Fas-mediated Apoptosis in Neuroblastoma Requires Mitochondrial Activation and Is Inhibited by FLICE Inhibitor Protein and bcl-2

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ABSTRACT

Fas-mediated apoptosis proceeds through mitochondria-dependent or -independent pathways and is deficient in drug-resistant cells. Neuroblastoma, a common pediatric malignancy, often develops drug-resistance and has a silenced caspase 8 (FLICE) gene, which has been associated with Fas- and drug-resistance. We report that besides caspase 8, which was absent in approximately one-third of 26 neuroblastoma cases in this study, other proteins such as bcl-2 and FLICE-inhibitory protein (FLIP), are equally important in conferring Fas-resistance to neuroblastoma cells. Both bcl-2 and FLIP were frequently expressed in neuroblastoma tissues. Our in vitro studies showed that FLIP was recruited to the death-inducing signaling complex and interfered with the recruitment of caspase 8 in neuroblastoma cells. bcl-2 inhibited the activation of the mitochondria; but it also lowered the free cytoplasmic levels of caspase 8 by binding and sequestering it, thus acting through a novel antiapoptotic mechanism upstream of the mitochondria. In vitro down-regulation of bcl-2 with antisense oligonucleotides allowed the release of cytochrome c from mitochondria and the activation of caspases 8 and 3 upon Fas activation as well as sensitized neuroblastoma cells to Fas-mediated apoptosis. Downregulation of FLIP had only a modest apoptotic effect because of the coexistent mitochondrial block. However, combined treatment with bcl-2 and FLIP antisense oligonucleotides had a statistically significant synergistic effect reversing Fas-resistance in neuroblastoma cells in vitro. These data indicate that Fas-mediated apoptosis in neuroblastoma cells is mitochondria-dependent and inhibited both at the mitochondrial level and at the level of caspase 8 activation. Thus, gene-targeting therapies for bcl-2 and FLIP may reverse Fas-resistance and prove useful in the treatment of drug-resistant neuroblastomas.

INTRODUCTION

NB, the most common extracranial solid pediatric tumor, exhibits biological heterogeneity. High-risk NB is characterized by advanced clinical stage and MYCN amplification, and the patient fares poorly despite current intensive therapy because of relapses and the development of chemoresistance (1). Chemotherapeutic agents kill tumor cells through apoptosis (2) using mitochondrial or death-receptor pathways (3, 4). The Fas-receptor pathway has recently been recognized as an important pathway in drug-induced apoptosis (5–8), and its deficient activation has been implicated in the development of drug-resistance (9, 10). We hypothesized that drug-resistance in high-risk NB may be attributable to a defective Fas pathway and investigated this pathway in Fas-resistant NB cell lines. Proteins that emerged as possible candidates of Fas resistance were studied further in NB tissues.

Ligation of the Fas (CD95/APO-1) receptor on the cell surface results in its trimerization and recruitment of the adaptor molecule FADD and procaspase 8 (FLICE) to form the DISC. Procaspase 8 is activated at the DISC by autocatalytic cleavage. Cells expressing and recruiting sufficient amounts of procaspase 8 at the DISC (type I cells) undergo apoptosis through direct activation of caspase 3 by active caspase 8 (11). In contrast, cells with limited amounts of active caspase 8 at the DISC (type II cells) cannot proceed through this direct pathway and depend on the mitochondria to transmit the apoptotic signal (12). Specifically, in type II cells, caspase 8 cleaves Bid, a cytoplasmic member of the bcl-2 family, which induces the release of cytochrome c from the mitochondria (13, 14). Cytochrome c, with the help of apoptotic protease activating factor 1, activates caspase 9, which in turn activates caspase 3 (15). The mitochondrial pathway is also activated in type I cells and is inhibited by bcl-2 in both cell types (12, 16). However, only in type II cells, bcl-2 inhibits caspase 8 and caspase 3 activation as well as apoptosis (12, 16). Fas-mediated apoptosis is also inhibited by FLIP. Because FLIP prevents the recruitment and cleavage of caspase 8 at the DISC, which is a common initial step in both type I and II cells, it inhibits apoptosis in both cell types (16).

In this study we demonstrate that NB cells are mitochondria-dependent with regard to their Fas pathway and develop Fas resistance by inhibition of the mitochondrial pathway but also by inhibition of caspase 8 activation. In agreement with a previous report showing that the caspase 8 gene is frequently inactivated in NB (17), we found that caspase 8 is absent in approximately one-third of NB cases. However, we also found that even caspase 8-expressing NB cells develop Fas resistance, either because they express high levels of FLIP, which interferes with the activation of caspase 8 at the DISC, or because they express high levels of bcl-2, which blocks the mitochondrial pathway and also the caspase 8 activation step. We show that inhibition of caspase 8 activation by bcl-2 is accomplished through a novel mechanism, i.e., through binding and sequestration of caspase 8 by bcl-2. Treatment with bcl-2 and FLIP ASOs, especially when used in combination, reversed Fas resistance in NB cells. These data indicate that strategies aiming at lowering bcl-2 and FLIP may improve the efficacy of traditional therapeutic schemes in selected high-risk NB cases.

MATERIALS AND METHODS

Cell Lines. Seven previously published NB cell lines (IMR-32, KCNR, SMS-SAN, CHP-126, SK-N-SH, SY5Y, and SK-N-AS; Refs. 18–20) and a line established in our laboratory from a high-stage NB tumor with MYCN amplification (TC-378) were used in this study. The SK-N-MC line that was originally reported as NB (20), but was subsequently reclassified as an ESFT, because it was found positive for the ESFT-specific (11;22) translocation (21), was used as a control. All cells were grown in DMEM (Bio Whittaker, Walkersville, MD) with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% FCS (Life Technologies, Inc., Gaithersburg, MD).

NB Tissues. Primary or metastatic, paraffin-embedded NB tissues obtained from 26 patients during surgery (23 cases) or autopsy (3 cases) were used for the immunocytochemical studies. Seventeen cases were retrieved from the archives of the Laboratory of Pathology at the National Cancer Institute and nine cases were obtained from the National Cancer Institute Cooperative Human Tissue Network, Columbus, OH. Tumor tissue obtained before treatment was available in 12 cases, and tumor tissue obtained after treatment was available in 8 cases; the treatment status was unknown in the remaining 6
cases. Information on the treatment status, clinical stage, and patient age was obtained from the pathology records. All cases were unlinked from personal patient identifiers. For the purposes of this study, the patients were classified into low-, intermediate-, and high-risk groups on the basis of stage, age, MYCN status, and histological category, as described previously (1). Intermediate- and high-risk groups were considered together in the analysis. The MYCN status was evaluated by immunostaining, as described below.

Antibodies. The goat anti-FLIP<sub>P</sub> (C19; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-caspase 8 (Upstate Biotechnology, Lake Placid, NY), mouse anti-bcl-2 (Dako, Carpinteria, CA), and rabbit anti-bcl-2 (Santa Cruz Biotechnology) antibodies were used for immunoperoxidase/immunofluorescence staining and immunoblotting. The mouse anti-MYC antibody (Oncogene Research, Boston, MA) was used for immunoperoxidase staining. The goat anti-FLIP L (C19; Santa Cruz Biotechnology) and mouse monoclonal anti-cytochrome c (clone 65981A, Pharmingen, San Diego, CA) antibodies were used in the immunoblot experiments. Cleavage of caspase 3 was evaluated with the rabbit anti-caspase 3 antibody (Upstate Biotechnology). The cytototoxic anti-Fas monoclonal CH11 antibody (Panvera, Madison, WI) was used for the cytotoxic cell assays and the APO-1, biotin-labeled murine antihuman Fas antibody IgG1 (Kamiya Biomedical Company, Seattle, WA) was used for the DISC immunoprecipitation experiments.

Survival and Death Assays. (a) Cell survival was evaluated with the MTT colorimetric assay. The cells were incubated for 4 h at 37°C in DMEM medium containing 1 mg/ml MTT (Sigma Chemical Co., St. Louis, MO), as described previously (22). Dye absorbance was measured at 570 nm wavelength. Apoptotic cell death was evaluated with the TUNEL method. Air-dried cells were labeled with the in situ cell death kit-fluorescein (Roche Molecular Biochemicals, Indianapolis, IN), following the instructions of the manufacturer, and were viewed with a Zeiss standard fluorescence microscope equipped with an FITC filter.

Western Blotting. Cells (1 × 10<sup>6</sup>) were lysed for 30 min on ice in a lysis buffer [50 mM Tris-HCl (pH 8.0), containing 120 mM NaCl and 1% Igepal], supplemented with the Complete mixture of protease inhibitors (Boehringer-Mannheim, Indianapolis, IN). The samples were cleared by centrifugation (14,000 rpm for 30 min at 4°C) and assessed for protein concentration with the bicinchoninic acid protein assay (BCA; Pierce, Rockford, IL). SDS-PAGE (12%; Invitrogen Corporation, Carlsbad, CA) was performed (30 µg of protein/lane) and the proteins were electroblotted onto nylon membranes. After a 1-h incubation in blocking solution consisting of 20% IgG-free normal horse serum in PBS/Tween (0.5% Tween 20 (Sigma Chemical Co.) in PBS), the membranes were exposed to the primary antibody (1:500 for anti-FLIP<sub>P</sub>, anti-FLIP<sub>L</sub>, and anti-bcl-2 and 1:200 for caspase 8 and caspase 3) overnight at 4°C. After washing in PBS supplemented with 1% Triton X-100 (Sigma Chemical Co.), the peroxidase-labeled secondary antibodies were added at concentrations 1:10,000 (antimouse; Amersham Pharmacia Biotech, Piscataway, NJ), or 1:5,000 (antigoat; Santa Cruz Biotechnology) for 40 min at room temperature. The proteins were visualized with the enhanced chemiluminescence technique (Amersham Pharmacia Biotech). Transfection with ASO. Phosphorothioate single-stranded ASO directed against the human bcl-2 translation initiation site and the subsequent 15 bases (sequence, 5'-TCTCCAGCGTGCCGGCAT-3'), as well as a 2-base mismatch CO sequence, 5'-TCTCCAGCGATGGCCAT-3'; Ref. 23) were purchased from BIOMOL Research Labs, Inc. (Plymouth Meeting, PA). Phosphorothioate single-stranded ASO directed against the human FLIP translation initiation codon (sequence, 5'-GACTTCAAGCACATCCTCAG-3') and the control nonsense phosphorothioate oligodeoxynucleotides (sequence, 5'-TGGATCGCGACATGTCCGAGA-3'); 24) were synthesized by the Molecular Technology Laboratory at the National Cancer Institute-Frederick Cancer and Development Center (Frederick, MD). Human NB cells grown to 70% confluency in serum-containing DMEM medium with 5% calf serum) at 37°C. Subcellular Fractionation and Western Blotting for Cytochrome c. The cells were washed in HBSS once, harvested in 100 µl of isotonic buffer [210 mM mannitol, 70 mM sucrose, 1 mM EDTA, and 10 mM HEPES (pH 7.5)] supplemented with the Complete protease inhibitors cocktail and homogenized with a Dounce homogenizer. Samples were centrifuged originally at 1,000 × g to remove the nuclei and subsequently at 10,000 × g for 30 min at 4°C to separate the heavy-membrane, mitochondria-enriched pellet from the cytoplasmic supernatant. Both pellet and supernatant were subjected to Western blotting, as described above.

Cytochrome c ELISA. Relative cytochrome c levels in mitochondrial and cytoplasmic extracts were assessed with ELISA (R&D Systems, Inc., Minneapolis, MN). IMR-32 and SMS-SAN cells were treated as indicated and subjected to subcellular fractionation as described above. Equal amounts of protein from both the mitochondrial and cytoplasmic fractions were loaded onto a 96-well plate coated with an antibody against cytochrome c and subjected to ELISA according to the manufacturer’s instructions.

DISC Immunoprecipitation Experiments. Immunoprecipitation of the Fas DISC was carried out as described previously (25). Briefly, 10<sup>5</sup> cells were either first stimulated with biotin-labeled anti-Fas APO-1 antibody (2 µg/ml) for 10 min and then lysed in a lysis buffer [20 mM Tris-HCl (pH 7.4), 1% Triton X-100, 10% glycerol, and 150 mM NaCl] supplemented with protease inhibitors (Complete) or first lysed and then incubated overnight with the biotin-labeled anti-Fas-APO-1 antibody (unstimulated cells). The Fas receptor and the associated proteins (DISC) were subsequently precipitated for 2 h at 4°C with streptavidin-agarose beads (Life Technologies, Inc.). After immunoprecipitation, the beads were washed five times with 1 ml of lysis buffer. For Western blot analysis, 20 µg of protein were separated by 4–20% gradient PAGE (Innogenet) and immunoblotted with mouse anti-caspase 8, or goat anti-FLIP<sub>P</sub> at 4°C, as described above. The Super Signal Dura (Amerham Pharmacia Biotech) chemiluminescence reagent was used to visualize the proteins.

Precipitation with Agarose-conjugated bcl-2. The cells were lysed in a lysis buffer (20 mM Tris-HCl (pH 7.4), 1% Triton X-100, 10% glycerol, and 150 mM NaCl) supplemented with protease inhibitors and incubated overnight with 10 µl of bcl-2-conjugated agarose beads (5 µg beads/ml lysis; Upstate Biotechnology, Inc.). After precipitation, the beads with the associated proteins were washed five times with 1 ml of lysis buffer. The samples were evaluated by Western blot analysis, as described above, using an antibody against caspase 8.

Caspase 8 and Caspase 3 Activation Assays. Cells (5 × 10<sup>6</sup>) unstimulated or stimulated with the Fas-activating antibody CH11 for 10 min (for caspase 8) or for 12 h (for caspase 3) with or without prior treatment with bcl-2 CO or APO-1 were washed in HBSS and harvested by centrifugation at 800 × g for 5 min. Caspase 8 and 3 enzymatic activities were measured in arbitrary units using the ApoAlert caspase 8 colorimetric (Clontech; Palo Alto, CA) and the caspase 3 colorimetric kits (BIOMOL Research Labs, Inc.). Caspase 3 and 8 activation was also assessed by Western blotting and DISC immunoprecipitation (presence or absence of cleavage products), as described above.

Transfection of ESF and NB Cells with the Caspase 8-CD8 Molecular Chimera. NB cells (IMR-32) and ESF cells (SK-N-MC) were grown to 70–80% confluence, washed once with HBSS, and incubated for 4 h with a caspase 8-CD8 construct or a mutant (inactive) caspase 8-CD8 construct (gifts of Dr. M. J. Lerado; Ref. 26) in the presence of Superfect (Qiagen, Inc., Valencia, CA). Subsequently, the medium was removed, and the cells were grown overnight in DMEM supplemented with 10% FCS. Cell death was assessed 16 h later with MITT.

Immunohistochemical Detection of Caspase 8 and FLIP<sub>P</sub> in NB and ESFT and of MYCN in NB Tissues. Five-µm paraffin sections were deparaffinized in xylene and rehydrated in alcohol. Endogenous peroxidase activity was quenched for 30 min in methanol containing 0.5% H<sub>2</sub>O<sub>2</sub>. The sections were then subjected to antigen retrieval by incubation in Dako Antigen Retrieval solution (Dako) in a pressure cooker (FLIP<sub>P</sub>, MYCN, and bcl-2) or microwave oven (caspase 8). The sections were washed in PBS and immersed for 1 h in a blocking solution consisting of 20% normal donkey or normal goat serum (Santa Cruz Biotechnology), 0.4% Tween 20, and 2% BSA (Sigma Chemical Co.) in PBS. The primary antibodies were applied overnight at concentrations of 1:100 (anti-FLIP<sub>P</sub> and anti-N-myc), 1:50 (anti-caspase 8), and 1:20 (bcl-2). Subsequently, the sections were washed in PBS and incu-
bated for 1 h at room temperature with a biotinylated donkey antigoat (Santa Cruz Biotechnology) or goat antimouse (Dako) antibodies (1:500), respectively. After washing with PBS, the sections were covered with the avidin-biotin complex reagent (Dako) for 30 min. The peroxidase reaction was developed with 3,3-diaminobenzidine and the slides were counterstained with hematoxylin. Positive staining was evaluated by two independent observers (95% interobserver agreement).

**Two-Color Immunofluorescence and Confocal Microscopy.** Air-dried cytospins from cultured IMR-32 cells were fixed in 4% paraformaldehyde for 15 min, blocked with a cocktail of 10% normal goat serum and 10% normal horse serum containing 0.4% Triton X-100 in PBS, washed in PBS, and incubated overnight at 4°C in a cocktail of anti-caspase 8 (mouse, 1:5) and anti-bcl-2 (rabbit, 1:10) antibodies containing 2% normal goat serum, 2% normal horse serum, and 0.4% Triton X-100 in PBS. After washing in PBS, a cocktail of FITC-conjugated goat antirabbit IgG (1:100), and Texas red-conjugated horse antismouse IgG (1:100; Vector Laboratories, Inc., Burlingame, CA) was applied for 1 h at room temperature. Subsequently, the cells were rinsed, coverslipped using Vectashield mounting medium with 1.5 μg/ml 4,6-diamidino-2-phenylindole (Vector Laboratories, Inc., Burlingame, CA) for nuclear counterstaining and viewed with a confocal fluorescence microscope equipped with argon and argon-krypton laser sources (Model TCS4D/DMIRBE; Leica, Heidelberg, Germany). A series of optical sections were acquired at 0.5-μm increments. Images were digitized using Adobe Photoshop 5.0 software.

**Statistical Analyses.** The potential correlation of FLIP expression with disease stage or treatment was evaluated by the χ² test. To determine whether the combined in vitro treatments with bcl-2 ASO, FLIP ASO, and CH11 had a synergistic effect, we performed a two-way ANOVA as recommended by Slinker (27). All other comparisons were carried out with the one-factor ANOVA method. Statistical significance was set at 0.05.

**RESULTS**

**Caspase 8 Expression in Fas-resistant NB Cell Lines and in NB Tissues.** We have shown previously that NB cell lines with MYCN amplification (IMR-32, SMS-SAN, KCNR, CHP-126, and TC-378; Refs. 28 and 29) are resistant to Fas-mediated apoptosis despite the presence of a functional Fas receptor (30). In this study, we have identified and used additional Fas-resistant NB lines (SK-N-AS and SK-N-SH and its neuroblastic clone SH-SYSY; data not shown), which have been established from high-stage NB but lack MYCN amplification (19, 20). In a recent report, the resistance of NB cells to Fas-mediated apoptosis or doxorubicin was attributed to suppressed caspase 8 activity with an enzymatic colorimetric method that showed caspase 8 activation only in Fas-stimulated SK-N-MC and not in IMR-32 cells (Fig. 1D). Three additionally studied Fas-resistant NB cell lines with relatively high levels of caspase 8 expression (KCNR, TC-378, and SMS-SAN) also lacked caspase 8 activation, by the enzymatic colorimetric method (Fig. 1E).

**Cytochrome c Is Not Released from the Mitochondria of NB Cells upon Fas Activation.** Because Fas-induced apoptosis is accompanied by mitochondrial activation that results in the release of cytochrome c from mitochondria (33, 34) in both type I and type II cells (12), we evaluated cytochrome c levels in mitochondria-enriched extracts from IMR-32 and SK-N-MC cells before and after activation with the Fas-agonistic antibody CH11. We found that cytochrome c remained in the mitochondria-enriched extracts of the NB cells, in contrast with the ESFT cells (Fig. 2A), indicating the absence of mitochondrial activation in the former.

**FLIP Is Expressed in NB Cell Lines and Is Recruited to the DISC.** Absence of caspase 8 activation at the DISC of the IMR-32 NB cell line suggested the presence of an inhibitory protein such as FLIP. FLIP is expressed as a long (FLIPL) Mᵣ 55,000 and a short (FLIPS) Mᵣ 28,000 isoform. FLIPₗ is a more potent inhibitor of cell death than FLIPₛ (21), and is processed at the DISC into a p43 intermediate product that forms a tight complex with cleaved caspase 8. The FLIP/caspase 8 complex remains bound to the receptor and can no longer be replaced by new molecules of caspase 8, thus preventing its further recruitment and activation (32). Using Western blotting, we studied the expression of FLIPₗ and FLIPₛ in whole cell lysates and DISC immunoprecipitates prepared from the IMR-32 and SK-N-MC cell lines. Although the Mᵣ 28,000 FLIPₛ was equally expressed in both lines, the Mᵣ 55,000 FLIPₗ was present exclusively in the IMR-32 and not in the SK-N-MC cells (Fig. 2B). More importantly, the p43 cleavage product of FLIPₗ was present exclusively at the DISC of IMR-32 and not SK-N-MC cells upon Fas stimulation (Fig. 2C). Among the eight NB cell lines in this study, FLIPₗ protein was expressed at high to moderate levels in five (Fig. 2D).

**Table 1** Immunocytochemical detection of caspase 8 and FLIP in NB tissues in relation to N-myc expression, clinical stage, age, and risk group

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<th>Stage</th>
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<th>FLIP</th>
<th>N-myc</th>
<th>bcl-2</th>
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$^a$ L, low risk; I, intermediate risk; H, high risk.

$^b$ Before treatment.

$^c$ After treatment.
FLIP Expression in NB Tissues. To exclude the possibility that FLIP expression in NB is a random trait acquired from prolonged propagation of tumor cells in vitro, we studied its expression by immunohistochemistry in 26 NB specimens. We found positive staining in 15 of 26 NB tissues (57.7%; Table 1 and Fig. 2E), and a statistically significant correlation of FLIP expression with higher disease stage \( (P = 0.002) \). The percentage of FLIP-positive neuroblastic cells varied from 5% to 100% in a given tumor. Also, there was a higher frequency of FLIP-positive cases in tissues obtained after treatment when compared with those obtained before treatment, i.e.,
seven of eight posttreatment specimens were FLIP-positive, versus 4 of 11 pretreatment specimens ($P < 0.05$).

**A Constitutively Active Form of Caspase 8 Kills NB Cells.** Because our findings indicated defective caspase 8 activation, we studied whether bypassing this step with transfection of a constitutively active caspase 8 construct would induce cell death in NB cells. We used a CD8/caspase 8 molecular chimera in which the caspase domain of caspase 8 is genetically fused to the transmembrane and extracellular portion of the human CD8 chain. The latter forms disulfide-linked homodimers in the membrane, leading to caspase 8 oligomerization and autoactivation (26). As shown in Fig. 3A, both the IMR-32 and the SK-N-MC cells that were transfected with the CD8/caspase 8 construct had lower survival than the ones transfected with a mutant caspase 8 construct. Although the MTT assay used reflects cell viability rather than apoptosis, it provided an overall estimate of tumor cell death in active versus mutant caspase 8 transfectants and showed that active caspase 8 is capable of inducing cell death not only in the SK-N-MC, but also in the IMR-32 cells, suggesting that the downstream components of the apoptotic machinery are in place.

**Treatment with bcl-2 ASO Results in Cytochrome c Release and Activation of Caspase 3 in Fas-stimulated NB Cells.** Because high levels of activated caspase 8 could induce apoptosis through direct activation of caspase 3 (12), the induction of cell death in caspase 8-transfected NB cells does not provide information as to whether the mitochondrial step is functional. Apoptosis-specific mitochondrial activities are blocked by bcl-2 overexpression (33–37), and bcl-2 is frequently expressed in NB tissues, as shown previously (28, 38–42) and in this study (Table 1). To investigate whether bcl-2 inhibits the mitochondrial pathway in NB, we down-regulated its intrinsic levels with bcl-2 ASO and studied cytochrome c release and caspase 3 activation before and after stimulation with CH11. We found that treatment with bcl-2 ASO led to down-regulation of the bcl-2 protein (Fig. 3B). Immunoblotting and ELISA revealed that cytochrome c translocated from the mitochondria-enriched to the cytoplasmic fraction in the IMR-32 and SMS-SAN NB cells after treatment with bcl-2 ASO and additional Fas stimulation with CH11. In contrast, bcl-2 ASO-treated, unstimulated IMR-32 and SMS-SAN cells showed no change in their mitochondrial and cytoplasmic levels of cytochrome c, similarly to cells treated with bcl-2 CO, irrespective of the presence or absence of Fas stimulation (Fig. 3, C and D). Similarly, caspase 3 was activated only in NB cells treated with bcl-2 ASO and CH11 and not in those treated with ASO, CO, or CO and CH11, as shown by the colorimetric enzymatic method (Fig. 3E) and by the appearance of the p17 cleavage product on Western blotting (Fig. 3F). These data indicated that mitochondrial activation as well as caspase 3 activation is inhibited by bcl-2 in NB cells. Because bcl-2 inhibits caspase 3 activation exclusively in type II cells, these data also indicated that NB cells are type II and therefore depend on mitochondria for Fas-mediated apoptosis.

**Treatment with bcl-2 ASO Results in Rapid Activation of Caspase 8 in Fas-stimulated NB Cells.** Using a caspase 8 activation assay, we showed that NB cells treated with bcl-2 ASO also showed activation of caspase 8 upon Fas stimulation. Because activation of caspase 8 is inhibited by bcl-2 only in type II and not type I cells, these data would provide additional support for NB cells being type II. However, in type II cells, activation of caspase 8 occurs downstream of mitochondria and follows slow kinetics, occurring no earlier than 60 min after Fas stimulation (12). In the bcl-2 ASO-treated IMR-32 and SMS-SAN cells, caspase 8 activation occurred within 10 min after Fas stimulation, suggesting its activation at the DISC. Caspase 8 activation did not occur in Fas-stimulated IMR-32 and SMS-SAN cells treated with CO or in the same nonstimulated cells treated either with bcl-2 ASO or CO (Fig. 4A).
bcl-2 Binds and Sequesters Caspase 8 in NB Cells. The finding of rapid caspase 8 activation in Fas-stimulated NB cells after bcl-2 down-regulation suggested that bcl-2 may sequester caspase 8 by binding. To investigate whether this is true, we incubated cell lysates from the IMR-32 and other NB cell lines (CHP-126, KCNR, and TC-378) with bcl-2-coated agarose beads and after precipitation, and blotted them with an anti-caspase 8 antibody. The SK-N-MC line was used as a control. The \( M_c \) 54,000 band of procaspase 8 was detected only in the IMR-32, CHP-126, KCNR, and TC-378 and not in the SK-N-MC cell precipitates (Fig. 4B). Treatment with CH11 did not alter the amount of caspase 8 that was precipitated from the IMR-32 cells with the bcl-2-coated agarose beads.

According to the model proposed above, down-regulation of endogenous bcl-2 results in the release of caspase 8 from bcl-2/caspase 8 complexes.

**Fig. 4.** A, caspase 8 is activated only after combined treatment with bcl-2 ASO and CH11 and not with ASO alone or CO with and without CH11, as shown with a colorimetric enzymatic method in the IMR-32 and SMS-SAN NB cells. B, the IMR-32, CHP-126, KCNR, and TC-378 NB and SK-N-MC ESFT cells were incubated with bcl-2-coated agarose beads, electrophoresed, and immunoblotted with an anti-caspase 8 antibody. The exogenously added bcl-2-coated beads precipitated caspase 8 only in the IMR-32, CHP-126, KCNR, and TC-378 cells and not in the SK-N-MC cells, suggesting that bcl-2 is able to bind caspase 8 only in NB cells. The phenomenon was observed in Fas-stimulated (CH11) and unstimulated (control, C) NB cells alike. C, treatment of IMR-32 cells with bcl-2 ASO increased the amount of caspase 8 that was precipitated by the bcl-2-coated agarose beads, as compared with the amount of caspase 8 that was precipitated in the untreated (U), or CO-treated IMR-32 cells. This finding supports that down-regulation of endogenous bcl-2 results in the release of caspase 8 from bcl-2/caspase 8 complexes. The total amount of caspase 8, as detected by Western blotting of untreated (U) and CO- or ASO-treated NB cells, remained unchanged, indicating that the observed increased amount of bcl-2 agarose-bound caspase 8 represents the fraction of caspase 8 that was released from the bcl-2 complex and not a change in the total amount of caspase 8. D, the image shows an optical cut of a representative cell aggregate from the IMR-32 cell line. Fluorescein (bcl-2), Texas red (caspase 8), and 4',6-diamidino-2-phenylindole (nuclei) emissions were acquired separately. Areas of colocalization of green and red fluorescence appear yellow in the computer-generated image (×600).
DISCUSSION

A previous study has shown that NB tissues frequently lack caspase 8 mRNA expression because of inactivation of the caspase 8 gene by methylation or deletion, leading to Fas- and drug-resistance (17). In accordance with these data, we found absence of caspase 8 protein in 34.6% of NB tissues by immunocytochemistry and minimal or no expression in three of eight NB cell lines by Western blotting. Variable expression of caspase 8 in some NB cell lines used in the present and other studies (43) could be attributed to differences in subclones and passages. However, in the present material, we found that absence of caspase 8 expression did not correlate with MYCN overexpression in NB tissues, in contrast with the previous study (17). The reason for this discrepancy is unclear. Treatment or other biological parameters may have contributed to the different findings in our and previous studies. One could argue that our study material was skewed toward high-risk NB, or that our immunocytochemical method of MYCN protein detection is not used for patient risk group assignment, and other methods detecting MYCN gene copy number are used instead (1). However, we have recently obtained similar results in a larger study of 71 NB cases in which MYCN copy number was evaluated by fluorescence in situ hybridization or differential PCR and which were clinically representative of all NB with regard to stage- and risk-group assignment (44). Because increased

the bcl-2-coated beads in IMR-32 cells treated with bcl-2 ASO or bcl-2 CO and in IMR-32 cells receiving no oligonucleotide treatment. We found that the amount of procaspase 8 that was precipitated with the bcl-2 beads was indeed higher in cells treated with bcl-2 ASO compared with the untreated or the CO-treated cells, whereas the total amount of procaspase 8 in the same cell lysates remained unchanged (Fig. 4C). This finding provided additional support for a fraction of caspase 8 in NB cells being sequestered through binding with bcl-2 and released upon down-regulation of bcl-2. The above data were corroborated by the finding of bcl-2/caspase 8 colocalization in the cytoplasm of IMR-32 cells using two-color immunofluorescence staining and confocal microscopy (Fig. 4D).

**bcl-2 Antisense and Combined bcl-2/FLIP Antisense Treatments Reverse Fas-resistance in Caspase 8-expressing NB Cells.** In agreement with the above findings, bcl-2 ASO in combination with the Fas agonistic antibody CH11 induced apoptosis in the Fas-resistant IMR-32 NB cells (∼29% of cell death) in contrast with the bcl-2 CO that did not induce apoptosis either alone or in combination with CH11. Treatment with bcl-2 ASO without CH11 also did not induce apoptosis (Fig. 5, A–D). Combined bcl-2 ASO and CH11 treatments induced a comparable degree of cell death not only in the IMR-32 cells but also in two other caspase 8-expressing NB cell lines that we studied (Fig. 5, E–G; P < 0.001), indicating that the phenomenon is not unique to the IMR-32 cells but also occurs in other NB cells with comparable biological characteristics. However, the bcl-2 ASO/CH11-induced cell death occurred only in NB cell lines with high caspase 8 expression, because the SH-SYSY cell line with minimal caspase 8 levels remained nonresponsive (Fig. 5H).

Treatment with FLIP ASO also sensitized the IMR-32 NB cells to Fas-mediated apoptosis, but this effect was relatively modest (15% of cell death; Fig. 5I), probably attributable to the additional block imposed by bcl-2 downstream of FLIP. This interpretation was supported by our finding of strong sensitization to Fas cross-linking (48%) after combined bcl-2 and FLIP ASO treatment. This combined effect was stronger than the mere additive effect of each one of the ASOs and was found to be statistically significant by a two-way ANOVA analysis (P < 0.001). These data support that in addition to the mitochondrial block imposed by bcl-2, the IMR-32 cells also have a block at the DISC, imposed by FLIP.
MYCN expression has been found to sensitize NB cells to receptor-mediated or drug-induced apoptosis (45, 46), NB cells with MYCN amplification may develop mechanisms leading to impairment of their apoptotic pathways. Direct down-regulation of caspase 8 may be one such mechanism, but as we show in this study, other mechanisms, such as overexpression of FLIP and/or bcl-2 resulting in sequestration and/or inhibition of activation of caspase 8 may also lead to Fas resistance in MYCN-amplified NB cells.

FLIP was first described as a viral product that inhibited Fas and TNF-mediated apoptosis (47, 48). The cellular homologue FLIP also functions as an antiapoptotic molecule blocking apoptosis induced by death receptors (48–52). Specifically, when present at elevated levels, FLIP is recruited to the DISC and processed into a p43 intermediate product, which prevents the recruitment and activation of caspase 8 (32, 49). Our finding of the p43 FLIP cleavage product at the DISC of NB cells, instead of caspase 8, indicated that FLIP prevented caspase 8 activation in these cells. This was supported further by the finding that down-regulation of FLIP led to partial reversal of Fas resistance in NB cells. In fact, this effect was even more pronounced when the coexistent mitochondrial block was also removed by concomitant down-regulation of bcl-2. FLIP was expressed in 57.7% of NB tissues and at high to moderate levels in five of eight NB cell lines in this study. Solid tumors reported to express FLIP are melanoma (49) and late stage Kaposi sarcomas (53). Here we report that NB is yet another tumor with frequent expression of FLIP. The statistically significant association of FLIP expression with high-stage NB patients in this study indicated that FLIP may confer a growth advantage to NB cells. Such a conclusion is supported by experiments in which overexpression of FLIP in human or murine tumors led to their immune escape and progression in mice (54, 55). FLIP was also detected more frequently in NB tissues obtained after chemotherapy. This was statistically significant, suggesting that FLIP overexpression may contribute to the development of drug-resistance in NB cells.

Unlike FLIP, bcl-2 is a known inhibitor of mitochondrial apoptotic activities (33–37). In accordance with this, we found that bcl-2 down-regulation induced cytochrome c release from the mitochondria of NB cells upon Fas stimulation. However, we also found that bcl-2 inhibited the activation of caspase 8 in these cells. Inhibition of caspase 8 activation by bcl-2 has recently been reported in type II cells (12) and in conjunction with stimuli that induce mitochondrial apoptotic activities in a Fas-independent fashion (5). In type II cells, caspase 8 activation occurs downstream of the mitochondrion and is delayed significantly, occurring no earlier than 60 min after Fas stimulation (5, 12). Our finding of caspase 8 activation within 10 min after Fas stimulation does not support its activation downstream of mitochondria and is suggestive of its activation at the DISC (12). Other investigators have shown that the recruitment of procaspase 8 to the DISC is inhibited in bcl-2 overexpressing transformants (56) but have given no explanation as to how this is accomplished. In this study, we show that bcl-2 acts through a novel mechanism lowering the free cytoplasmic levels of caspase 8 by formation of bcl-2/caspase 8 complexes. This leads to reduction of the relative caspase 8:FLIP ratio and inhibition of the recruitment of caspase 8 to the DISC (57). Caspase 8 binding to bcl-2 was specifically observed in the Fas-resistant NB cell line and was reversed by bcl-2 ASO treatment. Although bcl-2 and other bcl-2 family members associate with and target several apoptosis-related proteins to the mitochondrial membranes (58–62), association of bcl-2 with caspase 8 has not been previously reported. BCL2, a recently identified protein, exhibits domains interacting with both bcl-2 and procaspases and in cotransfection experiments bridged procaspase 8 and bcl-2 into a protein complex (63). It is therefore possible that Fas-resistant NB cells express high levels of BCL2, or a BCL2-like protein, leading to formation of caspase 8/bcl-2 complexes.

bcl-2 is frequently expressed in NB cell lines and tissues, as shown in our previous studies (28, 35, 38–42), and its expression correlates inversely with apoptosis (38, 64). Also, up-regulation of bcl-2 with differentiating agents or by transfection with a bcl-2 expression vector led to drug-resistance in NB cells (65, 66). In this study we show that the intrinsically expressed bcl-2 protein inhibits Fas-mediated apoptosis in NB cells because its down-regulation reversed their Fas resistance. Because bcl-2 inhibits Fas-mediated apoptosis only in the mitochondria-dependent type II cells (12, 16), this finding provided support for Fas-mediated apoptosis in NB cells as mitochondria-dependent. Reversal of Fas-resistance occurred only in caspase 8-expressing NB cells and was more pronounced in cells subjected to combined bcl-2 and FLIP ASO treatment, indicating that Fas-mediated apoptosis is blocked both at the mitochondrial level and upstream of mitochondria in NB cells. We have chosen an ASO approach for bcl-2/FLIP down-regulation, because bcl-2 ASOs have been widely used in preclinical studies in severe combine immunodeficiency mice (23, 67, 68) and in humans (69) and have shown no toxicity. However, other gene silencing strategies (70) or compounds specifically targeting bcl-2 (71) may become available in the future.

In conclusion, we have shown that caspase 8-expressing NB cells develop Fas-resistance by expression of FLIP and bcl-2, which block both the initial step of caspase 8 activation, as well as the subsequent step of mitochondrial activation. The latter is important, because NB cells are mitochondria-dependent in regards to their Fas-mediated apoptosis (type II cells). These data indicate that therapies aiming at down-regulating FLIP and/or bcl-2 have serious promise and should be explored in the treatment of high-risk, drug-resistant NB cases with caspase 8 expression.

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