Interferon- γ Sensitizes Resistant Ewing's Sarcoma Cells to Tumor Necrosis Factor Apoptosis-Inducing Ligand-Induced Apoptosis by Up-Regulation of Caspase-8 Without Altering Chemosensitivity

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Ewing's sarcoma cells are highly susceptible to apoptosis via tumor necrosis factor apoptosis-inducing ligand (TRAIL). Resistance to TRAIL has been linked to deficient expression of caspase-8 *in vitro*. Here, we report on the status of caspase-8 expression in tumors from patients with Ewing's sarcoma, the effect of interferon- γ on caspase-8 expression and apoptosis, and the role of caspase-8 for TRAIL- and chemotherapy-mediated apoptosis in Ewing's sarcoma. Using immunohistochemistry, we show that low expression of caspase-8 is seen in about 24% of tumors. Interferon- γ induces expression of caspase-8 at concentrations achievable in humans and sensitizes cells to TRAIL. Transfection of wild type but not mutant caspase-8 into caspase-8-deficient Ewing's sarcoma cells restored sensitivity to TRAIL, indicating that up-regulation of caspase-8 is sufficient to restore TRAIL sensitivity. In contrast, no role for caspase-8 in chemotherapy-induced apoptosis was identified, because 1) transfection of caspase-8 or treatment with interferon- γ did not alter the sensitivity of caspase-8-deficient cells to chemotherapeutics, 2) application of chemotherapy did not select for caspase-8-negative tumor cells *in vivo*, and 3) the caspase-8 status of tumors did not influence survival after chemotherapy-based protocols. In conclusion, our data provide a rationale

for the inclusion of interferon- γ in upcoming clinical trials with TRAIL. (*Am J Pathol* 2007, 170:1917–1930; DOI: 10.2353/ajpath.2007.060993)

Ewing's sarcoma (ES) is the second most common bone tumor in children and adolescents.¹ Although the cell of origin is not known for ES, tumors can show signs of neural differentiation and are therefore classified into the group of neuroectodermal tumors.^{2,3} Unfortunately, the prognosis for patients with metastatic or relapsed ES is poor,⁴ underlining a clear need for more effective treatment.⁵ The induction of apoptosis via activation of death receptors *in vitro* and in mice offers a new direct way of triggering apoptosis, even in cells resistant to chemotherapeutic drugs.^{6,7} For translation into the clinic, however, the safety of death ligands has to be ascertained. Although Fas ligand rapidly induces death by hepatic failure in mice,⁸ the application of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL; also called Apo2L)^{9,10} remains promising, although there is controversy about the hepatotoxicity of this death ligand.^{11–13} TRAIL induces apoptosis by crosslinking TRAIL receptor 1 (also called DR4;¹⁴) and TRAIL-receptor 2 (also called DR5/TRICK-2/KILLER^{7,15–20}). Crosslinking of the TRAIL receptor leads to a conformational change of intracellular death domains, which are then capable of binding the adaptor protein FADD. The adaptor protein then

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Table 1. Characteristics of Patients with Ewing's Sarcoma

Characteristics	n = 47	%
Age at diagnosis (median, range)	18.1	1.0 to 32.8
<10 yrs	7	15
11 to 20 yrs	23	49
>20 yrs	17	36
Sex		
Male	27	57
Female	20	43
Primary tumor site		
Extremities	16	34
Chest	10	21
Pelvis	10	21
Other	11	23
Primary tumor type		
Skeletal	40	85
Extraosseous	7	15
Extent of disease		
Localized	39	83
Metastatic	8	17
Outcome (%; confidence interval)		
10-year overall survival	60	40 to 73
10-year disease-free survival	57	40 to 69

binds to procaspase-8 and/or -10, thereby activating the caspase cascade.²¹⁻²³

We have previously shown that ES cells are highly susceptible to TRAIL-mediated apoptosis *in vitro* and that TRAIL is also able to kill ES cells in a mouse xenograft model.^{24,25} For the ultimate success of TRAIL-based therapies, overcoming TRAIL resistance, however, will be of major importance. Resistance to TRAIL-mediated apoptosis in tumor cells has been described on various levels, including the lack of TRAIL-receptor expression, overexpression of FLIP, or deficient expression of caspase-8.²⁶⁻³¹ In ES cell lines, lack of expression of caspase-8 has been found to be linked to TRAIL resistance.^{24,32} Although expression of caspase-8 protein is absent in about 20% of ES cell lines studied, its expression has not yet been systematically examined in tumors from patients with ES.²⁴ Interferon- γ (IFN- γ), which influences the expression of various pro- and anti-apoptotic genes in tumor cells, has been shown to up-regulate caspase-8 in TRAIL-resistant ES cell lines and to sensitize cells *in vitro* to TRAIL-mediated apoptosis.^{24,25,32} In addition, IFN- γ decreased the incidence of metastatic disease in the mouse xenograft model of Ewing's sarcoma, making it an attractive candidate for combination therapy with TRAIL.²⁵

In this report, we demonstrate that low expression of caspase-8 occurs in tumors from patients with Ewing's sarcoma, that IFN- γ sensitizes resistant ES cells to TRAIL

at concentrations achievable in humans, and that re-expression of caspase-8 is necessary and sufficient for sensitizing caspase-8-deficient ES cells to TRAIL but not to chemotherapeutics.

Materials and Methods

Patients and Tissue Samples

A total of 54 formalin-fixed, paraffin-embedded ES tumor specimens were available for immunohistochemical analysis. The samples were obtained during the period from 1986 to 2001 from 47 patients treated at the Pediatric Oncology Branch of the National Cancer Institute ($n = 38$) and from patients treated at the Children's Hospital of the University of Freiburg, Germany ($n = 9$). For four patients, samples were available both at initial diagnosis and at relapse; for one patient, at two subsequent relapses; and for another patient, at both initial diagnosis and two subsequent relapses. The majority of samples ($n = 52$) were derived from diagnostic biopsies at initial presentation or relapse. Two specimens were from tumors removed at surgery as part of multimodal treatment. The morphology of all tumor specimens was homogeneous and consisted of small round tumor cell aggregates in lobular or sheet-like arrangement. Rosette formation was present only in one case. Immunohistochemical staining (desmin, 12E7, and leukocyte common antigen) and/or detection of EWS/Flt-1 fusion transcripts by reverse transcriptase-polymerase chain reaction (RT-PCR) (14 cases) was performed to differentiate the tumor from other round cell tumors. Patients' clinical data were analyzed by retrospective review of medical charts and are summarized in Table 1. Patients were treated after informed consent on Institutional Review Board-approved protocols such as 86-C-169 ($n = 25$), Euro-E.W.I.N.G. 99 ($n = 9$), 98-C-0037 ($n = 6$), and others ($n = 7$), including 83-C-73, 87-C-10, and 93-C-0125. All protocols contained vincristine, doxorubicin, cyclophosphamide, ifosfamide, and etoposide with the exception of 83-C-73, which only contained vincristine, doxorubicin, and cyclophosphamide. Informed consent for immunohistochemical analysis of archived tissues was waived because the patient identities were anonymized with regard to the investigators on this study.

Cell Lines

ES cell lines A4573, JR (NCI-EWS 94), SB (NCI-EWS 95), SK-N-MC, TC32, TC71, and 5838 were selected for study

Table 2. Characteristics of ES Cell Lines Used

Cell line	Origin	Gene rearrangement	Caspase-8 protein ²⁴
A4573 ³³	ES of bone	EWS-FLI1	—
JR (NCI-EWS 94) ³⁶	ES of bone	EWS-FLI1	—
SB (NCI-EWS 95) ³⁵	ES of bone	EWS-FLI1	+
SK-N-MC ³⁴	Askin's tumor	EWS-FLI1	+
TC32 ³⁷	Peripheral neuroepithelioma	EWS-FLI1	+
TC71 ³⁴	ES of bone	EWS-FLI1	+
5838 ³³	ES of bone	EWS-ERG	+

based on their different expression of caspase-8 (Table 2).²⁴ All cell lines have been previously characterized.^{33–37} They were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and 100 U/ml penicillin, 100 μ g/ml streptomycin, and 4 mmol/L glutamine.

Reagents

Recombinant human TRAIL (rh TRAIL) and a crosslinking anti-TRAIL antibody were obtained from Alexis (Grünberg, Germany). Mouse monoclonal anti-caspase-8 antibody was purchased from Biocheck (Muenster, Germany), mouse anti- β -actin antibody was from Sigma (Muenchen, Germany), and goat anti-mouse antibody was from Santa Cruz (Heidelberg, Germany). IFN- γ and caspase inhibitor benzyloxycarbonyl-Ile-Glu-Thr-Asp(OMe)-fluoromethylketone (Z-IETD-fmk) were obtained from R&D Systems (Wiesbaden, Germany).

Immunohistochemistry

For immunohistochemical detection of caspase-8, paraffin sections of 5 μ m were deparaffinized in xylene and rehydrated in alcohol. Endogenous peroxidase activity was quenched for 10 minutes at room temperature in methanol containing 1.5% hydrogen peroxide. After washing twice with water, sections were subjected to antigen retrieval by incubation in Dako Antigen Retrieval solution, pH 6.2 (Dako Corporation, Carpinteria, CA), for 15 minutes in a microwave oven. The sections were then washed in phosphate-buffered saline (PBS) and incubated for 1 hour with a blocking solution consisting of 10% normal goat serum (Vector Laboratories, Burlingame, CA) 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. Subsequently, the sections were washed with PBS and incubated with goat anti-mouse immunoglobulins conjugated to peroxidase-labeled dextran polymer (Dako Envision⁺ Peroxidase) for 30 minutes at room temperature. The peroxidase reaction was developed with 3',3'-diaminobenzidine (Dako), and the slides were counterstained with hematoxylin. The sections were evaluated for intensity of staining and percentage of positive cells. Evaluation of tumor cell percentage was performed on the entire tumor section. Because all samples except two were derived from diagnostic biopsies consisting of small pieces of tissue, evaluation for caspase-8 staining was performed in one representative tumor section. Sections were evaluated for intensity and distribution of staining. The biopsy specimens were evaluated by a pathologist and two other investigators (M.T., M.E.R.) who examined the sections at the same time using a two-head microscope. The latter reviewers were blinded to clinical information. Equivocal cases were discussed, and a consensus score was reached. The intensity was scored as no signal, weak signal, or medium to strong signal. Distribution of staining

was evaluated on the entire tumor section using a 20 $\times$ lens. The percentage of positive cells within various fields was determined, and a mean score was calculated.

RNAse Protection Assay

Total RNA from cell lines was prepared according to the manufacturer's instruction (PqLab, Erlangen, Germany). A RiboQuant Multi-Probe RNAse Protection Assay System (Pharmingen, Hamburg, Germany) was used per manufacturer's instructions. The hAPO5c probe set containing DNA templates for caspases 1 to 10, L32, and glyceraldehyde-3-phosphatase dehydrogenase was used for T7 polymerase direct synthesis of [α ³²-P]UTP-labeled antisense RNA probes. The probes were hybridized with 5 μ g of RNA of the above-described ES cell lines. Samples were then digested with RNase to remove single-stranded (nonhybridized) RNA. Remaining probes were resolved on denaturing 5% polyacrylamide gels.

Immunoblots

Protein was extracted from cells by detergent lysis with a buffer containing 10% sodium dodecyl sulfate. The lysates were boiled for 10 minutes, and cellular debris was then removed by centrifugation (10 minutes at 14,000 rpm). The protein concentration then was measured using the Bio-Rad protein assay (Bio-Rad Laboratories, Munich, Germany). Forty micrograms of protein was boiled for 10 minutes before loading onto a 12% sodium dodecyl sulfate-polyacrylamide gel. Proteins were transferred to nitrocellulose membranes and then blocked for 30 minutes in PBS containing 5% dry milk and 0.1% Tween 20 (Sigma). Membranes were incubated with anti-caspase-8 antibody, at a 1:1000 dilution for 4 hours in blocking buffer, washed three times in PBS/Triton X-100, incubated for an additional 30 minutes with a secondary antibody conjugated to horseradish peroxidase at a 1:2000 dilution, washed three times with PBS/Triton X-100, and then developed using enhanced chemiluminescence (Amersham, Braunschweig, Germany).

Apoptosis

Apoptotic cells were determined by the propidium iodide method.³⁸ In brief, ES cells were plated overnight in a 24-well plate (Falcon Microtest; Becton Dickinson, Heidelberg, Germany) at a concentration of 5×10^4 cells/well in 10% fetal calf serum. The following morning, IFN- γ was added where indicated. After indicated time frames, rh TRAIL was added at a final concentration of 200 ng/ml, together with a crosslinking anti-TRAIL-antibody at a final concentration of 2 μ g/ml or chemotherapeutics doxorubicin or etoposide at various concentrations. Twenty-four hours after the addition of TRAIL and 48 hours after the addition of doxorubicin and etoposide, cells in suspension and adherent cells were collected in 12- $\times$ 75-mm Falcon Polystyrene tubes and centrifuged at 200 $\times$ g. The cell pellet was resuspended in 400 μ l of a hypotonic buffer (50 μ g/ml propidium iodide and 0.1% sodium ci-

trate plus 0.1% Triton X-100) and placed at 4°C in the dark overnight. Flow cytometric analysis was performed using a FACScan analyzer (Becton Dickinson). The propidium iodide fluorescence of individual nuclei was measured in the red fluorescence, and the data were registered in a logarithmic scale. At least 10^4 cells of each sample were analyzed.

Transfection Studies

Cells were plated in triplicate in flat-bottom wells at 1×10^5 cells/well in a 24-well microtiter plate. After an overnight incubation, cells were transiently cotransfected with 1 μ g of the empty vector pcDNA3, the pcDNA3-wild-type (wt)-caspase-8 vector, or the pcDNA3-mutant (mut)-caspase-8 vector together with 0.8 μ g of pEGFP (Clontech Laboratories, Heidelberg, Germany), using Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany). The wt caspase-8 vector was kindly provided by V. Castle (University of Michigan, Ann Arbor, MI); and the mut caspase-8 vector, by V. Kidd (St. Jude Children's Research Hospital, Memphis, TN). Twenty-four hours after transfections, cells were treated with rh TRAIL (100 ng/ml) or with various concentrations of doxorubicin. Where indicated, the caspase-8-selective inhibitor Z-IETD-fmk was added at the time of transfection. For immunofluorescence, cells were analyzed with the Axiovert 200 fluorescence microscope (Carl Zeiss, Jena, Germany) 14 hours after the addition of TRAIL. The percentage of transfected cells was determined by flow cytometry, 36 hours after the addition of TRAIL, using a BD FACScan and the CellQuest software. Here, 10,000 cells per sample were analyzed for the fluorescent intensity of enhanced green fluorescent protein (EGFP) in the FL-1 channel. Cells were counted as transfected if their fluorescence intensity was above a cutoff fluorescent intensity level, which was defined as being the 99.9% percentile value of the fluorescent signal of nontransfected cells. Transfection rates for the empty vector plasmid (control) were around 10% for cell line JR and 20% for cell line A4573. Relative transfection rates were calculated as the ratio of the percentage of transfected cells of a given sample to the percentage of transfected cells of untreated controls. Experiments were done in triplicates. For immunoblotting of caspase-8, protein extracts were obtained 24 hours after the addition of TRAIL. Cells were cotransfected as described above with pEGFP and either the empty vector pcDNA3 or the pcDNA3-wt-caspase-8 plasmid, both of which contain a neomycin resistance gene, and stable transfectants were obtained by selection in geneticin (final concentration of 1 mg/ml).

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Assay

Cells were plated in quintuplicates in flat bottom wells at 30,000 cells per well in 96-well microtiter plates and allowed to attach to the plate. The following day, rh TRAIL was added at a final concentration of 200 ng/ml, together with a crosslinking anti-TRAIL-antibody at a final concen-

tration of 2 μ g/ml. Eighteen hours later, cell viability of stably transfected cells was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium dye reduction assay as described previously.

Statistical Analysis

Overall survival was defined as the time from date of diagnosis to death or last follow-up. The life-table calculations were performed according to the Kaplan-Meier method, and comparisons between probabilities in different groups were performed using the log-rank test. χ^2 test was used to compare differences in percentages for groups. Because normal distribution cannot be assumed, median values and ranges were reported, and nonparametric statistics were used to test differences in continuous variables (Kruskal-Wallis rank test with adjacent post hoc Mann-Whitney *U*-test). All *P* values were two-sided, and values less than 0.05 were considered statistically significant. *P* values greater than 0.1 were reported as nonsignificant, whereas those between 0.05 and 0.1 were reported in detail. Statistical analysis was performed using SPSS for Windows 14.0.1 (SPSS Inc., Chicago, IL).

Results

Expression of Caspase-8 in Tumors from Patients with Ewing's Sarcoma

We have previously shown that ES cell lines with low expression of caspase-8 are resistant to TRAIL-induced apoptosis and that incubation with IFN- γ could sensitize them to apoptosis via TRAIL.²⁴ To examine whether low expression of caspase-8 was a phenomenon restricted to cell lines or whether it was also encountered in ES tumors, we examined ES tumor samples for caspase-8 expression by immunohistochemistry. Of the 54 specimens examined, 40 were derived from diagnostic biopsies of patients who had not previously received chemotherapy. The 14 remaining samples were obtained from patients with Ewing's sarcoma who had been treated with chemotherapy. Of these specimens, eight had been from patients who were receiving chemotherapy for a period between 1 and 16 months. The other six specimens were taken from diagnostic biopsies at relapse in patients who had been off chemotherapy between 3 months and 4 years. Caspase-8 was expressed in 50 of 54 tumor specimen analyzed; 23 (43%) tumor samples exhibited weak staining, and 27 (50%) exhibited medium to strong staining, as shown in a representative pair of tumors in Figure 1, a and b. Interestingly, within tumor samples, there was remarkable heterogeneity with respect to the percentage of caspase-8-positive cells (Figure 1, a and b). In 41 of 54 (76%) tumors examined, caspase-8 expression could be detected in 60 to 100% of tumor cells, whereas in the remaining 13 tumor specimens, caspase-8 was expressed in 0 to 50% of cells (referred to as low expression) (Figure 1c). This suggests that a substantial proportion of cells that compose Ewing's tumors will be primarily resistant toward apoptosis via TRAIL because of absent

Figure 1. Heterogeneity of caspase-8 expression in ES tumors. **a:** Strong expression of caspase-8 in >70% of cells of an ES tumor. **b:** Weak expression of caspase-8 in approximately 40% of tumor cells of an ES tumor. **c:** Distribution of percentage of caspase-8-positive tumor cells in 54 ES tumor specimens.

or low expression of caspase-8. Up-regulation of caspase-8 therefore could be a means of restoring sensitivity to TRAIL.

Up-Regulation of Caspase-8 by IFN- γ

Because IFN- γ has been shown to up-regulate caspase-8 expression *in vitro* in various cell line systems and because we have previously demonstrated that IFN- γ could sensitize ES cell lines with low expression of caspase-8 against apoptosis via TRAIL,^{24,39-42} we investigated the regulation of caspase-8 in two TRAIL-resistant, caspase-8-deficient ES cell lines by IFN- γ . Using an RNase multitemplate protection assay for human caspases 1 to 10, we demonstrate up-regulation of caspase-8 mRNA in both TRAIL-resistant and caspase-8-deficient cell lines (Figure 2a). In addition, *de novo* induction of caspase-1 could be noted in the two cell lines. Expression of caspase-10, which can substitute for caspase-8 as an initiator caspase in the TRAIL signaling pathway, could not be detected, nor was it induced by treatment with IFN- γ . By immunoblot, we were able to demonstrate that IFN- γ increases caspase-8 expression starting at a dose of 20 U/ml (Figure 2b). This is of importance, because in humans, serum levels of IFN- γ up to 80 U/ml are readily achieved and tolerated.⁴³ For induction of caspase-8 expression, 12 to 18 hours of preincubation were required (Figure 2c).

Sensitization of Caspase-8-Deficient ES Cell Lines to TRAIL-Mediated Apoptosis by IFN- γ

To show whether the dosage of IFN- γ needed to up-regulate caspase-8 expression could also sensitize caspase-8-deficient ES cell lines to apoptosis by TRAIL, cells of the caspase-8-deficient and TRAIL-resistant ES cell lines A4573 and JR were incubated

with increasing dosages of IFN- γ for 48 hours and then treated with rh TRAIL. Apoptosis of cells was determined by flow cytometry 24 hours later. As shown in Figure 3a, IFN- γ sensitized cells to TRAIL-mediated apoptosis starting at concentrations of 10 U/ml, the same range required for induction of caspase-8. Whereas up-regulation of caspase-8 was observed already after 12 to 18 hours of incubation with IFN- γ , sensitization to TRAIL-mediated apoptosis only started at 24 hours (Figure 3b), suggesting either that the amount of caspase-8 protein synthesized at earlier time points was not sufficient or that additional proapoptotic proteins regulated by IFN- γ could contribute to the TRAIL-sensitizing effect.

Transfection of Caspase-8 Is Sufficient for Restoring Sensitivity to TRAIL in Caspase-8-Deficient ES Cell Lines

Because IFN- γ has been shown in different cell systems to modulate the expression of a variety of apoptosis-relevant genes,^{39,42,44} we sought to determine whether induction of caspase-8 expression alone was sufficient to restore sensitivity to apoptosis by TRAIL or whether other IFN- γ -regulated genes in addition to caspase-8 were required. To answer this question, caspase-8 was transiently cotransfected with an EGFP-carrying plasmid into caspase-8-deficient ES cell lines. Twenty-four hours after transfection, cells were treated with rh TRAIL and then analyzed for intensity of green fluorescence as a marker for cell viability. Transfection of both cell lines with wt caspase-8 sensitized cells to apoptosis via TRAIL, whereas no effect on TRAIL-mediated apoptosis was seen when cells were transfected either with vector or a mut caspase-8 containing plasmid (Figure 4, a and b). In cell line A4573, the percentage of cells transfected with wt caspase-8 was lower than that of cells transfected with vector or mut caspase-8, probably reflecting increased

Figure 2. Induction of caspases by IFN- γ in TRAIL-resistant and caspase-8-deficient ES cell lines. **a:** RNase protection assay for caspases 1 through 10. The marker lane represents RNA probes before hybridization with RNA. House-keeping genes L32 and glyceraldehyde-3-phosphatase dehydrogenase serve as normalized controls. HeLa cells were included as a control. In both ES cell lines, A4573 and JR, mRNA expression of caspase-1 and -8 is induced after 1 day of treatment with 2000 U/ml IFN- γ . In addition, expression of caspase-4 and -7 is increased in both cell lines. **b:** Induction of caspase-8 protein expression at low concentrations of IFN- γ . Caspase-8 expression is induced at concentrations as low as 20 U/ml in both cell lines, as shown by immunoblot. **c:** Time course of induction of caspase-8 protein expression. IFN- γ induces caspase-8 expression after 12 to 18 hours of incubation in both caspase-8-deficient ES cell lines, as demonstrated by immunoblot. β -Actin is included as a loading control.

spontaneous apoptosis due to the introduction of caspase-8. This is supported by the observation that addition of the caspase-8-selective inhibitor Z-IETD-fmk blocked not only TRAIL-mediated apoptosis but also spontaneous apoptosis in these cells. In cells transfected with wt caspase-8, cleavage into the active caspase (p19) was observed, whereas in cells transfected with mut caspase-8, only procaspase-8 (p57/55) and the product of the first activation cut (p45/43) were noted (Figure 4c). Sensitization to TRAIL-mediated apoptosis was also noted in cells stably transfected with caspase-8 (Figure 4d). Thus, up-regulation of caspase-8 is necessary and sufficient to restore sensitivity to TRAIL in the TRAIL-resistant ES cell lines examined. Although other effects of IFN- γ on the intracellular apoptotic pathway may also augment apoptosis in response to TRAIL, these

results demonstrate that the effects of IFN- γ on caspase-8 are necessary to restore sensitivity of ES cells to TRAIL-mediated death.

Treatment with Chemotherapy Does Not Select for Caspase-8-Negative Ewing's Sarcoma Tumors in Vivo

For the timing of any future therapy with TRAIL in ES, it will be of importance to know whether application of chemotherapy alters the degree of caspase-8 expression in tumors. The significance of caspase-8 in chemotherapy-induced apoptosis has been controversial. Activation of caspase-8 has been found in various cell systems to be required for apoptosis induced by chemotherapeutics,

Figure 3. Sensitization of caspase-8-deficient ES cell lines to TRAIL-mediated apoptosis by IFN- γ . **a:** Dose response. Cells were incubated with increasing concentrations of IFN- γ for 48 hours and then treated with 200 ng/ml rh TRAIL. Apoptosis was measured by flow cytometry of propidium iodide-stained nuclei. IFN- γ sensitizes TRAIL-resistant ES cell lines to TRAIL-mediated apoptosis at similar concentrations needed for the induction of caspase-8 expression, starting at 10 U/ml IFN- γ . IFN- γ itself induces some apoptosis in both cell lines. The means of three replicates are indicated; bars = SD. Similar results were obtained in three different experiments. **b:** Time course. Cells were preincubated with 1000 U/ml IFN- γ for the times indicated and then treated with 200 ng/ml rh TRAIL for an additional 24 hours. In both cell lines, sensitization to apoptosis by TRAIL is seen when cells are preincubated for 24 to 48 hours.

especially doxorubicin.⁴⁵ However, in most cells, chemotherapy induces apoptosis via the activation of the intrinsic pathway, with caspase-9 as initiator caspase.⁴⁶ Here, activation of caspase-8 can occur downstream of the activation of effector caspase-3, -6, and -7 and is not necessarily required for the induction of apoptosis.⁴⁷ If expression of caspase-8 was necessary for chemotherapy-induced apoptosis in ES, treatment of tumors with chemotherapy should select for cells not expressing caspase-8, rendering therapy with TRAIL less effective. We therefore compared the percentage of caspase-8-positive cells between tumor samples that were obtained before patients had received any chemotherapy ($n = 14$) and those taken while patients were on or after chemotherapy ($n = 37$). No significant difference was seen between the percentages of caspase-8-expressing cells in the two groups (Table 3). In fact, in tumor samples obtained from patients after chemotherapy, only one sample had less than 60% of tumor cells expressing caspase-8, underlining a trend toward higher expression of caspase-8 in tumors that were treated with chemotherapy. In four patients, tumor specimens were available from both the initial diagnostic biopsy and the biopsy at relapse. Again, no differences in the percentages of caspase-8-expressing cells were observed between

these pre- and postchemotherapy specimens (data not shown). Thus, chemotherapy for Ewing's sarcoma does not appear to select for caspase-8-negative tumor cells, suggesting that the expression of caspase-8 was not influencing chemosensitivity.

Expression of Caspase-8 in ES Does Not Affect Survival

Because response to chemotherapy has been one of the most important factors linked to survival in ES,⁴⁶ the status of caspase-8 expression could be of prognostic value if caspase-8 deficiency was associated with chemoresistance. We therefore retrospectively analyzed the overall and disease-free survival of the 40 ES patients of whom caspase-8 expression of tumors was known before chemotherapy. No significant difference in a Kaplan-Meier analysis for overall and disease-free survival was observed between patients whose tumors had a low percentage of caspase-8-positive tumor cells and patients whose tumors expressed more than 50% caspase-8-positive tumor cells (Figure 5, a and b), suggesting that the expression of caspase-8 in tumor cells was not required for sensitivity to ES-like chemotherapy. No differ-

Figure 4. Transfection of caspase-8 is sufficient to restore sensitivity to TRAIL-mediated apoptosis. Cells of caspase-8-deficient and TRAIL-resistant ES cell lines A4573 and JR were transiently cotransfected with EGFP and empty vector, mut caspase-8, or wt caspase-8 using Lipofectamine. Where indicated, 50 μ mol/L caspase-8-selective inhibitor Z-IETD-fmk was added at the time of transfection. Twenty-four hours after transfection, cells were incubated with rh TRAIL at a final concentration of 100 ng/ml and analyzed as follows. **a:** Immunofluorescent microscopy of A4573 cells, 14 hours after the addition of TRAIL. Cells transfected with mut caspase-8 are attached to the bottom and polygonally shaped, whereas cells transfected with wt caspase-8 and treated with TRAIL show signs of cell death, being small, round, and detached from the bottom. **b:** Flow cytometric analysis of transfection rates. Transfection rates were determined by measuring the intensity of EGFP 36 hours after the addition of TRAIL. Relative transfection rates were calculated as the ratio of the percentage of transfected cells of a given sample to the percentage of transfected cells of untreated controls. Higher percentage of transfected cells in samples transfected with empty vector or mut caspase-8 compared with cells transfected with wt caspase-8 suggests spontaneous apoptosis involving wt caspase-8. Reduction of percentage of transfected cells after treatment with TRAIL in samples transfected with wt caspase-8 but not with empty vector or mut caspase-8 reflects loss of cells transfected with wt caspase-8 by TRAIL-mediated apoptosis; the addition of the caspase-8-selective inhibitor Z-IETD-fmk blocks TRAIL-mediated as well as spontaneous apoptosis in cells transfected with wt caspase-8. The means of three replicates are indicated; bars = SD. **c:** Expression and cleavage of caspase-8 by immunoblot 24 hours after the addition of TRAIL. Expression of caspase-8 is induced in cells transfected with either wt caspase-8 or mut caspase-8 but not empty vector. Treatment of transfected cells with TRAIL leads to cleavage of mut caspase-8 into fragment p45/p43 but not the p19 form, which reflects active caspase-8. In contrast, treatment of cells transfected with wt caspase-8 leads to the generation of p45/p43 and p19 forms. **d:** A4573 cells stably transfected with wt caspase-8 are sensitized to TRAIL-mediated apoptosis. Cells stably transfected with either wt caspase-8 or the vector plasmid were incubated with rh TRAIL at a final concentration of 200 ng/ml for 18 hours. Cell survival was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium reduction assay. Means of quintuplicates are indicated; bars = SD. Expression of caspase-8 by cells stably transfected with wt caspase-8 is demonstrated by immunoblot.

Table 3. Expression of Caspase-8 in Tumor Specimens According to Clinical Characteristics

Variable	Caspase-8-positive tumors*		P value
	n	%	
Chemotherapy before biopsy (n = 54)			
No (n = 40)	28	70.0	0.09
Yes (n = 14)	13	92.9	
Age (n = 54)			
≤15 years (n = 23)	17	73.9	n.s.
>15 years (n = 31)	24	77.4	
Metastases at diagnosis (n = 54)			
Yes (n = 12)	11	91.7	n.s.
No (n = 42)	30	71.4	
Tumor site (n = 54)			
Extremities (n = 20)	14	70.0	n.s.
Chest (n = 14)	10	71.4	
Pelvis (n = 10)	9	90.0	
Other (n = 10)	8	80.0	

*Caspase-8-positive tumors: >50% tumor cells stain positive for caspase-8; n.s., nonsignificant.

ences in the expression of caspase-8 within subgroups reflecting known prognostic factors for Ewing's sarcoma such as age, the presence of metastases, and tumor location were identified.

Figure 5. Kaplan-Meier estimate of disease-free (a) and overall (b) survival according to expression of caspase-8 in 40 patients who had not been previously treated with chemotherapy.

Figure 6. Expression of caspase-8 does not correlate with sensitivity to doxorubicin-induced apoptosis in ES cell lines. Cells from seven different ES cell lines with known caspase-8-protein expression were incubated with doxorubicin at a concentration of 0.5 $\mu\text{g/ml}$ for 48 hours. Apoptosis was measured by flow cytometry of propidium iodide-stained nuclei. No correlation between caspase-8 expression and sensitivity to doxorubicin is seen.

Expression of Caspase-8 Does Not Correlate with Chemosensitivity in ES Cell Lines

To analyze further the contribution of caspase-8 to chemosensitivity *in vitro*, we first examined the chemosensitivity of a panel of seven ES cell lines with different caspase-8 status toward doxorubicin which has known efficacy against ES and is considered as part of standard therapy in current ES protocols.⁴⁷ In addition, doxorubicin has been shown to induce apoptosis in various cell systems via the extrinsic pathway.⁴³ Cells were incubated with doxorubicin for 48 hours, and apoptosis was measured by flow cytometry. No association of sensitivity to doxorubicin and caspase-8 status was seen (Figure 6). In fact, the caspase-8-deficient cell line A4573 was the second most sensitive cell line to induction of apoptosis via doxorubicin. No induction of caspase-8 or -10 expression by doxorubicin was observed (data not shown). Therefore, in ES cell lines, expression of caspase-8 is not a requirement for chemosensitivity to doxorubicin.

Transfection of Caspase-8 Does Not Modulate Sensitivity to Doxorubicin in Caspase-8-Deficient ES Cell Lines

To determine whether caspase-8 might modulate doxorubicin-mediated apoptosis, caspase-8-deficient ES cell lines were transfected with either wt caspase-8 or vector using transient transfection as above. Transfection of cells with wt caspase-8 did not alter chemosensitivity of either cell line to various concentrations of doxorubicin, including concentrations achieved therapeutically in humans (up to 0.02 $\mu\text{g/ml}^{21}$) (Figure 7). Therefore, caspase-8 is not involved in doxorubicin-mediated apoptosis in ES. Similar results were obtained when transfected cells were treated with etoposide (data not shown).

Figure 7. Transient transfection of caspase-8 into caspase-8-deficient ES cells does not alter chemosensitivity to doxorubicin. Cells of caspase-8-deficient and TRAIL-resistant ES cell lines A4573 and JR were transiently cotransfected with EGFP and either vector or wt caspase-8 using Lipofectamine. Twenty-four hours after transfection, cells were incubated with doxorubicin at the indicated concentrations for 36 hours. Relative transfection rate was determined by fluorescence-activated cell sorting analysis. There is no increasing difference in the transfection rates with higher concentrations of doxorubicin between samples containing cells transfected with vector and samples containing cells with wt caspase-8 excluding a sensitizing effect of caspase-8 toward doxorubicin-mediated apoptosis. Lower transfection rates of samples containing cells transfected with wt caspase-8 compared with samples containing cells transfected with vector are seen and probably reflect the induction of apoptosis by autocatalytic activity of caspase-8 in some of the cells. The means of three replicates are indicated; bars = SD.

IFN- γ Does Not Alter Sensitivity to Chemotherapy-Induced Apoptosis in ES Cell Lines

Because IFN- γ could be of potential clinical use in sensitizing caspase-8-deficient ES cells to apoptosis via TRAIL, we were interested in the effects of IFN- γ on the induction of apoptosis by chemotherapeutics used in ES. Whereas IFN- γ modulates the expression of a variety of apoptosis-relevant genes in a proapoptotic fashion, it also acts as an antiproliferative agent, thereby decreasing the target population of cell cycle-specific drugs that act through S to M phase.⁴⁸ ES cells were incubated with IFN- γ for 24 hours and then exposed for a further 48 hours to various concentrations of doxorubicin and etoposide. Apoptosis was measured by flow cytometry. IFN- γ had no significant effect on the sensitivity of the cell lines to the two drugs (Figure 8a). A small increase in apoptosis was seen at some concentrations; however, it was not higher than the sum of apoptosis by the chemotherapeutic and IFN- γ . In addition, no major change in the rate of apoptosis was seen when cells were incubated with different concentrations of IFN- γ before adding the chemotherapeutic drug (Figure 8b). This suggests that IFN- γ does not interfere with the apoptotic effect of chemotherapeutics such as doxorubicin and etoposide in ES and that the combination of TRAIL and IFN- γ with standard chemotherapeutics in ES could be feasible.

Discussion

The death ligand TRAIL is considered a promising agent for cancer therapy, because of its selectivity for transformed cells.⁴⁹ In ES, the majority of cell lines studied are sensitive to TRAIL,^{24,50–52} and TRAIL induces tumor regression in a mouse xenograft model of ES.²⁵ Resistance to TRAIL-induced apoptosis, however, has been de-

scribed and has been linked in many cases to deficient expression of caspase-8.^{27–30} The development of approaches to overcome TRAIL resistance is likely to emerge as a central issue for optimizing TRAIL receptor agonist therapies. In this report, we demonstrate that caspase-8 deficiency is not restricted to ES cell lines but is also encountered in tumors from patients with ES and that IFN- γ up-regulates caspase-8 and sensitizes cells to TRAIL at concentrations achievable in humans. In addition, we show that up-regulation of caspase-8 is necessary and sufficient to restore TRAIL sensitivity in ES cells but does not alter the sensitivity of caspase-8-deficient cells to chemotherapeutics.

Absent or low expression of caspase-8 protein has been described in cell lines derived from neuroectodermal tumors such as neuroblastoma, medulloblastoma, and ES in up to 73, 50, and 20%, respectively.^{32,53,54} In neuroectodermal tumor specimens, caspase-8 protein expression has only been systematically studied in neuroblastoma and medulloblastoma. Absent or low expression of caspase-8 protein has been detected in up to 60% of neuroblastomas and in 40 to 80% of medulloblastoma.^{32,53,55} In ES, the caspase-8 status of tumors has been characterized by investigation of the methylation status of the caspase-8 gene, because hypermethylation of intron 2 leads to silencing of the caspase-8 gene.²⁹ Results, however, are discrepant, with hypermethylation seen in 13 of 20 tumors in one study and 0 of five tumors in another study.^{32,53} Using immunohistochemistry, we could demonstrate not only differences in the degree of caspase-8 expression between tumor samples but also marked heterogeneity of expression within one tumor. In 24% of tumor samples, caspase-8 expression was confined to 50% or fewer of tumor cells. Because low expression of caspase-8 in ES has been associated with TRAIL resistance, treatment of such tumors with TRAIL would be predicted to lead to the emergence of TRAIL resistance.

Up-regulation of caspase-8 by IFN- γ has been described in various cell systems.^{40,42,44} IFN- γ is approved

Figure 8. IFN- γ treatment does not alter the sensitivity of caspase-8-deficient ES cell lines to apoptosis induced by doxorubicin and etoposide. **a:** Cells were treated with IFN- γ at 1000 U/ml for 24 hours; then different concentrations of doxorubicin or etoposide were added for a further 48 hours. Apoptosis was measured by flow cytometry of propidium iodide-stained nuclei. **b:** Cells were incubated with different concentrations of IFN- γ for 24 hours; then doxorubicin or etoposide was added at the indicated concentration for a further 48 hours. The means of three replicates are indicated; bars = SD.

by the FDA for the treatment of patients with chronic granulomatous disease and severe osteopetrosis.⁵⁶ Pharmacokinetic studies have shown that mean serum concentrations of up to 83 U/ml IFN- γ can be tolerated in humans.⁴³ We could demonstrate that concentrations as low as 20 U/ml IFN- γ induced expression of caspase-8 and sensitized caspase-8-deficient ES cells to TRAIL-mediated apoptosis. This suggests that clinically achievable therapies with IFN- γ could play a potential role in sensitizing caspase-8-deficient ES tumor cells *in vivo* to TRAIL-mediated apoptosis.

The sensitization of ES cells to TRAIL was not only associated with the induction of expression of caspase-8 but also with increased expression of caspase-4 and -7 and induction of expression of caspase-1. Therefore, we could not exclude the possibility that changes in addition to the induction of caspase-8 expression were necessary for restoring TRAIL sensitivity. Transfection of caspase-8 into both caspase-8-deficient ES cells, however, showed that expression of caspase-8 alone was sufficient for restoring TRAIL sensitivity. In addition, a higher rate of spontaneous apoptosis could be observed in untreated cells, suggesting that the introduction of caspase-8 at nonphysiological levels into these cells led to autocatalytic activation of the caspase system.

Most chemotherapeutics induce apoptosis in tumor cells via the activation of the intrinsic pathway.⁴⁶ Here, the release of cytochrome c from mitochondria together with APAF1 leads to the activation of the initiator caspase-9, which in turn activates effector caspases, such as caspase-3, -6, and -7, which promote apoptosis. Activation of caspase-8 can also be observed in the intrinsic pathway as a result of cleavage by activated caspase-3 and -6. Although such caspase-8 activation may serve to amplify apoptotic signals, it is not necessarily required for drug-induced apoptosis.⁴⁷ In some cell systems, drug-induced apoptosis is thought to be mediated by activation of the CD95/CD95L-system, for which caspase-8 is required as an initiator caspase.⁴⁵ This, however, is controversial. Whereas up-regulation of caspase-8 by IFN- γ was required for sensitization of tumor cells from various neuroectodermal cell lines to doxorubicin by one group, activation of caspase-8 did not prove to be necessary for drug-induced apoptosis in neuroblastoma and glioma cells as shown by other groups.^{40,57,58} In our experiments, the expression of caspase-8 in caspase-8-deficient ES cell lines did not alter their sensitivity to the chemotherapeutics doxorubicin and etoposide. In addition, there was no correlation between the expression of caspase-8 in ES cell lines and their sensitivity to doxorubicin. These results are supported by our observation that application of chemotherapy did not increase the percentage of caspase-8-negative tumor cells and that the caspase-8 expression does not influence survival or disease-free survival in our retrospective analysis. This supports the hypothesis that expression and activation of caspase-8 is not required for apoptosis induced by chemotherapeutics in ES.

Today, most chemosensitive tumors are treated with a combination of agents with different mechanisms of action to prevent the evolution of resistance. In Ewing's

sarcoma, the most effective chemotherapeutic regimens consist of blocks of different combinations of vincristine, actinomycin D, doxorubicin, cyclophosphamide, ifosfamide, and etoposide.^{5,59,60} Almost all patients of this study received these agents. Unfortunately, there has not been any major improvement in survival using different schedules or dosages of these drugs in the past 2 decades. If TRAIL proves to be effective in the treatment of Ewing's sarcoma in phase II studies, its integration into regimens with known efficacy against ES would be warranted. Preliminary results from phase I trials suggest that TRAIL does not have major hematopoietic toxicity.⁶¹ If these results hold, TRAIL could be administered between cycles of conventional chemotherapy, with the advantage of increasing treatment intensity and using a different mode of action other than cytotoxic therapeutics. Because we show that low expression of caspase-8 is observed in tumors from patients with ES, IFN- γ could be included in future protocols. Because IFN- γ slows down cell proliferation and most chemotherapeutics act on dividing cells,⁴⁸ we investigated the effect of IFN- γ on the apoptotic effect of doxorubicin and etoposide, which predominantly affect cells in the G2 phase of the cell cycle. No inhibition of chemotherapy-induced apoptosis was noted. This is in accordance with observations in breast cancer cells where IFN- γ also did not alter the sensitivity of cells to doxorubicin.⁴² Further potential benefit for the role of IFN- γ in the treatment of ES comes from our observation in the mouse xenograft model, where IFN- γ reduces the incidence of lung metastases.²⁵ Recently, loss of caspase-8 has been shown to promote the formation of metastases in a neuroblastoma mouse model by rendering tumor cells refractory to integrin-mediated cell death.⁶¹ This suggests that up-regulation of caspase-8 by IFN- γ could play a role in preventing the formation of metastases in our ES mouse xenograft model. Further studies to test this hypothesis will be conducted.

In conclusion, our results demonstrate that caspase-8 deficiency occurs in tumors from patients with ES and that IFN- γ sensitizes TRAIL-resistant caspase-8-deficient ES cells to TRAIL-induced apoptosis by up-regulation of caspase-8 at concentrations achievable in humans without affecting chemosensitivity. Therefore, combined application of TRAIL with IFN- γ in upcoming phase II studies of TRAIL in ES would be warranted. Furthermore, because IFN- γ does not affect sensitivity of ES cells to chemotherapeutics, integration of TRAIL and IFN- γ into therapy protocols with current chemotherapeutics used in ES would appear to be a promising approach.

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