells that might locally enhance the therapeutic effect of TRAIL.

In conclusion, IFN-γ transcriptionally induces caspase 8 in NB cell lines and in NB tumors in patients. NB tumor tissues lack TR1 and TR2 expression, and combination of caspase 8 induction and TR2 restoration sensitizes NB cell lines to TRAIL. As a clinically applicable strategy, the chemotherapy/IFN-γ/TRAIL combination appears able to turn a nonresponder into a responder *in vitro*. Additional studies are needed to determine the clinical feasibility of this approach.

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[4] X. Yang and C. Thiele, unpublished data.

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