Aberrant DNA hypermethylation and apoptotic defects in pediatric neuroblastomas
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Chapter 3

The N-myc paradox: N-myc overexpression in neuroblastomas is associated with sensitivity as well as resistance to apoptosis

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Abstract

Neuroblastomas are characterized by defects in TRAIL induced apoptosis, especially down-regulation and methylation of CASP8. This defect is associated with amplification of N-myc. However, N-myc has also been implicated in induction of apoptosis, especially activation of CASP9 mediated apoptosis. Here we found that ectopic N-myc expression induces TRAIL susceptibility, both by CASP8 and CASP9 mediated apoptosis. N-myc did not modify CASP8 expression and methylation. CASP8 defects therefore represent an independent event in neuroblastoma, counteracting the N-myc induced susceptibility to apoptosis. Analysis of the CASP9 mediated route in a series of neuroblastoma cell lines, we found normal expression and no aberrant methylation of four apoptotic intermediates, including CASP9 itself.
Introduction

Apoptotic defects play a major role in the development of cancer. Pediatric neuroblastomas are characterized by the inability to respond to the apoptosis inducing ligand TRAIL (TNF-Related Apoptosis Inducing Ligand) which leads to a decreased apoptotic potential\(^1,2\). Analysis of the death receptor induced apoptotic cascade has revealed epigenetic repression of one or more intermediate proteins. Normally, TRAIL can bind four receptors, death receptors DR4 and 5 and decoy receptors DcR1 and 2. Activation of the death receptors leads to activation of CASP8 through an intracellular intermediate protein called FADD (Fas-associated protein with Death Domain). Activated CASP8 directly activates the executioner caspase, CASP3, which invariably leads to programmed cell death. The decoy receptors are homologous to the death receptors, but lack a functional intracellular domain. TRAIL binding to the decoy receptors therefore does not lead to activation of the apoptotic cascade. The decoy receptors most probably function as modifiers of the TRAIL response. The best-known apoptotic defect in neuroblastoma cell lines and tumors is down-regulation of CASP8, which strongly correlates with TRAIL unresponsiveness. The lack of expression was associated with hypermethylation of the promoter in 70-80% of cases and is associated with aggressive, N-myc amplified neuroblastomas\(^3\). Demethylation was able to restore CASP8 expression and apoptosis in cell lines\(^4\). We recently showed that also the TRAIL receptors were down-regulated by promoter hypermethylation in neuroblastoma cell lines\(^5\). The decoy receptors were down-regulated in the majority of cell lines and tumors, which was associated with promoter hypermethylation in up to 80% of the non-expressing cell lines and 25 % of the tumors. Down-regulation by hypermethylation of DR4 and DR5 was found in a few neuroblastoma cell lines, but not in neuroblastoma tumors. The functional role of the lack of DcR1 and 2 expression is not clear. We argued that it may represent an early anti-tumor response in cancerous cells to increase the sensitivity for apoptotic stimuli\(^5\). The data imply that in neuroblastomas the death receptor mediated route to apoptosis is affected in multiple ways and epigenetic regulation of gene expression plays an important role.

Alternatively, apoptosis can also develop through the so-called intrinsic pathway, which involves the activation of CASP9 through the release of mitochondrial cytochrome c (cyt c) into the cytosol. Regulation and initiation of this process relies on the balance between the anti-apoptotic Bcl2 and the pro-apoptotic BAX family of proteins in the mitochondrial membranes. A variety of stimuli can shift the balance towards increase of BAX family of proteins. When BAX is increased it induces release into the cytosol of cyt c and other pro-apoptotic proteins like Smac/DIABLO (Second Mitochondria-derived Activator of Caspase). When cyt c is released, it binds Apaf-1 (Apoptotic Protease Activating Factor) and pro-CASP9 under the formation of the apoptosome\(^6,7\). The result is cleavage of inactive pro-CASP9 into active CASP9, which can activate the executioner caspase, CASP3. Most stimuli that increase BAX and release cyt c are related to DNA damage and increased p53 and the intrinsic pathway to apoptosis is therefore considered to be important in intracellular
control of cell homeostasis. Induction of apoptosis by chemotherapeutic drugs is also considered to be activated through the mitochondria\(^8,9\). Lastly, the mitochondrial route is also connected to the death receptor mediated route. Activated CASP8 can cleave the cytosolic Bid protein (BH3-Interacting Domain Death Agonist), which can activate the BAX family of proteins in the mitochondria\(^10,11\). While the CASP8 route to apoptosis clearly shows defects in neuroblastoma, much less is known of the role of the intrinsic apoptotic pathway. Neuroblastomas with amplification of N-myc have been reported to have an increased expression of Bcl2 and survivin (an inhibitory protein of the apoptosome), suggesting that in high-risk neuroblastomas this apoptotic route is less active\(^12\).

The association between amplification of N-myc and decreased apoptosis through the CASP8 mediated and perhaps CASP9 mediated pathways is in sharp contrast with the findings that N-myc and its close relative c-myc can also induce apoptosis\(^13,14\). The most important pro-apoptotic effect of N-myc and c-myc is probably their potential to increase the levels of BAX and the cytosolic cyt\(^c\)\(^6,7,15\). Here, we investigated the association between N-myc and apoptosis in neuroblastoma cell lines. We analyzed both a cell line panel with amplification of the endogenous N-myc gene, as well as a neuroblastoma cell line with ectopic expression of N-myc. Both the CASP8 and CASP9 routes to apoptosis were induced by N-myc, suggesting that defects in these routes in neuroblastoma tumors function to neutralize this effect of N-myc.

**Materials and methods**

**Cell Culture**

Cell lines were cultured at 37 \(^0\)C, 5% \(\text{CO}_2\), using Dulbecco's Modified Essential Medium (DMEM; Gibco) containing 10% Fetal Calf Serum, 292 \(\mu\)g/ml L-Glutamine, 1% 100x MEM (Non-essential amino acids medium, Gibco) and 0,5% Penicillin/Streptomycin solution. Fresh media were applied twice weekly.

**TRAIL treatment, propidium iodide staining and FACS analysis**

The irreversible protease inhibitors Z-IETD-FMK (CASP8 inhibitor) and Z-LEHD-FMK (CASP9 inhibitor) were obtained from Alexis Biochemicals, Switzerland. They were kept as stock solutions of 0,2% in DMSO and added directly to cell cultures in a final concentration of 20 mM. For apoptosis studies, cell cultures were pretreated for 1 hour with protease inhibitors, before addition of TRAIL. TRAIL was obtained from R&D systems Europ Ltd., UK and used in a final concentration of 20 ng/ml. Treatment with TRAIL was performed in 24-well plates (1-2 \(\times\) 10\(^6\) cells in 1 ml medium). Cells were harvested after 24 hours of TRAIL addition, washed once with PBS and centrifuged at 200 x g. The propidium iodide method was used for detection of apoptotic cells, as described elsewhere\(^16\). Briefly, 500 \(\mu\)l of a hypotonic buffer (50 \(\mu\)g/ml propidium iodide in 0.1 % sodium citrate plus Triton X-100; Sigma) was added directly to the cell pellet. The tubes were placed at 4 \(^0\)C in the dark overnight before flow cytometry analyzes. The propidium iodide fluorescence of individual
nuclei was measured using a FACSscan flow cytometer (Beckman). At least $1 \times 10^4$ cells of each sample were analyzed in duplicates or triplicates for each sample. Apoptotic nuclei appeared as a broad hypodiploid DNA peak, as compared to the diploid DNA peak (G0 or resting cells) or hyperdiploid DNA peak (G2 or dividing cells). Induction of apoptosis after stimulation with TRAIL was defined as a two-fold induction of base line apoptosis or more.

**RT-PCR detection of mRNA**

Total RNA was isolated from cell lines using RNAzol™ B (Cinna, Biotecx Laboratories Inc) according to manufacturer’s protocol. First strand cDNA synthesis was performed on 2 μg of total RNA in a volume of 20 μl using Superscript™ II (GibcoBRL) and oligo (dT). The specific primers used for mRNA amplification were as follows (in 5’ to 3’ orientation):

- **Caspase 8** (accession nr GI4502582) F(514): GGAAAGGGAACTTCAGACACC; R(869): TCAGCAGGCTCTTGTGATTIT, PCR product length 356 bp.
- **Caspase 9** (accession nr GI1532150) F(92): GTGGAAGAGCTGGAGGTGG; R(294) GTCTGT GCCTGTGCTCTCTA, PCR product length 201 bp. **APAF-1** (accession nr. AF013263) F(589): AGCTCGAAATTGTTTGCTTCA; R(921): 5-AAAACAACTGGCCTCTGTGG, PCR product length 333 bp. **Smac/DIABLO** (accession nr GI11094502) F(24): GAAATCAGAGCCTCATTCCCT; R(344) GCTGCCATCTCTGAAGACC, PCR product length 321 bp. **VDAC1** (accession nr GI11417131) F(66): CGACATGGATTTCGACATTG; R(396) CACTTTagccgagaagcag, PCR product length 331 bp.

Analysis of expression was performed in a 25 μl PCR reaction containing 1 μl of cDNA, 1 μl dNTP’s (2.5 mmol/l each), 0.5 μl each of the specific primers (150 ng/μl), and 0.25 μl Taq DNA polymerase (5 U/μl; Boehringer). PCR conditions were as follows: 1 cycle, 5 min/95 °C; 35 cycles, 30 sec/95 °C, 1 min/60 °C, 1 min/72 °C, and one cycle 5 min/72 °C. PCR products were loaded on a 4% agarose gel (Metaphor; BioWhittaker Molecular Applications, USA), stained with ethidium bromide and directly visualized under UV illumination.

**Methylation specific PCR (MSP)**

Genomic DNA was isolated from cell lines and primary tissues, using standard procedures. Approximately 1 μg of DNA was bisulfite-modified, as described\(^5\). PCR reactions are hot started at 95 °C for 15 min, by using 0.25 μl (5 U/μl) of HotStarTaq DNA polymerase (Qiagen). Reactions were performed at 60 °C annealing temperature. Each PCR reaction was loaded on a 6 % non-denaturing polyacrylamide gel, stained with ethidium bromide and directly visualized under UV illumination. Genomic DNA treated with Sss1 methylase (New England Biolabs; as instructed by manufacturers protocol) and after bisulfite modification was used as positive control for methylated DNA. Primer sequences for CASP8 as described earlier\(^3,5\), other primer sequences on request.
# Results

**TRAIL induction of apoptosis in neuroblastoma cell lines**

The relationship between N-myc expression and apoptosis susceptibility was analyzed in a panel of 11 neuroblastoma cell lines. The cell lines were treated with TRAIL and analyzed by FACS for the percentage of apoptotic nuclei. Only three neuroblastoma cell lines, SK-N-AS, GI-ME-N and SJNB-8, were responsive to TRAIL, with a 2-5-fold induction of apoptosis (see table 1). Responsiveness was strongly correlated with expression of CASP8, since all three cell lines expressed CASP8, while CASP8 was switched off in 8/10 TRAIL-resistant cell lines. The two unresponsive cell lines that expressed CASP8, LA-N-2 and SK-N-FI, both expressed DR5. SK-N-FI also expressed DR4 (data not shown), suggesting that these cell lines have the potential to bind TRAIL. In this panel, we observed a clear tendency for a correlation between TRAIL resistance and N-myc amplification. Of the seven cell lines with endogenous N-myc amplification, 6 cell lines were TRAIL resistant. Of the four cell lines without N-myc amplification, two were TRAIL sensitive. Six out of seven N-myc amplified cell lines have a methylated CASP8 promoter, which strongly correlates with CASP8 down-regulation (Table 1). However, also two of the four N-myc single copy cell lines show promoter methylation and silencing of CASP8.

<table>
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<th>NBL Cell lines</th>
<th>TRAIL Apoptosis</th>
<th>NMA</th>
<th>Casp8 Met</th>
<th>Exp</th>
<th>Casp9 Met</th>
<th>Exp</th>
<th>Apaf-1 Met</th>
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**Blocking of apoptosis**

To assess the role of N-myc in TRAIL induced apoptosis more directly, we used an N-myc transfected cell line (a kind gift of M. Schwab). The SHEP cell line has no N-myc amplification and expression, nor c-myc expression. A tetracycline-regulated N-myc expression vector has been introduced into these cells (SHEP-21N clone). SHEP-2 is a mock-transfected control clone. Background apoptosis,
Figure 1. TRAIL induced apoptosis in the N-myc transfected SHEP-21N cell line.
A. FACS analysis of apoptotic nuclei after 24 hr induction with TRAIL, with or without inhibitors for caspase 8 and 9.
B. Quantification of apoptotic nuclei. 21N: SHEP-21N control, +8inh: addition of CASP8 inhibitor, +9inh: addition of CASP9 inhibitor, 8/9inh: addition of both CASP8 and CASP9 inhibitors.
as assessed by propidium iodine staining followed by FACS analysis was for both cell lines 5-8%. SHEP-2 cells were unresponsive to TRAIL. However, SHEP-21N cells showed a strongly increased sensitivity to TRAIL resulting in 72% (range 60-74) apoptosis (Fig 1).

We used the SHEP-2/SHEP-21N system to analyze the roles of the CASP8 and the CASP9 routes to N-myc induced apoptosis. We determined the contribution of CASP8 and CASP9 in apoptosis by using specific inhibitors for CASP8 (Z-IETD-FMK) and CASP9 (Z-LEHD-FMK) \(^{19,20}\). Z-IETD-FMK and Z-LEHD-FMK did not enhance background apoptosis (Fig 1b). Addition of Z-IETD-FMK reduced TRAIL-induced apoptosis in SHEP-21N from 72% to 38% (range 34-41). Z-LEHD-FMK reduced apoptosis to 57% (range 50-60), and the combination of the 2 inhibitors reduced apoptosis to 31% (range 29-37) (Fig 1a and 1b). This suggests that the N-myc induced sensitivity to TRAIL-dependent apoptosis is mediated mainly by CASP8 and also to a lesser extent by CASP9. These analyses confirm that N-myc can induce susceptibility to apoptosis and show that both the CASP8 and CASP9 routes are involved. This strongly contrasts with the observed correlation between N-myc expression and resistance to apoptosis and down-regulation of CASP8, as observed in the cell line panel and previously\(^3\). We therefore directly analyzed whether N-myc can down-regulate the expression of CASP8 in the SHEP-21N cells. CASP8 was expressed in both SHEP-2 and SHEP-21N cells (Table 1.). Methylation analysis showed no detectable methylation changes in SHEP-2 or SHEP-21N (data not shown). This implies that N-myc is not directly involved in the down-regulation or methylation of CASP8 in high-risk neuroblastomas. The defect in CASP8 expression in neuroblastoma cell lines has therefore most probably been induced by genes or mechanisms other than the oncogene N-myc itself. The defects in the CASP8 route to apoptosis possibly function to neutralize the N-myc induced susceptibility for CASP8 mediated apoptosis.

Methylation and expression of genes of the intrinsic route to apoptosis
Since high expression of N-myc also increases the susceptibility for CASP9 mediated apoptosis, we wondered whether N-myc amplified cell lines have defects in the CASP9 route to apoptosis. We analyzed epigenetic regulation of a series of genes of the CASP9 mediated apoptotic pathway. Four proteins down-stream of the activation of BAX were selected. The genes analyzed were VDAC1, a Voltage Dependent Anion Channel protein involved in the mitochondrial pore protein complex, Smac/DIABLO, the inhibitor of the apoptosome inhibitory proteins (like survivin), and two proteins of the apoptosome, CASP9, and Apaf-1. We used the Methylation Specific PCR for methylation analysis. Each of the four genes contains a CpG island in the promoter. The analysis showed that all four genes were completely unmethylated without evidence of aberrant methylation in any of the cell lines from our cell line panel (data not shown). Subsequently, we analyzed their expression pattern by semi-quantitative RT-PCR. The four genes were all normally expressed in all cell lines of the cell line panel (Fig 2.). In conclusion, the expression profiles of the genes do not suggest that either one acts as a tumor suppressor gene in neuroblastomas.
The N-myc paradox

Discussion

Down-regulation and hypermethylation of CASP8 has become the hallmark of defective apoptosis in neuroblastomas. CASP8 is the initiator caspase of the death receptor mediated apoptotic route, but is also connected to the intrinsic apoptotic route through the intermediate protein Bid. Defects in CASP8 are primarily found in high-risk neuroblastomas, often characterized by amplification of N-myc. This model of tumorigenesis, which combines defective apoptosis and amplification of an oncogene, seems to fit the clinical behavior of high-risk neuroblastomas. The defective apoptosis could mirror the frequently observed chemoresistance, while amplification of N-myc is known to increase cell proliferation. The model seems to explain the poor outcome for this group of patients. However, the molecular mechanisms at the basis of the model are not altogether clear. N-myc and c-myc family members are also known to induce apoptosis. This apoptotic potential results from the ability to increase the levels of BAX and to increase the release of cyt c into the cytosol. Here, we assessed the paradoxical role of N-myc amplification in neuroblastoma cell lines, most of which have endogenous overexpression of N-myc. As expected, we confirmed the strong correlation between TRAIL resistance, down-regulation of CASP8 and amplification of N-myc. However, in a SHEP cell line with ectopic expression of N-myc we observed strong induction of TRAIL mediated apoptosis compared to the mock-transfectant control clone. Specific blocking of CASP8 or CASP9 showed that both CASP8 and CASP9 mediated apoptosis were involved in the TRAIL response. The potential of N-myc to induce both apoptotic routes suggests an effect at several levels of the pathways, but the precise mechanism is unknown. In addition, when we analyzed CASP8 in the SHEP cells, we found CASP8 expression without evidence of methylation changes in both the N-myc expressing and non-expressing cell line. Our data imply that the down-regulation of CASP8 in high-risk neuroblastomas is not a direct effect of N-myc, but an indirect effect of the biology of these tumors. In addition, we considered the possibility that also the CASP9 mediated route to apoptosis is less functional in neuroblastomas. This was suggested by several reports. We argued that multiple genes in the intrinsic route to apoptosis were candidate tumor suppressor genes for neuroblastomas. CASP9 is located at chromosome 1p36, a common
region of LOH in neuroblastomas and other tumors. Recently, it was shown that CASP9 was indeed located within the shortest region of overlap of these heterozygous deletions\textsuperscript{22}. However, the same report also showed that expression of CASP9 in neuroblastomas was normal. Apaf-1 is epigenetically down-regulated in melanomas and is considered as a potential tumor suppressor gene for this malignancy\textsuperscript{23}. Smac/DIABLO peptides are able to increase sensitivity for TRAIL induced apoptosis of various tumors, including neuroblastomas\textsuperscript{24}. These genes, together with the intermediate VDAC1 were tested for aberrant methylation and expression. All cell lines, including the two CASP8 expressing, TRAIL resistant cell lines were completely unmethylated for these genes and showed normal expression levels.

In conclusion we showed that N-myc induces both CASP8 and CASP9 mediated apoptosis. Epigenetic down-regulation of CASP8 in neuroblastomas is unlikely to be induced by overexpression of N-myc. Additional defects in apoptosis may be involved in the CASP9 route to apoptosis, but we found no evidence for (epigenetic) silencing of several selected key intermediates down-stream of the activation of BAX.
References


