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REVIEW

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Aggressive childhood neuroblastomas do not express caspase-8: an important component of programmed cell death

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Abstract Neuroblastomas that overexpress N-Myc due to amplification of the *MYCN* oncogene are aggressive tumors that become very resistant to treatment by chemotherapy and irradiation. to identify tumor suppressor genes in this group of neuroblastomas we analyzed the expression and function of both apoptosis-related cell cycle regulatory genes in cell lines and patient tumor samples. We found that in a high percentage of neuroblastoma cell lines and patient samples with amplified *MYCN*, caspase-8 mRNA is not expressed. The caspase-8 gene, *CASP8*, was deleted or silenced by methylation in the neuroblastoma cell lines while methylation of its promoter region was the predominant mechanism for its inactivation in the patient tumor samples. Reintroduction of caspase-8 into the neuroblastoma cell lines resensitized these cells to drug-induced and survival factor dependent apoptosis. Subsequently others have also shown that caspase-8 is silenced by methylation in neuroblastoma and peripheral neural ectodermal tumors, and that the caspase-9 regulator Apaf-1 is silenced by methylation in melanoma cell lines and patient samples. We conclude that caspase-8 acts as a tumor suppressor gene in neuroblastomas, that its silencing provides a permissive environment for *MYCN* gene amplification once the tumors are treated with chemotherapeutic drugs/irradiation, and that expression of this gene in these tumor cells may be of clinical benefit. We also discuss the possible significance of the neural crest cell progenitor cell origin and the silencing of important apoptotic regulators via methylation in both neuroblastoma and melanoma tumors.

Keywords Tumor suppressor · Neuroblastoma · Apoptosis · Caspase-8 · Methylation-specific polymerase chain reaction

Abbreviations *NB:* Neuroblastoma · *FADD:* Fas-associated death domain · *LOH:* loss of heterozygosity · *MEF:* Mouse embryo fibroblast · *PCR:* Polymerase chain reaction

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Introduction

Neuroblastoma (NB) is a childhood solid tumor that accounts for approximately 10% of pediatric cancers [1]. The tumor is of neural crest cell origin and can arise any-

where in the sympathetic nervous system, most frequently in the adrenal medulla and paraspinal ganglia. NB is a heterogeneous set of diseases, as various molecular events can give rise to the disease [2]. The best known genetic abnormalities are amplification of *MYCN*, which occurs in about one-third of patients, hemizygous deletions, or loss of heterozygosity (LOH), and translocations of several chromosomal regions, such as 1p36 (26% of patients), 2q33 (30%), 11q (24%), 14q (22%), and 18q (31%) [3]. Amplification of *MYCN* in NB patients is preceded by chromosome 1 band p36 LOH [4, 5]. NB patient tumors, which harbor the 1p36 LOH and *MYCN* amplification, are usually advanced-stage tumors that have been previously treated, and they subsequently respond poorly to chemotherapy/irradiation [6, 7, 8]. In general, NB can be treated by chemotherapy, but once the tumor reoccurs, it is often more resistant to chemotherapeutic drugs and the patient prognosis is poor. Our goal has been to identify tumor suppressor genes involved in NB. Although the chromosomal regions described above are known to be important in NB, the tumor suppressor genes that reside within these regions have not been identified. Known tumor suppressor genes such as $p16^{INK4a}$, pRb, p53, and $p14^{ARF}$ are not frequently deleted and/or mutated in NB, although the nuclear localization of the $p16^{INK4a}$ and p53 proteins is frequently altered in the cells of these tumors [8, 9, 10, 11]. We initiated these studies by examining 18 established NB cell lines [10, 12]. Most of these cell lines had *MYCN* gene amplification (15/18), and almost all (17/18) had 1p36 LOH or translocation.

Caspase-8 is deleted or silenced by methylation in NB cell lines with *MYCN* amplification and 1p36 LOH

RNase protection assays of a battery of apoptosis-related genes revealed that the majority of the NB cell lines did not express caspase-8 (also known as FLICE or MACH1) [12, 13, 14, 15]. In contrast, caspase-3 [16], Fas-associated death domain (FADD) [17, 18], and DR3 (a Fas-related death receptor) [10] mRNAs were present in all of the cell lines. As revealed by northern analysis, RNAse protection, and reverse transcriptase polymerase chain reaction (PCR) assays, only 5 of the 18 cell lines examined expressed caspase-8 mRNA. All of the 13 NB cell lines that did not express caspase-8 mRNA had amplified the *MYCN* oncogene and demonstrated 1p36 LOH. Immunoblot analysis of the NB cells that did not express the caspase-8 mRNA demonstrated, as expected, that these cells were devoid of the caspase-8 protein. All of the NB cell lines examined in this study did, however, express the related caspase-10 protein. The caspase-10 gene, *CASP10*, is located approx. 40 kb upstream from *CASP8* on chromosome 2 band q33 [20, 21], a region known to be associated with LOH in neuroblastoma as well as other tumors [22]. The fact that caspase-10 continued to be expressed in each of these cell lines indicated that the loss of caspase-8 expression did not involve a global event in this region. The fundamental question that needed to be addressed was whether the *CASP8* gene is aberrantly inactivated in NB, and if so, by what molecular mechanism(s).

Caspase-8 has an important and nonredundant role in receptor mediated apoptosis (Fig. 1) [14]. It forms a complex with the adapter molecule FADD and a receptor molecule such as FasR to establish what is known as the death-inducing signaling complex (Fig. 1A) [13, 23]. Once the complex is successfully formed, it transmits a signal from the receptor to effector caspases (e.g., caspase-3), resulting in the activation of a "caspase death cascade." The receptors that transmit a death signal through caspase-8 include Fas, TNFR1, DR3, DR4, and DR5 [24]. Thus, caspase-8 acts as an initiator caspase that can activate the downstream effector caspases in the apoptotic cascade in response to the activation of death receptors, eventually resulting in apoptosis. In addition, it has been shown that there is cross-talk between this death receptor pathway and a distinct mitochondrial-dependent form of apoptosis that utilizes caspase-9, Apaf-1, and cytochrome *c* to form an "amplification loop" (Fig. 1B) [25, 26, 27, 28]. Caspase-8 under certain circumstances preferentially activates the cytoplasmic protein Bid through cleavage of this pro-Bid isoform, resulting in a truncated Bid (tBid), which acts as a ligand to promote Bak tetramer formation in the mitochrondrial membrane (Fig. 1B) [29]. Once this occurs, holocytochrome *c* is released into the cytoplasm from the mitochondria, and the caspase-9/Apaf-1/cytochrome *c* "apoptosome" complex is activated. Furthermore, the proform of caspase-8 can interact with Bcl-2 related family members located in the mithochondrial membrane through a protein intermediate known as bifunctional apoptosis regulator [30]. In addition, it has recently been reported that the cytolinker plectin is a major early in vivo substrate for caspase-8 [25], a further indication that caspase-8 also acts as an executioner caspase. Thus the ability of caspase-8 to act as both an initiator and as an effector caspase in an "amplification loop" with caspase-9 might help to explain why it appears to be specifically targeted in tumors that overexpress Myc oncoproteins. Furthermore, the functional capacity of the caspase-8 enzyme may reflect cell-type differences as well (i.e., their need for such an amplification loop to facilitate cell death; see Fig. 1C).

To understand why caspase-8 was not expressed in the NB cell lines we investigated the structural organization of its corresponding gene in these cell lines. Employing a full-length caspase-8 cDNA probe on a Southern blot revealed that there is no hybridization signal and thus homozygous deletion of the caspase-8 gene in only one of the 18 NB cell lines tested (NB7). Using fiber fluorescent in situ hybridization showed that the deletion in line NB7 is limited to the *CASP8* genomic region (approx. 35 kb in size) on one allele, and to a genomic region containing *CASP8* and *CASP10* on the second allele. Conventional fluorescent in situ hybridization employed on all 18 NB

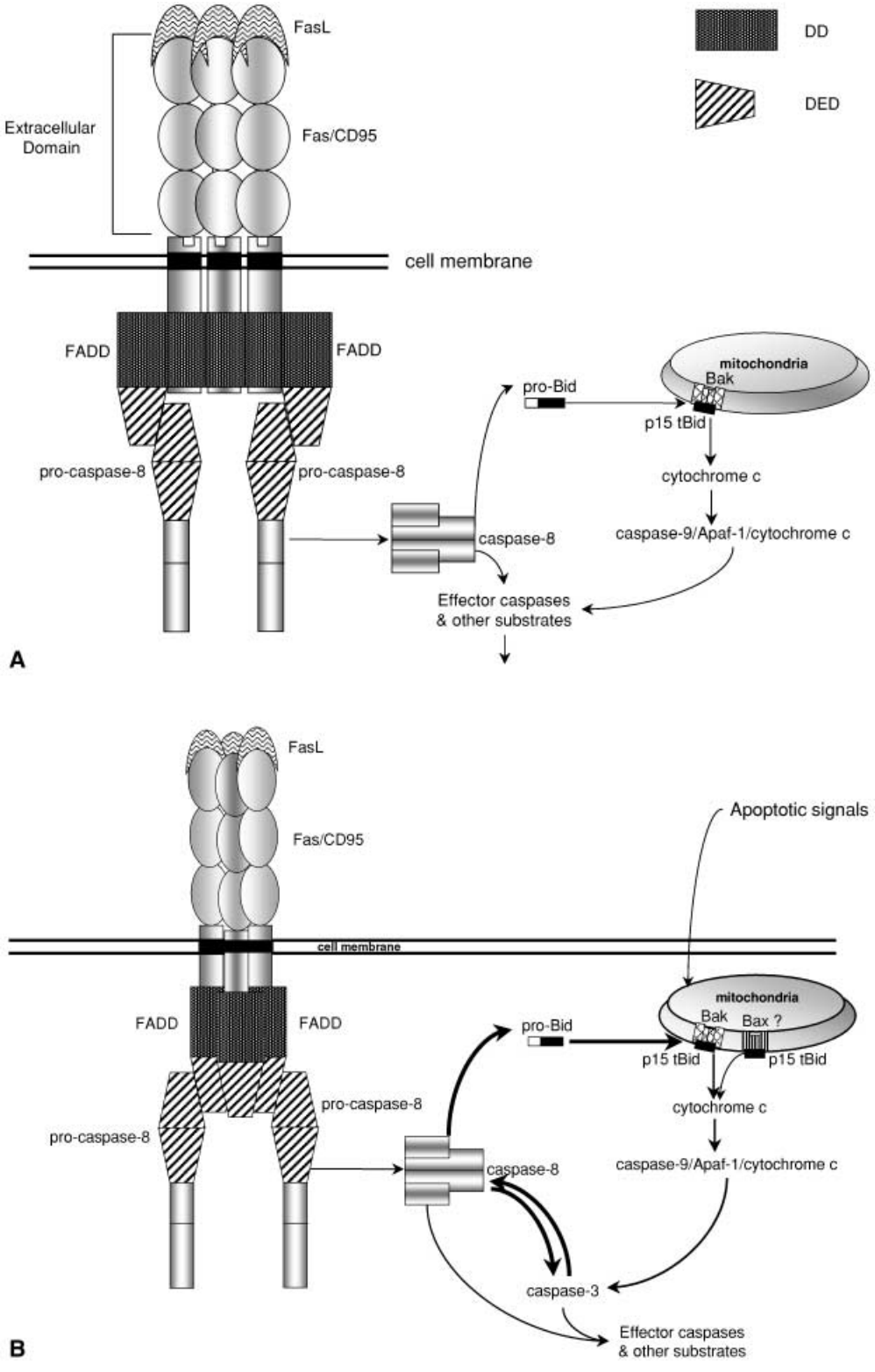

Fig. 1A,B Legend see page 431

cell lines with a bacterial artificial chromosome probe that contained *CASP8* and *CASP10* revealed that 5 of the NB cell lines have hemizygous deletion of a region containing the *CASP8* gene. These homozygous and hemizygous genomic deletions gave a partial explanation for why caspase-8 is not normally expressed in the NB cell lines, but it still did not answer the question of why the majority of the cell lines did not express the gene at all.

An alternative mechanism of tumor suppression is methylation of the promoter, as well as other DNA se-

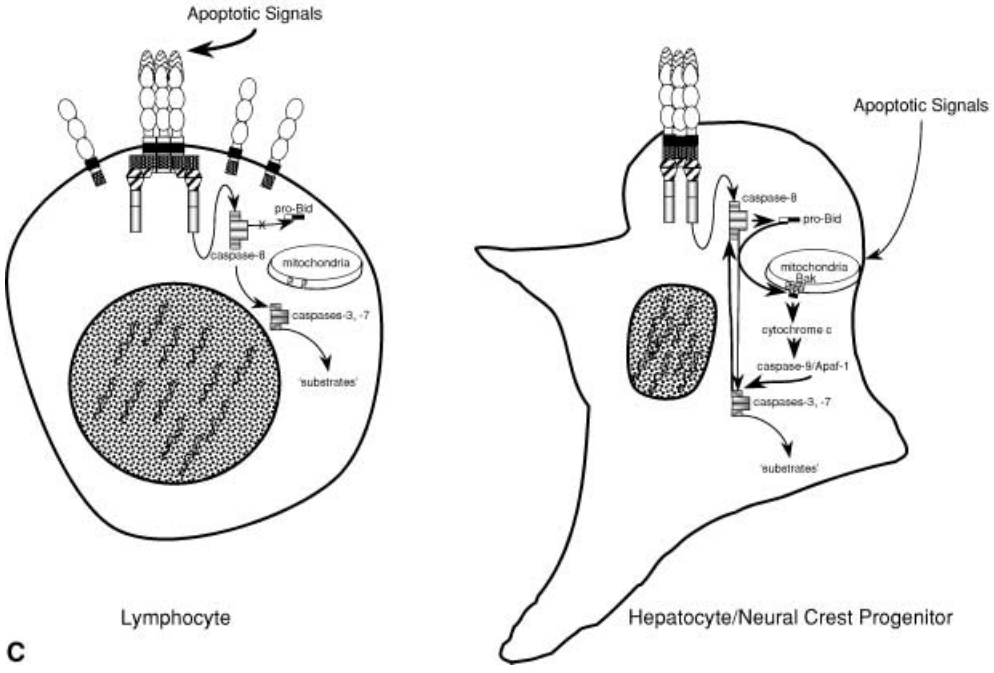

Fig. 1 **A** Schematic model demonstrating how Fas/CD95 is activated by its ligand through trimerization of the receptor, recruitment of the death domain adaptor molecule FADD, and the subsequent recruitment of pro-caspase-8 to this death-inducing signaling complex (*DISC*) through the interaction of its death effector domain (*DED*), and the DED of FADD. Current evidence suggests that pro-caspase-8 is activated by proximity to other pro-caspase-8 molecules. The activated caspase-8 then cleaves other substrates, including downstream effector caspases (e.g., caspase-3). Active caspase-8 also cleaves pro-Bid in the cytoplasm, and the truncated Bid product (*tBid*) translocates to the mitochondrial membrane where it acts as a ligand for the formation of functional Bak complexes, resulting in the release of holocytochrome *c* from mitochondria. The contribution of active caspase-8 to the former DISC-mediated cell death pathway, as compared to the latter tBid-regulated mitochondrial-mediated cell death-signaling pathway, is controlled by the rate of DISC formation and activation. **B** Schematic model demonstrating how active caspase-8 acts preferentially through tBid and the formation of Bak, and possibly Bax, tetramers to signal apoptosis through the release of cytochrome *c* from mitochondria and the subsequent formation of active caspase-9/Apaf-1/cytochrome *c* "apoptosomes." These apoptosomes then cleave downstream effector caspases, including caspase-3, caspase-6, and caspase-7, which then act upon other downstream substrates as well as pro-caspase-8. Once cleaved and activated, caspase-8 can also function as an "effector" caspase by amplifying what would otherwise be a weak mitochondrial death signal (as indicated by the *thickness of the arrows* in the various pathways) – a caspase-8/caspase-9/caspase-3 amplification loop. The ability of caspase-8 to attenuate the activity of both death receptor and mitochondrial-mediated apoptotic signals is unique. **C** Two different cell types, lymphocytes and hepatocytes (or neural crest progenitor cells), that utilize activated caspase-8 in different ways. As explained in the text, Bid gene knockouts are consistent with these models, suggesting that apoptotic signals are controlled in a cell type specific manner. These models also indicate that significant cross-talk between the death receptor- and mitochondrial-mediated apoptotic signaling pathways occurs in some, if not all cell types

quences of these genes [31, 32, 33]. Methylation of 5′ CpG islands of promoters of tumor suppressor genes has been shown in the past few years to be an important mechanism for silencing their expression in tumors. Examples include inactivation of $p16^{INK4a}$ in a variety of tumors, such as non-small cell lung, breast, prostate, renal, colon, and bladder carcinomas, and inactivation of p15 in leukemia and E-cadherin in breast and prostate carcinomas. Evidence that methylation plays a role in silencing the *CASP8* gene in the NB cell lines came from experiments in which the cells were treated with a known demethylating agent, 5-aza-cytidine [34]. In two of the NB cell lines, NB8 and NB10, treatment with 5-aza-cytidine induced marked expression of the caspase-8 mRNA and protein [12]. These two cell lines also underwent a much higher percentage of cell death in comparison to their control counterparts in which caspase-8 was not induced.

In order to verify that the caspase-8 promoter sequences are indeed methylated in the NB cell lines that do not express caspase-8, we employed a methylation-specific PCR method originally developed by Herman and colleagues [35]. In this method the total genomic DNA is bisulfite-treated and PCR-amplified with two specific primer-pairs that can amplify exclusively methylated or unmethylated sequences. The two primer pairs were designed to cover a 321- or 322-bp region in the 5′ untranslated CpG rich region of *CASP8* (55% G+C content). The primer sequences chosen and the relevant promoter region are given in Teitz et al. [12]. By employing these primers we observed a strict correlation between expression of the *CASP8* gene and the presence of a methylated or unmethylated band in the methylation-specific PCR. Thus, most of the NB cell lines (13/18) which did not express caspase-8 contained only the methylated PCR product band, while the 5 cell lines that did express

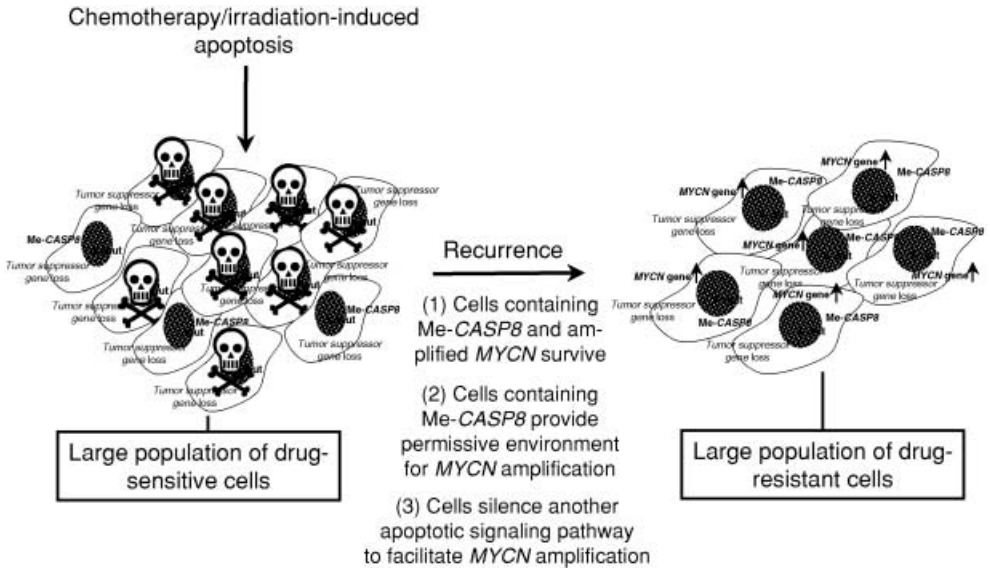

Fig. 2 Schematic showing our hypothesis regarding the "permissive" environment for *MYCN* amplification and overexpression in cells with methylated *CASP8* alleles. *Left* An initial neuroblastoma tumor mass consisting of heterogeneous cells, a small percentage of which has methylated *CASP8* alleles and 1p36 LOH. This mass of tumor cells contains a large population of drug-sensitive cells that will undergo apoptosis when exposed to either chemotherapy and/or irradiation. The cells sensitive to chemotherapy-induced apoptosis are primarily those that have not silenced *CASP8* by methylation, whereas those that continue to express caspase-8 undergo cell death (as indicated by the skull and cross-bone symbols). The few remaining tumor cells with 1p36 LOH and methylated *CASP8* alleles may now provide a "permissive" environment for *MYCN* gene amplification and overexpression, producing a population of highly proliferative drug-resistant cells that are much more aggressive than the initial tumor cells. Alternatively, a separate apoptotic signaling pathway may be silenced to accommodate amplification of *MYCN*. Three possible mechanisms are listed for the conversion of drug-sensitive NB tumor cells into drug-resistant NB tumor cells

the gene contained the unmethylated PCR product band. Cell lines NB3 and NB5, which had moderate levels of caspase-8, contained both the methylated and unmethylated PCR bands. As positive controls in this PCR assay we used the HeLa and Jurkat T-cells, both of which express caspase-8, and only the unmethylated PCR band was found, as expected. All of the 13 NB cell lines that did not express caspase-8 also contained amplified *MYCN*, while only one of the 5 cell lines that expressed caspase-8 (i.e., NB5) had amplified copies of *MYCN*.

Caspase-8 is silenced primarily by methylation of its promoter region in NB patients with *MYCN* amplification

In order to test whether inactivation of *CASP8* also occurs in primary NB patient tumors, 42 NB patient tumors were examined from two different sources: 15 from St. Jude Children's Research Hospital and 27 from the Pediatric Oncology Group. In all instances these tumor samples came from patients who had previously received some form of therapy (i.e., either chemotherapy or irradiation). Eleven tumors had complete methylation of all *CASP8* alleles, and these tumors did not express caspase-8 mRNA as determined by reverse transcriptase PCR [12]. Ten of these 11 patients also had *MYCN* amplification. In total, 10 of 16 NB patient tumor samples with amplified *MYCN* (63%), compared with only one of 26 NB patients without amplified *MYCN* (4%), contained completely methylated *CASP8* alleles. Complete methylation of *CASP8* did not occur in any of six stage 4 NB patient samples that did not contain amplified *MYCN* genes, whereas it did occur in all stage 4 tumors with *MYCN* amplification and in one stage 2 patient sample with *MYCN* amplification. This suggests that *MYCN* amplification is linked functionally to the complete methylation of all *CASP8* alleles. However, we stress that since partial methylation of *CASP8* occurred in early stage (i.e., stages 1–3) NB tumors without *MYCN* amplification, and that our current hypothesis is that methylation precedes *MYCN* amplification (Fig. 2). In addition, all of the patient samples analyzed were from patients who had been previously treated (i.e., chemotherapy and/or irradiation).

Our hypothesis is consistent with data that has recently come from other laboratories suggesting that methylation occurs prior to amplification of *MYC* oncogenes [36, 37]. We propose that the silencing of certain cell death signaling pathways provides a "permissive" environment for *MYCN* amplification and overexpression to occur, and that N-Myc overexpression is not a direct cause of methylation of these cell death genes. Inactivation of *CASP8* most likely represents only one mechanism, representing 63% of the patient tumors whom we examined, that allows tumor cells to survive once the *MYCN* gene is amplified and overexpressed, since 37% of *MYCN* amplified neuroblastoma tumors do not completely silence the *CASP8* gene via methylation and/or deletion. Alternatively, other apoptotic pathways could be silenced or

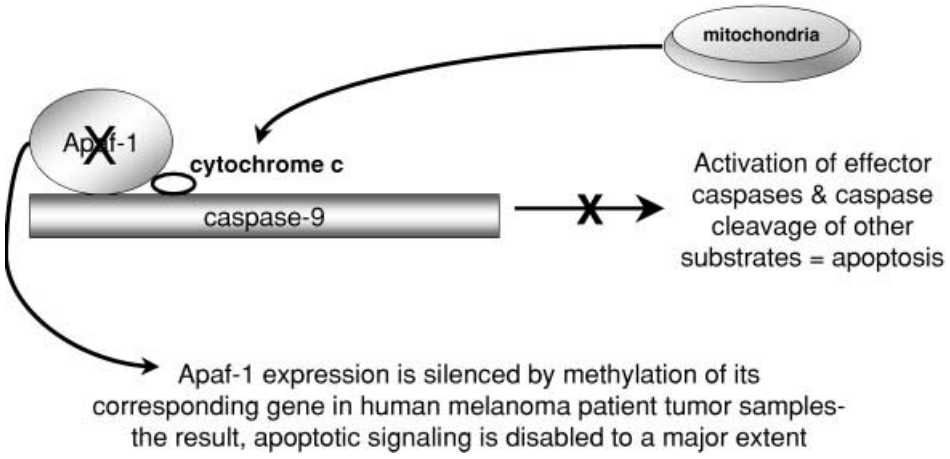

Fig. 3 Methylation of the *Apaf-1* gene in human melanoma, which results in the loss of Apaf-1 expression and the inactivation of the intrinsic apoptotic-signaling pathway in these tumor cells. This is based on the work of Soengas and colleagues [38] that was recently published. See the text for further details

Table 1 Doxorubicin-induced apoptosis in selected human neuroblastoma cell lines with or without caspase-8. Results represent three independent experiments [*N* less than 10% terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end-labeling (TUNEL) positive cells, *Y* greater than 80% TUNEL-positive cells, *N/A* not analyzed)

Cell line	DR3-induced apoptosis	FADD-induced apoptosis	DR3/FADD-induced apoptosis[a]	Caspase-8 protein	FasR protein	Doxorubicin-induced apoptosis[b]	Doxorubicin-induced apoptosis (cell line+caspase-8)[b]
NB3	N/A	N/A	N/A	Y	Y	52±4%	–
NB5	N/A	N/A	N/A	Y	Y	54±5%	–
NB7	N	N	N	N	N	24±3%	42±3%
NB8	N	N	N	N	Y	18±3%	38±2%
NB10	N/A	N/A	N/A	N	Y	12±3%	30±3%
NB16	Y	Y	Y	Y	Y	53±5%	–

[a] Apoptosis induced by overexpression of these proteins after microinjection of appropriate expression plasmids into NB7, NB8, and NB16. Apoptosis was assessed by TUNEL

[2] Apoptosis induced by treatment with 0.1 μg/ml doxorubicin for 48 hs in three independent experiments. Percentage of cell death of the stable transfected cell lines was determined by TUNEL

another gene in the same pathway could be silenced. Although Soengas and colleagues [38] found that the *Apaf-1* gene is silenced by methylation in melanoma (see Fig. 3), it is not inactivated in neuroblastomas with amplified *MYCN* (Teitz et al., submitted). As with neuroblastoma tumor cells, melanoma tumors are derived from neural crest progenitor cells [39], perhaps suggesting some common developmental mechanism linked to the silencing of apoptotic genes via methylation. In contrast to that which was observed in cultured neuroblastoma cell lines, the *CASP8* gene was not deleted frequently in patient tumor samples, as we found only one sample from the 42 examined with a hemizygous deletion of this genomic region.

Introduction of caspase-8 to the cells restores the Fas-induced apoptotic pathway and resensitizes the cells to chemotherapeutic drugs and the withdrawal of survival factors

Several of the NB cell lines that did not express any apparent caspase-8 protein (e.g., NB7, NB8, and NB10) were found to be more resistant to Fas-induced apoptosis than cell lines that did express functional protein (e.g., NB16). Cell microinjection experiments in which DR3, FADD, or DR3 plus FADD were introduced into the NB cell lines that do not express caspase-8 did not cause apoptosis, while microinjection of these same expression constructs into a cell line that expresses caspase-8 (NB16) caused cell death, as expected (Table 1). These experiments confirm that caspase-8, and not upstream genes in this pathway (e.g., DR3 and FADD), are defective in this NB cell line. Experiments in which caspase-8 was reintroduced into NB cells by transduction of a retroviral expression vector showed directly that expression of caspase-8 restores the Fas apoptotic pathway. These stable transfected cell lines have now reacquired the ability to undergo apoptosis in response to signals such as the agonistic FasR monoclonal antibody, or tumor necrosis factor α plus cycloheximide. In addition, as shown in Table 1, the NB cell lines that expressed caspase-8 after retroviral transduction (NB7 + caspase-8, NB8 + caspase-8, and NB10 + caspase-8) were almost twice as sensitive to the chemotherapeutic drug doxorubucin as compared to the corresponding parental cell lines, in which caspase-8 was not expressed (NB7, NB8, and NB10). Doxorubucin has been shown to up-regulate the expression of the FasR in neuroblastoma cell lines [40], and we indeed saw up-regulation

Table 2 Apoptosis-induced by the withdrawal of survival factors (low serum) in selected human neuroblastoma cell lines with or without caspase-8; apoptosis measured by Annexin V staining after 72 h

Cell line	Serum conc.	Apoptosis[a]
NB7	5%	18±2%
	0.05%	24±3%
NB7+caspase 8	5%	21±3%
	0.05%	55±6%

of this receptor in the NB7 cell line, which prior to treatment had no detectable levels of FasR mRNA or protein (Table 1). NB7 cells stably expressing caspase-8 also showed much higher levels of apoptosis in response to withdrawal of survival factors (i.e., low serum) than the parental cell line (Table 2). Thus NB cells that have regained expression of caspase-8 were resensitized to different death stimuli-drugs and serum starvation that are relevant to the in vivo environment of the tumor.

Caspase-9 and Apaf-1 are not affected in NB with *MYCN* amplification

We have assayed the expression of caspase-9 and Apaf-1 (Teitz et al., submitted; for review see [41]) in the NB cell lines and found that in contrast to caspase-8 the expression or activity of these proteins was not affected in these cells. Caspase-9 is an interesting potential tumor suppressor gene in neuroblastoma, in part due to its localization to human chromosome 1 band p36.1 [42] (Teitz et al., submitted). In addition, Soengas et al. [42]. have demonstrated that the caspase-9/Apaf-1 pathway sensitizes mouse embryo fibroblast (MEF) cells to p53-induced tumorigenicity. *Casp9* (–/–) MEF cells expressing c-Myc and Ras had greater tumorigenicity than c-Myc-Ras transformed MEFs in immunocompromised mice. Furthermore, a more recent report indicates that the *Apaf-1* gene is silenced by deletion or methylation in melanoma [38], similar to that which occurs with the *CASP8* gene in neuroblastoma [12, 36, 37]. Immunoblot analysis of the NB cell lines that we have used, most of which have 1p36 LOH and *MYCN* amplification, demonstrates that caspase-9 protein is expressed (Teitz et al., submitted), while reverse transcriptase PCR and DNA sequence analysis of caspase-9 mRNA shows no amino acid sequence changes, frame shifts, or deletions. Immunoblot analysis of Apaf-1 also revealed that this protein is expressed at high levels in all the NB cell lines.

Caspase-8 is not expressed in a subset of solid tumors

Another important question is whether the absence of caspase-8 expression is restricted to neuroblastoma tumors. The answer is no, as our recent results indicate that caspase-8 is not expressed in a variety of solid tumors derived from neural crest progenitor cells. We have found that no, or very low levels, of caspase-8 was expressed in seven of eight small cell lung carcinoma cell lines with *MYC* gene amplification, a brain tumor with *MYCN* amplification (BT3), and a rhabdomyosarcoma cell line with *MYCN* amplification (Rh5) (Teakautz, Teitz, Lahti, and Kidd, in preparation). Further analysis of a larger number of tumor samples should provide some understanding of the common dominator for the silencing of *CASP8* in these tumors and will hopefully provide an explanation for the specific inactivation of caspase-8 in this subset of tumors. Possible common features of these tumors are their neural crest cell lineage origin, *MYCN*, *c-Myc*, or *MYCL* gene amplification and chromosome 1p36 LOH. Whether combination of these or other features is necessary for inactivation of caspase-8 has not yet been determined.

Future prospects

In the past few years it has been shown that inactivation of apoptotic pathways is often critical for the pathogenesis of tumor cells and for their resistance to chemotherapeutic drug treatment and/or irradiation [43, 44, 45, 45, 47]. Future studies based on our current work will address the possible therapeutic applications of caspase-8 inactivation in neuroblastoma. Questions that can be answered include: Is the loss of caspase-8 expression a prognostic marker for NB with *MYCN* amplification? Are there neuroblastoma tumors containing a minority of cells (e.g., <1%) with completely methylated *CASP8* alleles that, when exposed to chemotherapy or irradiation, have a survival advantage and initially go into remission only to recur as extremely aggressive tumors that are now insensitive to treatment (Fig. 2)? Will the reactivation of caspase-8 in these NB tumor cells offer an effective therapeutic strategy for their treatment? Will use of specific demethylating agents in combination with chemotherapeutic drugs offer an advantage for the treatment of these NB patients? Finally, why is *MYCN* amplification associated with *CASP8* inactivation in neuroblastoma? Do the two events occur in these tumors concurrently, or does one event lead to the other? Does the inactivation of certain cell death pathways, particularly those involving caspase-8, provide a “permissive” environment for the amplification and overexpression of the *MYCN* gene? And finally, is the *CASP8* gene normally silenced by methylation at certain points of embryonic development in cells of neural crest origin? Experiments in which the *MYCN* gene is amplified in NB cell lines without *MYCN* amplification, or by tissue-specific overexpression of the *MYCN* gene in the neural crest progenitor cells of mice might help provide the answer. Ultimately, it will be important to investigate the effects of a *CASP8* gene knockout in cells of neural crest origin in an adult mouse model to further study the contribution of *CASP8*

inactivation to the development and progression of neuroblastoma, particularly in the presence of amplified *MYC* genes.

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