Loss of Caspase-8 Protein Expression Correlates with Unfavorable Survival Outcome in Childhood Medulloblastoma

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ABSTRACT

Purpose: **Escaping apoptosis is a hallmark of cancer. In medulloblastoma (MB) cell lines, resistance to tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis was recently shown to correlate with loss of caspase-8 mRNA expression, because of aberrant gene methylation (M. A. Grotzer *et al.*, Oncogene, *19:* 4604–4610, 2000). Loss of caspase-8 mRNA expression has been demonstrated in a subset of primary MB (T. J. Zuzak *et al.*, Eur. J. Cancer, *38:* 83–91, 2002). In this study, we analyzed primary MB samples to test whether loss of caspases correlates with survival outcome.**

Experimental Design: **We used immunohistochemistry to analyze the protein expression of the key initiator caspase-8 and caspase-9 in paraffin-embedded tumor samples from 77 well characterized MB patients and compared the expression levels of caspase-8 and caspase-9 with apoptosis indices, clinical variables, and survival outcomes.**

Results: **Weak expression of caspase-8 and caspase-9 was found in 16 and 24% of the MB samples evaluated, respectively. Weak expression of caspase-8 was an independent significant prognostic factor for unfavorable progression-free survival outcome and was more predictive than standard clinical factors. In contrast, caspase-9 expression was not a prognostic factor. Treatment of caspase-8-deficient MB cells with IFN-γ resulted in dose-dependent restoration of caspase-8 mRNA and protein expression and restoration of tumor necrosis factor-related apoptosis-inducing ligand sensitivity.**

Conclusions: **Loss of initiator caspase-8 is associated with an unfavorable survival outcome. Restoration of caspase-8 (*e.g.*, by treatment with IFN-γ) might, therefore, represent a novel experimental therapy in childhood MB.**

INTRODUCTION

Medulloblastomas (MBs) are the most common malignant central nervous system tumors in childhood and constitute >20% of all pediatric brain tumors (1). MBs are characterized by their aggressive clinical behavior and high risk of leptomeningeal dissemination. Most metastatic and recurrent childhood MBs are resistant to current therapeutic approaches, including high-dose chemotherapy with autologous hematopoietic stem cell rescue (2–4). To identify novel therapeutic targets, a better understanding of the pathogenesis of these tumors remains a major goal.

The ability of tumor cell populations to increase in number is determined not only by the rate of cell proliferation but also by the rate of cell death. Programmed cell death, apoptosis, is a major source of cell death. The apoptotic program is present in latent form in virtually every cell type of the body. Once triggered by a variety of physiological signals, this program unfolds in a precisely choreographed series of steps (5, 6). The apoptotic machinery can be broadly divided into sensors and effectors. The sensors are responsible for monitoring the extracellular and intracellular environment for conditions of normality or abnormality that influence whether a cell should live or die. Transmembrane sensors include cell surface receptors that bind death factors. Examples of these ligand/receptor pairs include the Fas ligand (CD95) binding the Fas receptor, tumor necrosis factor α-binding tumor necrosis factor receptor 1, and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) binding DR4 and DR5 (7–9). Intracellular sensors activate the death pathway when abnormalities are detected, such as DNA damage, signaling imbalance provoked by oncogene action, survival factor insufficiency, or hypoxia (10). Many of the signals that elicit apoptosis converge on the mitochondria, which respond to proapoptotic signals by releasing cytochrome *c*, a potent catalyst of apoptosis (11).

The ultimate effectors of apoptosis include an array of intracellular proteinases termed "caspases" (12). In apoptosis, caspases function in both cell disassembly (effectors) and in initiating this disassembly in response to proapoptotic signals (initiators). Caspase-9, a key initiator caspase, is activated when cytochrome *c* is released from mitochondria and binds to its adapter molecule Apaf-1 (9, 13). In contrast, caspase-8 is activated after ligation of death receptors such as the Fas receptor and the TRAIL receptors DR4/DR5 (14). Recruitment of caspase-8 leads to its proteolytic activation, which initiates a cascade of downstream effector caspases (including 3, 6, and 7; Ref. 15). In addition, caspase-8 also acts as an effector caspase in an "amplification loop" with caspase-9 (16).

Resistance to apoptosis can be acquired by cancer cells through a variety of means, including down-regulation of death

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receptors, presence of decoy receptors, and loss of downstream death-signaling pathway elements (17–19). In MB cell lines, resistance toward TRAIL-induced apoptosis was shown recently to correlate with loss of caspase-8 mRNA expression attributable to gene silencing by aberrant methylation (20, 21). In a pilot study of 26 tumors, we showed that caspase-8 mRNA is also lost in a subset of primary MB (21).

To characterize further the caspase pathways in primary MB, we used immunohistochemistry to analyze protein expression of caspase-8 and caspase-9 in formalin-fixed, paraffin-embedded tumor samples from 77 well characterized and relatively uniformly treated MB patients and compared the expression levels of caspase-8 and caspase-9 with apoptosis indices, clinical variables, and survival outcomes.

PATIENTS AND METHODS

Patients and Therapy. We studied MB samples from 77 children diagnosed at the Children's Hospital of Philadelphia between January 1981 and December 1998. The tumors were selected for the study based on the availability of a sufficient amount of tumor tissue to perform caspase-8 and caspase-9 immunostaining. All diagnoses were confirmed by histological assessment of the tumor specimen obtained at surgery by one neuropathologist (L. B. R.). Information available on the 77 patients included date of birth, date of diagnosis, extent of resection determined by operative reports (gross total resection, ≥90% removal of tumor; partial resection, ≥50% but <90% removal of tumor; biopsy, <50% removal of tumor), metastatic stage according to Chang *et al.* (22), therapy, follow-up, and survival outcomes. These data were obtained retrospectively from tumor registries, surgical reports, and clinical records. Outcome was characterized as survival and progression-free survival. The median age at diagnosis for all patients was 6.4 years (range, 0.3–21.8). The median follow-up period for the patients who remain alive and progression free at the time of this report was 8.8 years (range, 1.5- 17.4).

Table 1 lists the clinical characteristics of the 77 children in this study. Postoperative therapy included radiation and/or chemotherapy. Sixty-six of 77 patients (86%) were treated with ≥50 Gy of local radiation therapy. Sixty-five of these 66 patients received additional craniospinal radiation. Although 45 patients received craniospinal radiation at conventional doses (*e.g.*, 36 Gy), the doses were reduced in 20 younger patients. For 11 patients (14%), treatment was limited to radiation only. Chemotherapy was administered to 65 patients (84%): 43 patients according to a previously described protocol involving vincristine, lomustine, and cisplatin (23); 13 younger children according to infant brain tumor protocols (24); and an additional 9 patients according to other regimens. Approval to link laboratory data to clinical data has been obtained by the Institutional Review Board.

Table 1 Clinical characteristics of 77 patients with medulloblastoma

	Relative distribution
Sex	
Male	52 (68%)
Female	25 (32%)
Age at diagnosis	
<3 yr	22 (29%)
≥3 yr	55 (71%)
Metastatic stage	
M0	59 (77%)
M1–3	15 (19%)
M unknown	3 (4%)
Surgery	
Gross total resection	58 (75%)
Partial resection	19 (25%)
Biopsy	0 (0%)
Therapy	
XRT[a] ≥50 Gy + chemotherapy	55 (71%)
XRT ≥50 Gy alone	11 (14%)
Chemotherapy alone	10 (13%)
No chemotherapy + no XRT ≥50 Gy	1 (1%)

[a] XRT, local radiation therapy. Sixty-five of 66 patients with ≥50 Gy of XRT had ≥18 Gy of craniospinal radiation (98%).

Immunohistochemistry for Caspase-8 and Caspase-9. Sections (5 μm thick) were cut from Bouin's solution or formalin-fixed, paraffin-embedded blocks, mounted on 3-aminopropyltriethoxysilane-coated slides, and dried at 37°C overnight. Sections were deparaffinized with xylene, dehydrated, and then blocked with 1% hydrogen peroxide in methanol. After antigen retrieval with microwave [10 min at 1000 W in 10 mM citric acid buffer (pH 6.0)], washing with 0.1 M Tris and 2% horse serum, and treatment with serum-blocking solution (Zymed, San Francisco, CA), the tissue sections were incubated for 16 h at 4°C with either a monoclonal caspase-8 antibody (clone 5F7; Immunotech SA, Marseille, France) or a monoclonal caspase-9 antibody (Neomarkers, Fremont, CA), both at a 1:25 dilution. To detect reaction, the tissue sections were incubated with a FITC-conjugated secondary antibody (Zymed) for 10 min and then with a horseradish peroxidase-conjugated anti-FITC tertiary antibody (Zymed). The immune complex was visualized with the chromogenic substrate diaminobenzidine (Zymed). In negative controls, the primary antibody was omitted and replaced with nonimmune horse serum. In positive controls, human tonsils were used as described previously (25, 26).

Immunohistochemistry of primary tumor sections was scored blind on separate occasions by two observers (C. P-M., M. A. G.). Tumor sections were given a score based on the estimated percentage of cells demonstrating immunopositivity (1 = <25% of tumor cells showing positivity; 2 = 25–50% of tumor cells showing positivity; 3 = 50–75% of tumor cells showing positivity; 4 = >75% of tumor cells showing positivity) and on the intensity of the immunostaining (1 = no or weak staining intensity; 2 = moderate staining intensity; 3 = strong intensity; 4 = very strong intensity). A combined score was then calculated by adding both scores (weak immunostaining = score 2–3; moderate immunostaining = score 4–6; strong immunostaining = score 7–8). Discrepancies in the combined score were noted for six sections. These cases were reexamined using a multiheaded microscope, and a consensus was reached.

Statistical Analysis. The following variables were used to create multiple regression models to evaluate overall survival and progression-free survival: age at diagnosis, sex, metastatic stage, extent of surgical resection, treatment, caspase-8 expression, and caspase-9 expression. Relative risk of progression or death was calculated by univariate analysis using Cox regres-

sion models (27). Progression-free survival and overall survival were determined by Kaplan-Meier analysis (28), and differences between survival curves were calculated using the Mantel log-rank test (29).

Apoptosis Index (AI). The AI was determined by counting terminal deoxytransferase-mediated dUTP nick end labeled neoplastic cells in randomly chosen tumor fields, as described previously (30). In the present study, we included data from our previous studies and performed terminal deoxytransferase-mediated dUTP nick end labeling on an additional eight MB samples. Caspase-8 and caspase-9 expression was compared with AI using nonparametric tests.

Tumor Cells. DAOY human MB and PFSK human primitive neuroectodermal tumor cells were purchased from American Type Culture Collection (Manassas, VA). D341 and D425 human MB cells were a gift from Dr. Henry Friedman (Duke University, Durham, NC). DAOY, PFSK, D341, and D425 cells were cultured in Richter's zinc option medium/10% fetal bovine serum (1% nonessential amino acids was added to the medium of D341 and D425 cells). All cell cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2.

Total RNA Isolation and Reverse Transcription-PCR. Total RNA isolation, reverse transcription reactions, and semiquantitative PCR for caspase-8 were all performed as described previously (20, 21). The absence of contaminants was routinely checked by reverse transcription-PCR assays of negative control samples (H_2O control or reverse transcriptase omitted). To correct for variations in reverse transcription and PCR, the expression of the housekeeping gene *GAPD* was used as an internal control. Each PCR sample was analyzed in parallel with a molecular weight marker (Amersham, Arlington Heights, IL) on a 1.2% agarose gel.

Western Blot Analysis. Cells were lysed in 10% NP40, 20 mM Tris (pH 8.0), and 50 mM NaCl in the presence of COMPLETE protease inhibitor mix (Roche Diagnostics, Basel, Switzerland). Samples normalized for total protein content were separated by SDS-PAGE, electroblotted on nitrocellulose, and immunostained. Mouse anticaspase-8 monoclonal antibody (Neomarkers) was used at a concentration of 1 μg/ml. Immunocomplexes were detected using an enhanced chemiluminescence system (Amersham Biosciences, Dübendorf, Switzerland). To correct for variations in loading, the expression of the housekeeping gene β-*actin* was used as an internal control (mouse anti-β-actin monoclonal antibody; Abcam Ltd., Cambridge, United Kingdom).

Detection of Apoptosis. DAOY, PFSK, D341, and D425 cells (3×10^3 cells/well) were incubated with 0, 10, 100, or 1000 IU/ml IFN-γ (Roche Diagnostics) for 2 days. Thereafter, 200 ng/ml TRAIL and 1500 ng/ml potentiator (Upstate Biotechnology, Lake Placid, NY) or the same amount of H_2O were added to the cells for an additional 24 h. A photometric enzyme-immunoassay (Cell Death Detection ELISA^{PLUS}; Roche Diagnostics) was used for the quantitative determination of cytoplasmic histone-associated DNA fragments after TRAIL-induced cell death, as described previously (20). In brief, cell lysates of control and TRAIL-treated cells were placed in a streptavidin-coated microtiter plate. A mixture of biotin-labeled monoclonal histone antibody and peroxidase-conjugated monoclonal DNA antibody was then added and incubated for 2 h. After washing to remove unbound antibodies, the amount of nucleosomes was quantified photometrically. The enrichment of mononucleosomes and oligonucleosomes released into the cytoplasm was calculated using the following formula: absorbance (405 nm) of TRAIL-treated cells/absorbance (405 nm) of untreated cells.

RESULTS

Caspase-8 Expression in Primary MB. Immunoreactive caspase-8 was observed in neoplastic or stromal cells of all 77 MB samples studied. All caspase-8 immunostained MB sections were interpretable for quantification. Immunostaining was weak in 13 (17%) cases, moderate in 35 (45%) cases, and strong in 29 (38%) cases. There were no significant associations between caspase-8 immunostaining and metastatic stage, sex, or age (data not shown).

Caspase-9 Expression in Primary MB. Immunoreactive caspase-9 was observed in neoplastic or stromal cells of all 77 MB samples studied. All caspase-9 immunostained MB sections were interpretable for quantification. Immunostaining was weak in 18 (23%) cases, moderate in 41 (53%) cases, and strong in 18 (23%) cases. There were no significant associations between caspase-9 immunostaining and metastatic stage, sex, or age (data not shown).

Caspase-8 and Caspase-9 Expression Compared with Terminal Deoxytransferase-mediated dUTP Nick End Labeled Positivity in Primary MB. Terminal deoxytransferase-mediated dUTP nick end labeled-positive neoplastic cells with an apoptotic appearance were detected in 70 of 77 MBs. The median AI of all 77 specimens was 0.29% (range, 0–3.21). MBs with weak caspase-8 expression had a lower median AI (0.12%) than MBs with moderate (0.38%) or strong (0.19%) caspase-8 expression. However, the differences were statistically not significant (Kruskal-Wallis test, $P = 0.39$). MBs with weak caspase-9 expression had a lower median AI (0.14%) than MBs with moderate (0.34%) or strong (0.34%) caspase-9 expression. However, these differences were also statistically not significant (Kruskal Wallis test, $P = 0.30$).

Caspase-8 Expression and Survival. Twenty-one of 77 patients have died as a result of progressive disease, whereas 6 patients are still alive with progressive disease. Univariate Cox regression analysis using caspase-8 expression as a categorical variable (weak *versus* moderate/strong) showed that caspase-8 expression was a significant prognostic factor for predicting progression-free (hazard ratio, 3.16; $P = 0.004$) and overall survival (hazard ratio, 2.52; $P = 0.03$) outcome. When compared with the effect of age, metastatic stage, sex, extent of resection, or therapy, caspase-8 expression was the most robust prognostic factor, followed by age and metastatic stage (Table 2).

Five-year overall and progression-free survival of the 77 patients are summarized in Table 3. Multivariate Cox regression analysis with inclusion of the clinical factors age, metastatic stage, sex, extent of resection, therapy, and AI revealed that caspase-8 expression remained a significant prognostic factor for progression-free (hazard ratio, 2.60;

Table 2 Univariate and multivariate analyses of clinical and laboratory variables and progression-free survival in 77 patients with medulloblastoma

	Univariate analysis			Multivariate analysis		
Variable[a]	Hazard ratio	(95% CI[b])	*P*	Hazard ratio	(95% CI)	*P*
Caspase-8 expression	3.16	1.44–6.97	0.004	2.60	1.11–6.10	0.03
Caspase-9 expression	1.58	0.73–3.46	0.25			
Age	2.36	1.14–4.91	0.02	2.14	0.88–5.26	0.10
Metastatic stage	2.14	1.00–4.59	0.05	1.53	0.65–3.63	0.33
Sex	1.87	0.80–4.36	0.15	2.04	0.83–5.05	0.12
Surgery	1.67	0.78–3.58	0.19	1.93	0.80–4.66	0.15
Therapy	1.31	0.54–3.21	0.55	1.25	0.43–3.66	0.69
Apoptosis index	0.63	0.27–1.45	0.28	0.71	0.32–1.56	0.39

[a] The variables were compared as follows: caspase-8 expression, weak *versus* moderate/strong; caspase-9 expression, weak *versus* moderate/strong; age, <3 years *versus* ≥3 years; metastatic stage, M1–3 *versus* M0; sex, male *versus* female; surgery, extent of resection <90% *versus* ≥90%; therapy, local radiation therapy or chemotherapy alone *versus* local radiation therapy plus chemotherapy; apoptosis index, high *versus* low.
[b] CI, confidence interval.

$P = 0.03$) and overall survival (hazard ratio, 2.46; $P = 0.03$) outcome, indicating that caspase-8 expression is an independent prognostic factor in MB. The cumulative survival curves in the groups with weak and moderate/strong caspase-8 expression are shown in Fig. 1. The 5-year cumulative progression-free survival rate of the group with weak caspase-8 expression was 31%, significantly worse than the progression-free survival rate (73%) of the group with moderate/strong caspase-8 expression (log rank, $P = 0.003$).

Caspase-9 Expression and Survival. In contrast to caspase-8, caspase-9 protein expression was not a significant outcome predictor in our patient cohort (Table 2). The cumulative survival curves in the groups with weak and moderate/strong caspase-9 expression are not significantly different (Fig. 2).

IFN-γ Restores Caspase-8 mRNA and Protein Expression in MB Cells. Recent reports have demonstrated IFN-γ-mediated modulation of caspase-8 expression and death receptor-mediated apoptosis in various cancer cells (31–35). We, therefore, treated caspase-8-deficient D341 and D425 human MB cells (20) with IFN-γ at various concentrations for 48 h and examined caspase-8 mRNA expression by reverse transcription-PCR using the housekeeping gene *GAPD* as an endogenous control. Interestingly, treatment with IFN-γ resulted in a dose-dependent restoration of caspase-8 mRNA in D341 and D425 MB cells (Fig. 3*A*). We then examined caspase-8 protein expression by Western blotting using β-actin as an endogenous control. Treatment with IFN-γ for 48 h resulted in a dose-dependent up-regulation of caspase-8 protein (Fig. 3*B*). In DAOY human primitive neuroectodermal tumor cells previ-

Table 3 Survival and progression-free survival (PFS) in 77 patients with medulloblastoma

	5-year PFS		5-year survival	
Variable	%	95% CI[a]	%	95% CI
All patients	67	56–77	71	60–81
Sex				
Female ($n = 25$)	72	54–89	72	54–90
Male ($n = 52$)	64	51–78	70	58–83
Age at diagnosis				
<3 yr ($n = 22$)	52	31–74	58	37–79
≥3 yr ($n = 55$)	72	60–84	76	65–87
Metastatic stage				
M0 ($n = 59$)	71	59–83	79	69–90
M1–3 ($n = 15$)	44	21–67	53	29–77
Surgery				
≥90% resection ($n = 58$)	72	61–84	74	62–85
≥50% but <90% resection ($n = 19$)	50	27–73	62	40–84
Therapy				
XRT ≥50 Gy + chemotherapy ($n = 55$)	68	57–80	73	61–84
XRT ≥50 Gy or chemotherapy alone ($n = 21$)	58	30–86	62	35–88
Caspase-8 expression				
Weak ($n = 13$)	31	4–59	47	18–77
Moderate/strong ($n = 64$)	73	62–84	75	64–86
Caspase-9 expression				
Weak ($n = 18$)	56	33–79	61	39–84
Moderate/strong ($n = 59$)	70	58–82	74	62–85

[a] CI, confidence interval; XRT, local radiation therapy.

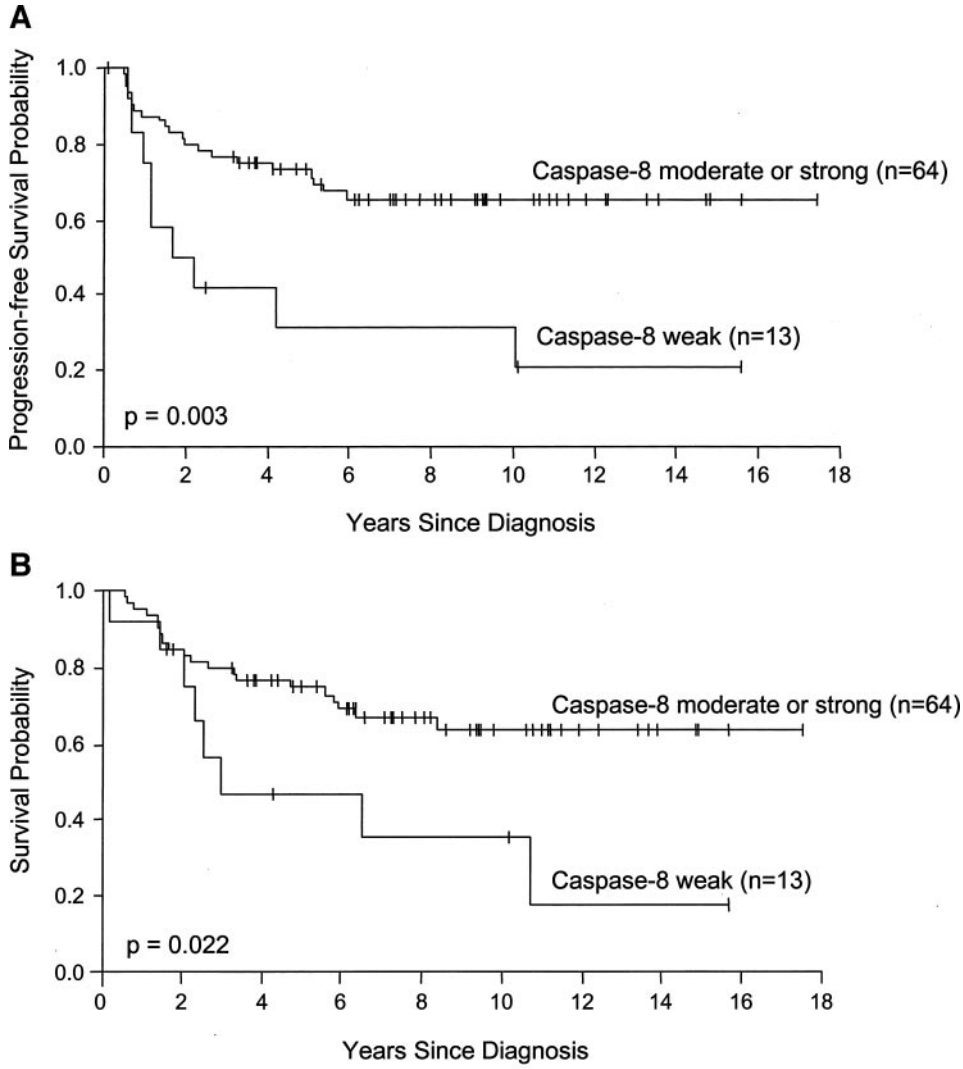

Fig. 1 Caspase-8 protein expression and survival. The Kaplan-Meier curves show the probability of progression-free survival (*A*) and overall survival (*B*) in terms of the level of expression of caspase-8 protein determined by immunohistochemistry. The survival curves were analyzed by the log-rank test.

ously shown to express caspase-8 mRNA and protein (20), IFN-γ treatment had no effect on caspase-8 mRNA or protein-expression.

IFN-γ Restores TRAIL Sensitivity in Formerly TRAIL-resistant MB Cells. We then treated TRAIL-resistant D341 and D425 MB cells (20) with IFN-γ for 48 h and examined TRAIL-mediated apoptosis (24-h TRAIL at 200 ng/ml) using a cell death ELISA. Treatment with IFN-γ for 2 days resulted in the restoration of TRAIL sensitivity (Fig. 4). In D341 human MB cells, 48-h pretreatment with IFN-γ resulted in a 50% (100 U/ml IFN-γ) and a 110% (1000 U/ml IFN-γ) increase of TRAIL-mediated apoptotic cell death relative to untreated cells. In D425 cells, the increase of TRAIL-mediated apoptotic cell death was 129% (100 U/ml IFN-γ) and 33% (1000 U/ml IFN-γ). In DAOY cells (caspase-8 positive; moderately TRAIL sensitive; Ref. 20) and PFSK cells (caspase-8 positive; TRAIL sensitive; Ref. 20), pretreatment with IFN-γ had no significant effect on TRAIL sensitivity. Taken together, these data suggest that IFN-γ treatment may overcome resistance to TRAIL-induced apoptosis in a subset of MB cells by up-regulating caspase-8. However, it remains to be seen whether treatment with IFN-γ also influences other genes involved in TRAIL-induced apoptosis.

DISCUSSION

In this study, we analyzed the protein expression of two key initiator caspases in MB. We found weak caspase-9 protein expression in 24% and weak caspase-8 expression in 16% of 77 primary tumors. Weak expression of caspase-8 correlated significantly with an unfavorable survival outcome in MB patients. Caspase-8 expression did not correlate with metastatic stage, age, or sex of MB patients. In multivariate analysis, correcting for clinical factors, the hazards ratio for caspase-8 expression remained significant, indicating that caspase-8 is of independent prognostic significance.

Caspase-8 expression represents one of five individual biological prognostic factors we have identified in MB (36–39).

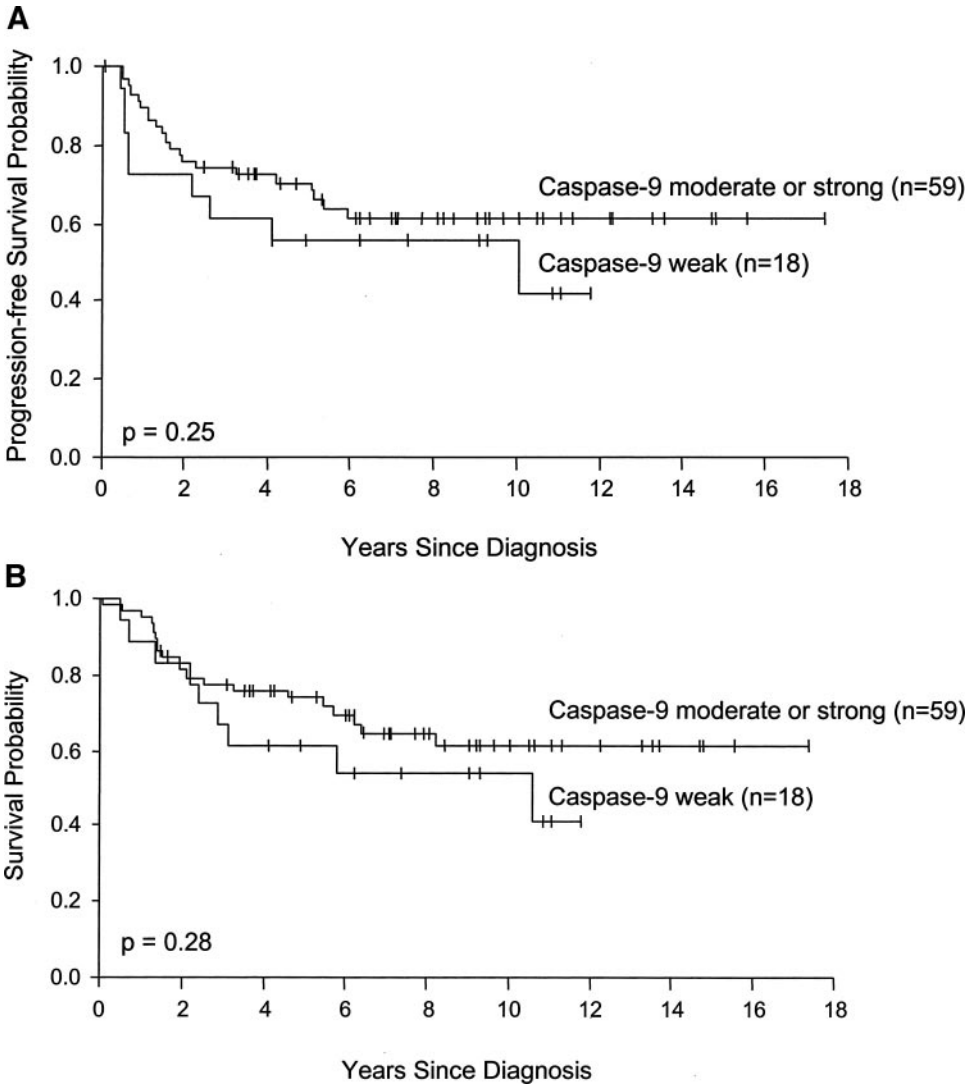

Fig. 2 Caspase-9 protein expression and survival. The Kaplan-Meier curves show the probability of progression-free survival (*A*) and overall survival (*B*) in terms of the level of expression of caspase-9 protein determined by immunohistochemistry.

Additional biological prognostic markers for MB include ErbB2 (40) and oligonucleotide microarray-based gene expression profiles (41). Because these molecular predictors were each developed retrospectively on different patient cohorts, it is not clear which will perform most accurately in future clinical studies. Before molecular outcome predictors can be used to stratify patients into low-risk, standard, or high-risk groups for clinical trials, it will be necessary to validate them in prospective studies. This is the goal of ongoing clinical trials of the Children's Oncology Group in the United States and the International Society of Pediatric Oncology in Europe. If these important prospective studies validate them, biological prognostic factors may be used together with clinical factors to define risk groups and help direct therapy decisions for children with MB. In MB with favorable biological factors and no evidence of leptomeningeal tumor dissemination, therapy with reduced craniospinal radiation might retain the efficacy but reduce the toxicity and, therefore, improve the quality of life for survivors.

Caspase-8 plays an essential role in apoptosis induced by activated death receptors but might also be involved in apoptosis induced by chemotherapeutic agents and irradiation (42–48). Drug resistance and/or resistance to irradiation has obvious selective advantages for tumor cells and might explain why patients whose MB express low levels of caspase-8 had a more unfavorable prognosis than patients whose MB express higher levels of caspase-8. Accordingly, restoration of caspase-8 might be of clinical benefit. We have shown previously that treatment with the methyltransferase inhibitor 5′aza-deoxycytidine restores mRNA and protein expression of caspase-8 in MB cells that lack caspase-8 expression attributable to aberrant caspase-8 gene methylation (20, 21). In this study, to investigate a more physiological and potentially more clinically useful method, we treated caspase-8-deficient MB cells with IFN-γ.

Interestingly, IFN-γ treatment of the caspase-8-negative MB cell lines D425 and D341 resulted in dose-dependent restoration of caspase-8 mRNA and protein expression and restoration of sensitivity to TRAIL-induced apoptosis. These results are consistent with data published by others who demonstrated

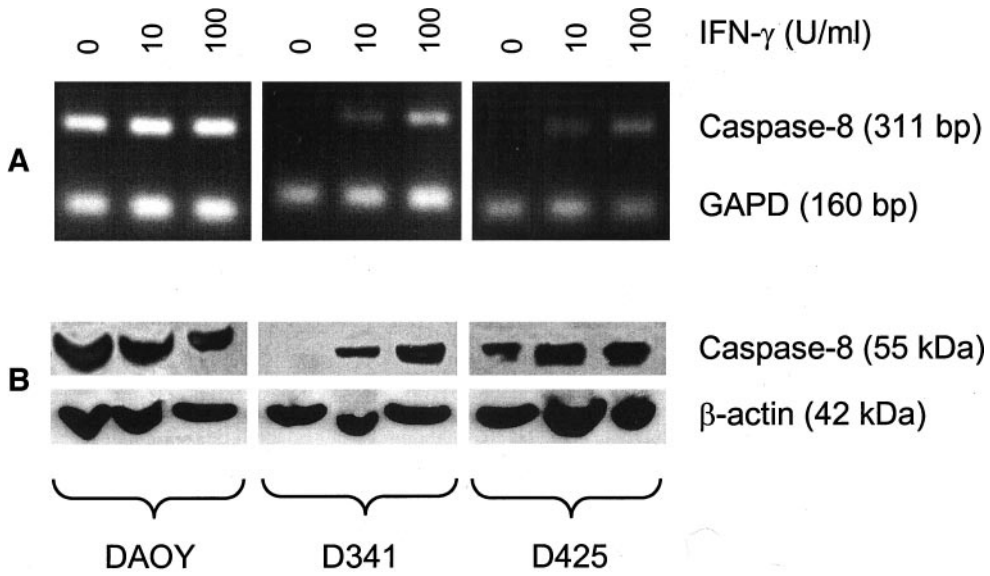

Fig. 3 Treatment with IFN-γ restores caspase-8 mRNA expression (*A*) and caspase-8 protein expression (*B*) in human MB cells D341 and D425 (DAOY cells serve as a control). Representative examples of semiquantitative reverse transcription-PCR with *GAPD* as an internal control (*A*) and Western blotting with β-actin as an internal control (*B*).

IFN-γ mediated up-regulation of caspase-8 in cell lines derived from neuroblastoma, Ewing's sarcoma, colon carcinoma, breast carcinoma, and cholangiocarcinoma and in D283 MB cells (32–35, 49). This effect of IFN-γ might be mediated through a Stat1/IRF1-dependent pathway without altering the methylation status of regulatory sequences of the *caspase-8* gene. Fulda and Debatin (35) reported recently that IFN-γ-mediated up-regulation of caspase-8 was blocked by overexpression of dominant-negative mutants of Stat1 or in Stat1-deficient U3A cells, whereas complementation of Stat1-deficient U3A cells with wild-type Stat1 restored the IFN-γ effect.

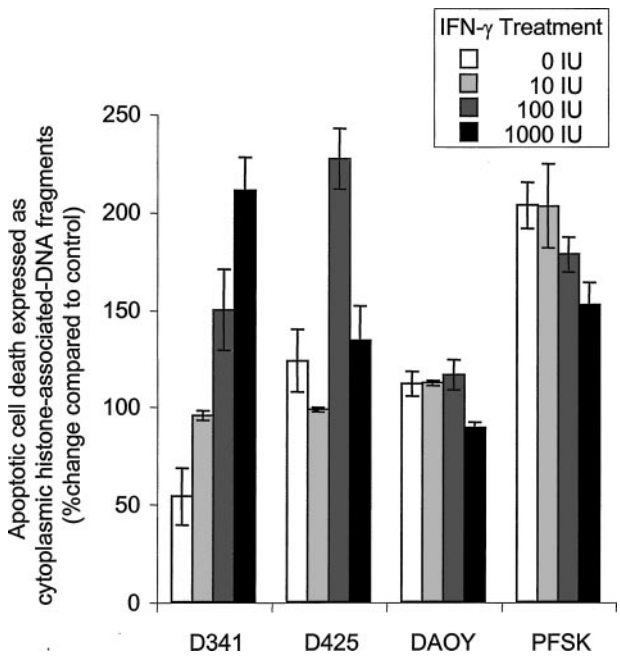

Fig. 4 Treatment with IFN-γ restores sensitivity to TRAIL-induced apoptosis in human MB cells D341 and D425. Apoptotic cell death is quantified using a cell death ELISA showing enrichment of nucleosomes in the cytoplasmic fraction of TRAIL (200 ng/ml)-treated (24 h) human MB cells D341 and D425. Values represent the mean percentage of increase compared with untreated cells ± SD ($n = 3$). DAOY human MB (moderately TRAIL sensitive) and PFSK human primitive neuroectodermal tumor (TRAIL sensitive) cells expressing caspase-8 serve as control.

Taken together, our findings indicate that subsets of MB do not express the key initiator caspases-8 and/or caspase-9 and might, therefore, avoid apoptosis. This might produce a more aggressive and more therapy-resistant tumor type in the case of caspase-8. Strategies to restore caspase-8 expression in MB cells with deficient caspase-8 expression (*e.g.*, by treatment with IFN-γ) are of great interest and deserve additional investigation. The *in vivo* effects of IFN-γ, and whether restoration of caspase-8 by IFN-γ influences the response of MB cells to chemotherapy and/or radiation therapy, are questions that remain to be answered.

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Clinical Cancer Research

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