Loss of Caspase-8 Expression in Highly Malignant Human Neuroblastoma Cells Correlates with Resistance to Tumor Necrosis Factor-related Apoptosis-inducing Ligand-induced Apoptosis1

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Abstract

Human neuroblastoma (NB) is a highly heterogeneous childhood cancer that is aggressively malignant or can undergo spontaneous regression that may involve apoptosis. NB-derived cell lines were tested for their sensitivity to apoptosis induced by the tumor-selective ligand tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Noninvasive S-type cell lines (NB cell lines of substrate adherent phenotype) are highly sensitive to TRAIL, whereas invasive N-type cell lines (NB cell lines of neuronal phenotype) are resistant. Whereas both S- and N-type cell lines express TRAIL-R2, FADD, and caspase-3 and -10, only S-type cells express caspase-8. Reduced levels of caspase-8 protein were also observed in a malignant stage IV NB tumor when compared with a benign ganglioglioma. The caspase-8 gene is not deleted in either N-type NB cell lines or high-stage NB tumors. Caspase-8 expression can be induced by demethylation with 5-aza-2-deoxycytidine, which enhances sensitivity to TRAIL. Therefore, caspase-8 expression is silenced in malignant NB, which correlates to tumor severity and resistance to TRAIL-induced apoptosis.

Introduction

NB3 is one of the most frequent solid tumors of childhood. Spontaneous regressions are common in infants and in early-stage tumors, whereas NB is extremely aggressive in older children with late-stage tumors that are often amplified for the N-MYC oncogene (1). Little progress has been made in improving the poor prognosis of patients with late-stage NB tumors because resistance to chemotherapy is common. Programmed cell death or apoptosis is associated with both drug-induced and spontaneous tumor remission (2), and resistance to apoptosis may explain the aggressive behavior of late-stage NB tumors.

TRAIL receptors TRAIL-R1/DR4 and TRAIL-R2/DR5/TRICK are members of the TNF death receptor family that trigger a cascade of events on binding to cell surface TRAIL involving caspase activation and resulting in DNA fragmentation and cell death (3). In contrast to FasL/CDD95L and TNF, which are both toxic after systemic administration, TRAIL has no adverse effects on normal tissues and can selectively kill implanted tumor cells (4). However, some tumor cells are resistant to TRAIL-induced apoptosis, with the expression of inhibitory molecules such as cFLIP (5) or decoy receptors TRAIL-R3/TRID/DcR1 and TRAIL-R4/DcR2 (6) being proposed as underlying mechanisms of TRAIL resistance. cFLIP overexpression blocks both Fas and TRAIL pathways by preventing the recruitment and activation of the initiator caspase-8 (7), whereas TRAIL decoy receptors are thought to block TRAIL signaling by competing with TRAIL-R1 and TRAIL-R2. These may not be the only existing mechanisms of resistance to TRAIL because no correlation was found between TRAIL resistance and cFLIP or TRAIL decoy receptor mRNA expression in human melanoma (3, 8).

In this study, we investigated the sensitivity of NB cell lines of invasive (N-type) and noninvasive (S-type) phenotypes (9, 10) to TRAIL and the underlying mechanisms used by invasive NB cells to evade apoptosis.

Materials and Methods

Cell Culture. The NB cell lines used in this study are described in detail elsewhere (9). Cells were cultured in RPMI 1640 and 10% FCS with 2 mM glutamine and 20 μg/ml gentamicin (Life Technologies, Inc.). AzaC was purchased from Sigma.

Primary Antibodies. Mouse antihuman Fas antibodies were from PharMingen (Becton Dickinson), agonistic mouse antihuman Fas antibodies (clone CH11) were from Upstate Biotechnology, mouse antihuman caspase-8 antibodies were obtained from Medical and Biological Laboratories, and rabbit antihuman caspase-8 antibodies were from Santa Cruz Biotechnology. Mouse anti-FADD and caspase-3 antibodies were obtained from Transduction Laboratories, and mouse anti-caspase-10 antibodies were obtained from Millennium Biotechnol- ogy. Anti-TRAIL-R2 (clone AL142) was generated in rabbits by injecting TRAIL-R2Fc (Alexis Biochemicals). Mouse anti-N-CAM (U313A) was kindly provided by Dr. John Kemshead (Bristol University, Bristol, United Kingdom), and HLA-ABC (B9-12-1) has been described previously (11).

Cell Viability Assays. Cells (100 μl; 105 cells/well in 96-well plates) were incubated in the presence of anti-Fas CH11 or soluble recombinant TRAIL and cross-linking mouse anti-FLAG antibody M2 (Alexis Biochemicals). After 16 h, tetrazolium dye solution from the Cell titer kit (Promega) was added, and the production of blue formazan product produced by viable cells was measured at an absorbance of 570 nm. Assays were performed in quadruplicate, mean cell viability was compared with untreated controls, and SDs were calculated. The percentage of cell viability was also evaluated by staining cells with 0.5% crystal violet (Fluka). Absorbance was measured at 570 nm. Each assay was performed at least three times, and the percentage of cell viability compared with untreated controls was calculated. The percentage of cell death was calculated as 100 – the percentage of cell viability.

RT-PCR Analysis. DNA-free RNA was prepared using the SV total RNA purification kit (Promega). RNA (1 μg) was used in RT-PCR reactions using the Promega Access RT-PCR kit. RNA samples were tested for DNA contamination by 40 cycles of PCR with each pair of primers used. Primers used to amplify Fas, TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4, and caspase-10 have been described elsewhere (12–14). Primers used to amplify human β-actin, cFLIP, and caspase-8 cDNA are as follows: (a) β-actin, (5′)

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3 The abbreviations used are: NB, human neuroblastoma; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TNF, tumor necrosis factor; RT-PCR, reverse transcription-PCR; MAb, monoclonal antibody; FACS, fluorescence-activated cell-sorting; AzaC, 5-aza-2-deoxycytidine; c-FLIP, cellular Flice inhibitory protein; FasL, Fas ligand; N-CAM, neural cell adhesion molecule; β-actin, class I molecules.
ABSENCE OF CASPASE-8 IN MALIGNANT NB CELLS

TGACGGGTCACCACAC TGGCCCATCTA and (3') CTAGAAG-
CATTGCGTGAGGTGGAGGG; (b) eFLIP, (5') GAGAGCCTAT-
GTCGCTGAACTC and (3') GGGAATTCTCTTGGCTCTG-
AAATGG; and (c) caspase-8, (5') TCTGAGCCTCAGCTCCTG-
and (3') CCTGCTGTGTTGAAGTT. RT-PCR reactions were performed using a thermal program of 48°C for 45 min; 94°C for 5 min; 40 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and then 72°C for 10 min. A total of 30% of the final PCR products were loaded onto a 1% agarose gel.

Flow Cytometric Analysis. Cells were washed in FACS buffer (RPMI 1640, 10% FCS, and 2 mM EDTA) and then stained with antihuman Fas MAb or rabbit antihuman TRAIL-R2 antibodies, followed by goat secondary antibodies conjugated to FITC (Caltag Laboratories). A total of 10,000 events were collected on a FACScan II (Becton Dickinson).

Western and Southern Blotting. Cellular 1% NP40 (30 µg) extracts were boiled in sample buffer and analyzed by 10% SDS-PAGE under reducing conditions and by Western blotting. Blots were saturated with 5% skim milk and 0.5% Tween 20 in PBS and revealed using mouse anti-FADD, anti-caspase-3, anti-caspase-8, or anti-caspase-10 MAb followed by incubation with peroxidase-conjugated secondary antibody (Jackson ImmunoResearch). Bound antibodies were detected using the enhanced chemiluminescence kit (Amersham International) according to the manufacturer’s instructions. Genomic DNA was isolated by lysis in a solution of 10 mM Tris-HCl (pH 10.5), 1 mM EDTA, 150 mM NaCl, and 0.5% SDS and digested twice for 90 min in 20 mg/ml protease K (Boehringer Mannheim) at 56°C, and then two chloroform/phenol extractions were performed. Southern blotting was then carried out as described previously (15), and nylon filters were hybridized with full-length caspase-8 cDNA or the pNB-1 NMYC probe kindly provided by Dr. M. Schwab (DFKZ, Heidelberg, Germany).

Caspase-3 Activity Assay. Caspase-3 activity was assayed by mixing 10 µl of post nuclear lysate (30–40 µg protein) with 10 volumes of reaction buffer [10 mM Tris-HCl (pH 7.4), 0.1% 3-(3-cholamidopropyl)dimethylam-no)-1-propanesulfonic acid, 2 mM MgCl2, 1 mM DTT, 5 mM EGTA, and 150 mM NaCl] containing 50 µM of a fluorogenic caspase-3 substrate (Ac-DEVD-AMC; Alexis Biochemicals). The mixture was incubated for 60 min in a black ELISA titer plate, and fluorescence was measured in a Fluoroskan ELISA reader (excitation, 355 nm; emission, 460 nm). The caspase-3 activity was expressed as a fold increase compared with nontreated cells, and background was deduced using the lysis buffer as a control. All values are normalized with respect to the protein content in the sample measured by the Bio-Rad protein assay.

Immunohistochemistry. Frozen sections (7 µm) were fixed in acetone, permeabilized for caspase-8 staining in 1% Triton X-100 in Tris-buffered saline, blocked in 2 g/liter BSA, and then incubated overnight with rabbit anti-caspase-8 antibodies, followed by biotinylated goat anti-rabbit antibody (DAKO) and avidin-alkaline phosphatase conjugate (ABCComplex, DAKO). Color was developed using Fast Red TR/Naphthol AS-MX (Sigma), followed by counterstaining with hematoxylin. For N-CAM and HLA class 1 staining, frozen sections were fixed and blocked as described above and incubated for 2 h in primary antibody followed by rabbit anti-mouse alkaline phosphatase and swine anti-rabbit antibody coupled to alkaline phosphatase (DAKO) before substrate development as described above.

Results and Discussion

We first tested the sensitivity of NB cell lines of invasive (N-type) and noninvasive (S-type) phenotypes to TRAIL-mediated apoptosis using cell viability tests. Cells were incubated with cross-linked soluble recombinant human TRAIL or with agonistic mouse anti-Fas antibody CH11, and cell viability tests were performed after 16 h. In contrast to Jurkat T cells, all NB cell lines were resistant to Fas-

![Fig. 1](cancersres.aacrjournals.org) Sensitivity of NB cell lines to Fas- and TRAIL-mediated apoptosis and expression of death receptors. A, NB cells incubated for 16 h with medium (■), 100 ng/ml anti-Fas MAb (□), or 100 ng/ml cross-linked soluble TRAIL (■). Tests were performed in quadruplicate, and means and SDs were calculated. B, percentage of cell viability of S-type cell lines SH-EP (□), SH-310 (○), and CA-2-E (●) and N-type cell line LAN-1 (■) after incubation with and without reverse transcriptase, respectively. C, RT-PCR analysis of human Fas, TRAIL-R1, TRAIL-R2, TRAIL-R3, and β-actin RNA expression in NB cell lines. Lanes 1–3 depict S-type NB cell lines SH-EP, SH-310, and CA-2-E; Lanes 4–6 represent N-type cell lines IMR-32, LAN-1, and SH-SY5Y. The first (Lane C) and last (Lane —) lanes represent RT-PCR reactions using Jurkat T-cell RNA with and without reverse transcriptase, respectively. D, flow cytometry of Fas and TRAIL-R2 expression. Cells were labeled with primary antibodies and FITC-conjugated goat secondary antibodies (black peaks). Cells stained with secondary antibodies alone represent negative controls (white peaks).
mediated apoptosis (Fig. 1A). N-type cell lines that have high tumorigenic indices in nude mice (10) were also resistant to TRAIL-induced cell death. In contrast, noninvasive S-type cells were extremely sensitive to TRAIL-induced cell death, even at doses as low as 1 ng/ml (Fig. 1B).

We investigated the mechanisms underlying N-type NB cell resistance to TRAIL and FasL by measuring the expression of death receptors and downstream signaling molecules. The expression of TRAIL-R1, TRAIL-R2, and Fas in addition to that of decoy receptors TRAIL-R3 and TRAIL-R4 was measured in NB cell lines by RT-PCR or FACS analysis. S-type cells expressed Fas mRNA and moderate levels of surface Fas protein, whereas no expression could be detected in any of the N-type cell lines (Fig. 1, C and D). Down-regulation of surface Fas expression has been described for other cancers (16) and may be a common mechanism of resistance to Fas-mediated apoptosis used by tumor cells. In contrast, TRAIL receptors could be detected on all cell types; S-type cells expressed mRNA specific for TRAIL-R1, TRAIL-R2, and TRAIL-R3, whereas N-type cells expressed only TRAIL-R2 mRNA (Fig. 1C). TRAIL-R4 mRNA was not detected in any of the cell lines, although RT-PCR of control TRAIL-R4 cDNA gave a positive signal (data not shown). FACS analysis demonstrated very high levels of surface TRAIL-R2 on S-type cells, whereas N-type cells expressed slightly lower levels that were comparable with TRAIL-R2 expression by Jurkat T cells (Fig. 1D). The findings that S-type cells express surface Fas and TRAIL receptors but are uniquely sensitive to TRAIL suggest that the Fas pathway is not functional in these cells. The lack of decoy receptor mRNA expression in N-type cells excluded the possibility that TRAIL-R3 or TRAIL-R4 was responsible for N-type cell resistance to TRAIL.

Expression of signaling molecules downstream of TRAIL death receptors was then examined. Caspase-3, caspase-8, and caspase-10 are involved in both Fas- and TRAIL-mediated apoptosis, with cFLIP being a negative regulator of both pathways. Adaptor protein FADD is essential for Fas-mediated cell death (17) and TRAIL-R2-mediated cell death (18). Both Jurkat T cells and S-type NB cells expressed cFLIP mRNA, whereas no cFLIP mRNA could be detected in N-type cells (Fig. 2A). It is unlikely that cFLIP inhibits TRAIL-mediated apoptosis in S-type cells because they undergo extensive cell death at low doses of TRAIL. In contrast, cFLIP may contribute to the resistance of S-type cells to Fas-mediated apoptosis because its expression is known to be more crucial in reducing sensitivity to Fas-induced cell death than reduced levels of Fas on the cell surface (7, 19). Expression of FADD and of caspase-3 and -10 was detected by Western blotting in both S-type and N-type cell lines at levels comparable to those in Jurkat T cells (Fig. 2B). Surprisingly, neither caspase-8 mRNA nor protein was present in N-type cells, although they were readily detectable in S-type cells (Fig. 2, A and B).

Caspases-3 and -8 were activated efficiently in S-type SH-EP cells after a 1-h incubation with TRAIL, when cleaved fragments of the activated caspases could be detected (Fig. 2C). In contrast, caspase-3 was not cleaved after incubation with TRAIL in N-type LAN-1 cells that do not express caspase-8. In agreement with these findings, caspase-3 activity after TRAIL treatment was found only in S-type cells (Fig. 2D). No caspase-10 processing was observed in either cell type (data not shown). Caspase-8 is essential for the initiation of the Fas and TNF apoptotic pathways (20), and we propose that it is also important for TRAIL-induced cell death in NB cells because caspase-8 cleavage occurs soon after the addition of TRAIL in S-type cells and coincides with caspase-8 cleavage and activation. We tested a total of 11 NB cell lines (4 S-type and 7 N-type cell lines) for TRAIL sensitivity and caspase-8 expression, and we found that all S-type cell lines expressed caspase-8 and were sensitive to TRAIL, whereas all N-type cell lines did not express caspase-8 and were TRAIL resistant (data not shown). The amplification of the N-MYC oncogene in these cell lines did not correlate with down-regulation of caspase-8 expression or TRAIL resistance, suggesting that N-MYC is not involved in this phenomenon.

The in vivo expression of caspase-8 by malignant NB tumors was investigated by immunocytochemistry comparing a malignant NB stage IV tumor with a benign ganglioneuroma (Fig. 3C). The expression of N-CAM, a neural adhesion molecule that is strongly expressed by NB and related neuroectoderm-derived tumors (21), and the MHC class I molecules (HLA-I), which are lacking in late-stage NB tumor cells (11), is also shown (Fig. 3, A and B). Sections of ganglioneuroma...
gave homogeneously positive stainings for N-CAM, HLA-I, and caspase-8. The malignant NB stage IV tumor was positive for N-CAM and negative for HLA-I and expressed only low levels of caspase-8, whereas regions of normal cells surrounding the tumor were observed to be N-CAM negative and HLA-I and caspase-8 positive. Therefore, as observed for N-type NB cells, caspase-8 expression appears to be reduced in late-stage NB tumors. In contrast, more differentiated ganglioneuromas are similar to S-type cells in that they express both caspase-8 and HLA-I. The fact that a subset of malignant NB tumors down-regulates the expression of caspase-8, which is a key initiator of death receptor-mediated apoptosis, could explain their highly aggressive behavior and their resistance to most treatment regimens (1).

Whereas our study is the first to provide evidence that caspase-8 expression is absent in aggressive neuroblastomas, lower levels of caspase-1 and -3 have been measured in high-stage NB tumors, compared with those of lower stages (22). In addition to the absence of HLA-I, the down-regulation of caspase expression could therefore be a general mechanism used by NB cells to evade immune attack.

The caspase-8 gene is located at chromosome locus 2q33 (15, 23), a region of loss of heterozygosity in several cancers including neuroblastoma (24). It was therefore possible that the absence of expression of caspase-8 observed in N-type cells and stage IV tumors was due to a homozygous deletion at this locus. However, for both N- and S-type cells, HindIII generated polymorphic DNA fragments of 4, 7 and 7.2 kb that hybridized to caspase-8 cDNA (Fig. 4A), as has been described previously (15). Genomic DNA derived from one localized stage II tumor sample and two stage IV tumor samples digested with EcoRI was also investigated for the presence of caspase-8. In all tumor samples, polymorphic fragments of 4 and 8 kb hybridized to the caspase-8 probe (Fig. 4B). Because no large homozygous deletions were detected in the caspase-8 gene, it seemed more likely that the absence of expression was due to the inhibition of caspase-8 mRNA synthesis. Indeed, incubation of N-type SH-SY5Y cells for 24 h with 3 μM AzaC, a demethylating agent, induced the expression of caspase-8 protein (Fig. 4C). This suggests that the caspase-8 promoter is hypermethylated, a mechanism of gene regulation that has been proposed for the down-regulation of Fas expression by oncogenic Ras (16). Treatment with AzaC induced cell death in SH-SY5Y cells (Fig. 4D), probably as a result of the expression caspase-8 or other methylated genes. However, preincubation of SH-SY5Y cells with AzaC did enhance their sensitivity to TRAIL-mediated cell death, suggesting that caspase-8 expression is required for the TRAIL pathway to function in NB cells.

In conclusion, malignant NB cells lack caspase-8 expression, which correlates to their tumorigenicity and resistance to TRAIL and may be due to hypermethylation of the caspase-8 promoter. In contrast, non-invasive NB cells express caspase-8 and are susceptible to TRAIL. Caspase-8 down-regulation may explain the aggressive behavior of high-stage NB, whereas the high sensitivity of noninvasive NB cells to TRAIL may account for the spontaneous regression of low-stage...
ABSENCE OF CASPASE-8 IN MALIGNANT NB CELLS


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