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MycN and IFNγ cooperate in apoptosis of human neuroblastoma cells

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Neuroblastomas undergo spontaneous regression at an unusually high rate. The mechanisms are not clear, but apoptosis may be involved. A large proportion of neuroblastomas is characterized by amplification of *MYCN*. Using human neuroblastoma cells harbouring tetracycline controlled expression of *MYCN* we have analysed the role of the MycN protein and IFNγ in cell death decision. Neither conditional expression of *MYCN* nor treatment with IFNγ alone was sufficient to trigger cell death. However, when acting in concert MycN and IFNγ efficiently triggered cell death, which was accompanied by DNA fragmentation and required caspase activity, two hallmarks of apoptosis. MycN and IFNγ may cooperate along at least two different pathways. First, IFNγ increased the CD95 cell surface expression while MycN enhanced the cellular susceptibility for the CD95 mediated death signal. Second, IFNγ treatment induced expression of *BAK* mRNA while MycN and IFNγ in combination increased the amount of Bax protein, another activator of apoptosis, without a concomitant increase in *BAX* mRNA. MycN also increased cell death in response to TRAIL and TNFα, suggesting that enforced *MYCN* expression in general increases the susceptibility of neuroblastoma cells towards a variety of death stimuli.

Keywords: oncogenes; amplification; APO-1; FAS; cytokines; CD95

Introduction

Neuroblastoma is a tumor of the sympathic nervous system developing in young children (Berthold, 1990; Schwab and Joshi, 1997). A subset of neuroblastomas, often accompanied by amplification of the *MYCN* gene (Schwab *et al*., 1995; Schwab, 1998), causes high mortality despite extensive therapy. Other tumors mature spontaneously *in vivo* to more benign ganglioneuroblastomas or ganglioneuromas or resolve without treatment such as neuroblastoma *in situ* or neuroblastoma stage 4S, a unique clinical presentation of apparently metastatic disease in infants. The incidence of spontaneous regression is greater in neuroblastomas than in any other human tumor, and it has been suggested that programmed cell death could account for this clinical feature (Pritchard and Hickman, 1994). In line with this, massive apoptosis has been detected in primary neuroblastoma (Ikeda *et al*., 1996). Additionally, expression of the apoptosis blocking *BCL2* in primary neuroblastoma was correlated with poor prognosis (Castle *et al*., 1993), while high level expression of ICE and CPP32, two proteases participating in the execution of apoptosis, was associated with a favourable outcome (Nakagawara *et al*., 1997).

Various cellular proteins have been discovered as participants in regulating apoptosis (for a review see White, 1996). Among these are the closely related transcription factors c-Myc, found to trigger apoptosis in serum-deprived rat embryo fibroblasts and antigen-stimulated T-lymphocytes (Askew *et al*., 1991; Evan *et al*., 1992; Shi *et al*., 1992, for a review see Henriksson and Lüscher, 1996) and MycN observed to cause apoptosis in B-lymphocytes when expressed from the immunoglobulin enhancer Eμ in transgenic mice (Zörnig *et al*., 1995). MycN accelerates cell cycle progression of neuroblastoma cells (Lutz *et al*., 1996), and targeted expression of *MYCN* causes neuroblastoma in transgenic mice (Weiss *et al*., 1997). However, in none of these systems a role of ectopic *MYCN* expression in apoptosis of neuroblastoma cells has been demonstrated.

Interferon-γ (IFNγ) inhibits proliferation and promotes differentiation of neuroblastoma cells and has been proposed as an agent in a differentiation-based therapy (Ponzoni *et al*., 1992). Neuroblastoma cells respond to activation of the IFNγ receptor not only with differentiation but also with apoptosis (Montaldo *et al*., 1997) suggesting a potential for this cytokine as a therapeutic agent.

To study the effect of MycN and IFNγ on neuroblastoma cells we used a human neuroblastoma cell line with conditional expression of *MYCN* (Lutz *et al*., 1996). Induced *MYCN* alone was unable to trigger cell death in neuroblastoma cells. However, induction of *MYCN* in combination with IFNγ treatment caused a dramatic increase in cell death. MycN and IFNγ mediated cell death was accompanied by DNA-fragmentation and required the activation of caspases. Cooperation between MycN and IFNγ was observed at two levels. IFNγ up-regulated the cell surface expression of CD95 (also termed Fas and Apo-1) while ectopic *MYCN* expression increased the susceptibility towards the CD95 mediated death signal. Previously, the CD95 system, one of the key ligand driven apoptosis pathways (for a review see Fraser and Evan, 1996), has been implicated in apoptosis of neuroblastoma cells (Fulda *et al*., 1997). The second level of cooperation was the apoptosis regulatory network of Bcl-2 related proteins, which either block (Bcl-2) or accelerate (Bak, Bax and Bad) cell death (Chittenden *et al*., 1995; Farrow *et al*., 1995; Kiefer *et al*., 1995; for a review see Kroemer, 1997).

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IFNγ induced expression of *BAK* and, in combination with MycN, increased Bax protein levels thereby shifting the balance of Bcl-2 related proteins towards death promoting family members.

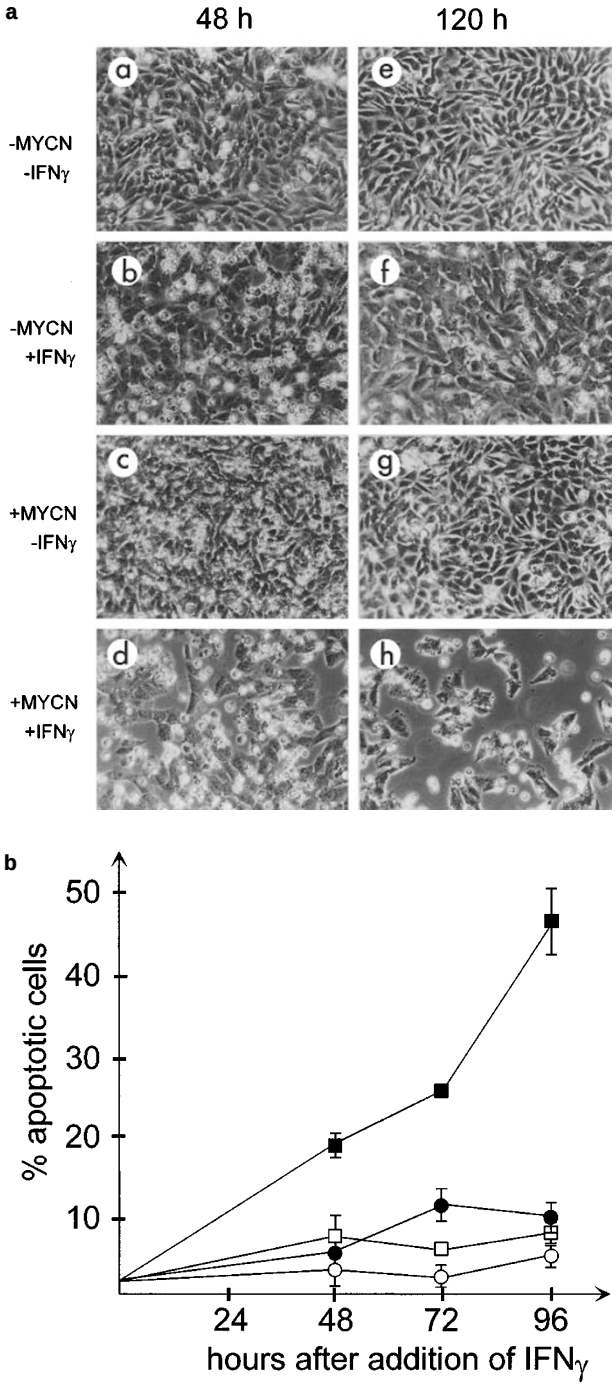


Figure 1 IFNγ and MycN cooperate to induce cell death in neuroblastoma cells. (**A**) Cells grown to confluence were either induced to express *MYCN* (c and g) or treated with 1000 U/ml of recombinant human IFNγ (b and f) or both (d and h) with a change of medium every other day. Control cells were neither induced to express *MYCN* nor treated with IFNγ (a and e). (**B**) Kinetics of cell death in response to IFNγ and/or MycN. At the time points indicated nuclei were prepared, stained with propidium iodide, and DNA content was measured by FACS analysis. The extent of cell death is given as the percentage of nuclei with a sub-diploid DNA content indicative of DNA fragmentation. ○: not expressing *MYCN*, not treated with IFNγ; ●: not expressing *MYCN*, treated with IFNγ; □: expressing *MYCN*, not treated with IFNγ; ■: expressing *MYCN* and treated with IFNγ. The standard deviation of three measurements is indicated

Results

MycN enhances IFNγ triggered cell death

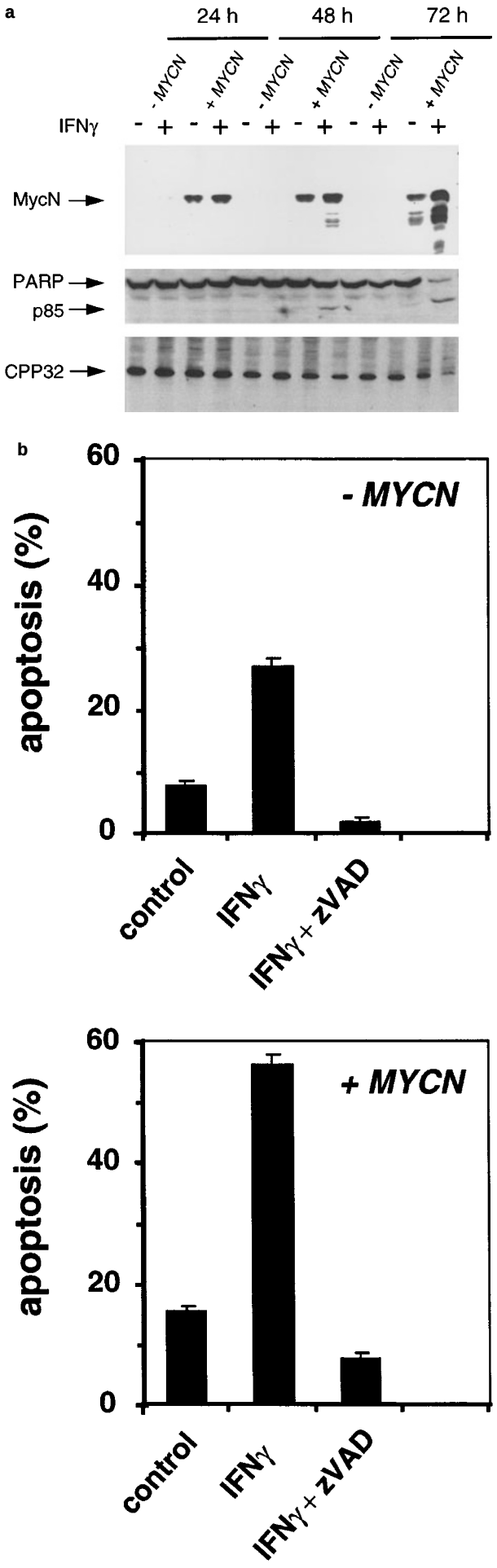
We have studied the ability of *MYCN* to induce apoptosis in neuroblastoma cells by using Tet-21/N cells, which are a derivative of the cell line SH-EP with tetracycline-controlled expression of *MYCN* (Lutz *et al*., 1996). Near-confluent cultures of Tet-21/N were cultivated in the presence (*MYCN* expression off; *MYCN*$^-$) and in the absence of tetracycline (*MYCN* expression on; *MYCN*$^+$) with a change of medium supplemented with 10% fetal calf serum every other day. Cell death was monitored by microscopic inspection. No significant cell death was seen during an observation period of 10 days regardless of *MYCN* expression status (Figure 1A; and data not shown). The same result was obtained when cells were cultivated in serum-free medium, a condition that leads to apoptosis in rat fibroblasts overexpressing c-*myc* (not shown; Evan *et al*., 1992). Only when confluent cultures were left without medium change for more than three days *MYCN*$^+$ cells responded with massive cell death, while *MYCN*$^-$ cells maintained an intact monolayer (not shown). We conclude that enforced *MYCN* expression alone is not sufficient to trigger apoptosis of neuroblastoma cells even in the absence of growth factors.

To test whether MycN can influence the cellular response to IFNγ *MYCN*$^+$ and *MYCN*$^-$ cells were treated with 1000 U/ml of IFNγ and monitored for cell death over a period of 5 days. A small fraction of cells treated with IFNγ in the absence of *MYCN* expression underwent death within 48 h after addition of IFNγ, but even after 5 days the majority of apparently unchanged cells adhered to the culture dish (Figure 1A; photographs b and f). In contrast, many of the *MYCN*$^+$ cells died within 48 h after addition of IFNγ, and only a minor fraction remained attached to the culture dish after 5 days (Figure 1A; d and h).

DNA fragmentation, determined by agarose gel electrophoresis, was seen at a low level in cells either expressing *MYCN* or treated with IFNγ, but to a much larger extent in cells expressing *MYCN* and simultaneously treated with IFNγ (data not shown). To quantitate the extent of apoptosis after addition of IFNγ and/or induction of *MYCN* cells were analysed by propidium iodide staining for the presence of hypodiploid (apoptotic) nuclei (Nicoletti *et al*., 1991). IFNγ treatment alone did not result in fragmentation within the first 48 h, and only 10% of the cells contained fragmented DNA after 96 h (Figure 1B). In contrast, IFNγ in concert with MycN induced fragmentation in 20% of the cells after 48 h, and almost 50% of the cells had a sub-diploid DNA content after 96 h (Figure 1B). We conclude that MycN and IFNγ efficiently cooperate to induce apoptosis in neuroblastomas.

Induction of cell death by IFNγ and MycN requires caspase activity

Caspases are a family of cysteine proteases involved in multiple apoptotic pathways (reviewed in Fraser and Evan, 1996). To determine if caspases play a role in MycN and IFNγ mediated cell death, the cleavage of



poly (ADP) ribose polymerase (PARP), a substrate of caspases with unknown function in apoptotis, was analysed by immunoblotting. Substantial levels of the 85 kD PARP fragment, which is generated by caspases through proteolytic cleavage of a 116 kD precursor, were detected in *MYCN*$^+$ cells treated with IFNγ (Figure 2a). Minute amounts of the 85 kD cleavage product were detected in *MYCN*$^+$ cells in the absence of IFNγ or in *MYCN*$^-$ cells treated with IFNγ (data not shown). PARP cleavage was not observed in control cells that were neither induced to express *MYCN* nor treated with IFNγ.

Caspase-3, known to cleave PARP, is a 17 kD protein generated by proteolytic cleavage of the 32 kD pro-caspase-3 (also named CPP32/Yama/apopain; Lazebnik *et al*., 1994). Immunoblotting was used to determine whether MycN and IFNγ triggered cell death is associated with cleavage of pro-caspase-3. The level of pro-caspase-3 declined within 48 h of treatment with IFNγ and/or induction of *MYCN*, indicating generation of the active form (Figure 2a). However, the decline of pro-caspase-3 was most pronounced in cells that were treated with IFNγ and simultaneously expressed *MYCN*, consistent with the previous observations.

To further determine the role of caspases in MycN and IFNγ induced cell death, zVAD-fmk, a broad range inhibitor of caspases, was added to cells exposed to IFNγ in the presence or absence of *MYCN*. Cell death was monitored by propidium iodide staining of nuclei followed flow cytometry. Cell death was almost completely blocked in the presence of 60 μM zVAD-fmk (Figure 2b).

MycN and IFNγ regulate death receptor signaling pathways

CD95 belongs to a family of cell surface receptors that trigger cell death upon binding of the ligand CD95-L (Trauth *et al*., 1989). To determine whether the CD95 death signaling system plays a role in MycN and IFNγ mediated apoptosis we analysed CD95 cell surface expression in response to IFNγ or to induction of *MYCN* expression by FACS analysis. IFNγ resulted in a twofold increase of CD95 cell surface expression that was not further increased by MycN (Figure 3a). IFNγ did not influence CD95-L expression as judged by Western-blotting using a CD95-L specific antibody (data not shown). In some experiments we observed a small increase in the amount of CD95-L in response to *MYCN* expression (data not shown). Further experiments will be necessary to clarify whether MycN regulates the level of CD95-L.

Figure 2 IFNγ and MycN triggered cell death requires caspase activity. (**a**) Western-blot with lysates of cells treated with 1000 U/ml IFNγ and/or induced to express *MYCN* for various periods. MycN, the caspase substrate PARP and pro-caspase-3 were detected using the corresponding antibodies. PARP is cleaved during cell death. Pro-caspase-3 (CPP32) disappears during cell death after 72 h. (**b**) Cell death is blocked by the caspase inhibitor zVAD-fmk. Differentially treated cells were cultivated in the presence of zVAD-fmk for 72 h. DNA fragmentation was determined by staining cells with propidium iodide and measuring fragmentation in a flow cytometer

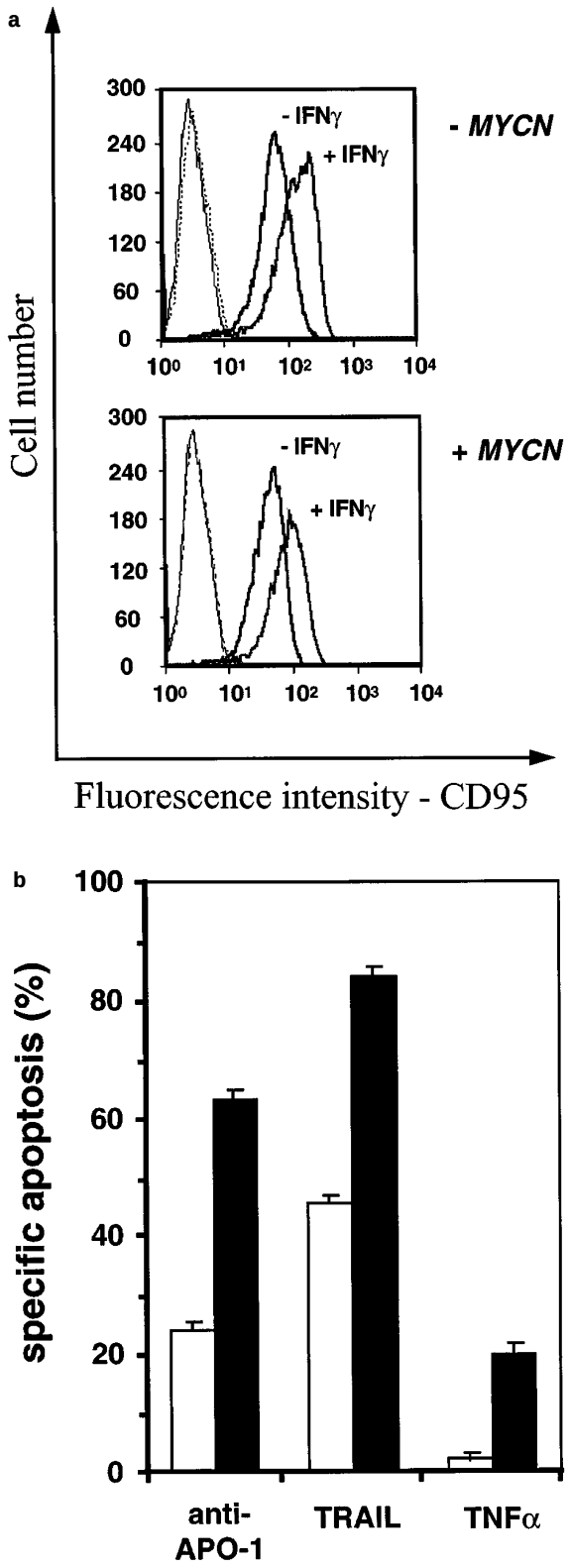


Figure 3 IFNγ and MycN regulate death receptor signaling. (**a**) FACS analysis of CD95 cell surface expression after 48 h of treatment with 1000 U/ml IFNγ and/or induction of *MYCN*. The background fluorescence obtained with a control antibody is also shown (**b**) Extent of cell death in response to various death ligands is dependent on *MYCN* expression. Cells were treated with 1 μg/ml anti-Apo-1, 10 μg/ml TRAIL, or 1 ng/ml TNFα in the presence (black bars) or absence (white bars) of *MYCN* expression for 72 h. DNA fragmentation was determined by staining cells with propidium iodide and measuring fragmentation in a flow cytometer. Specific cell death is given as the percentage of apoptotic cells after subtraction of the percentage of cells spontaneously undergoing apoptosis in control cultures

We next analysed whether *MYCN* expression could influence CD95 mediated apoptosis. Cells were treated with the monoclonal antibody anti-APO-1, which upon binding to the CD95 receptor mimicks CD95-L and initiates a signal cascade that eventually leads to apoptosis (Trauth *et al*., 1989). After 72 h cells were harvested and the extent of apoptosis was measured by FACS analysis of hypodiploid nuclei with propidium iodide. $MYCN^+$ cells showed an almost threefold increase in the proportion of cells undergoing anti-APO-1 triggered apoptosis (Figure 3b). *MYCN* expression likewise increased the sensitivity for apoptosis triggered by TRAIL and TNFα (Figure 3b). When cells were treated with IFNγ in combination with either anti-APO-1 or TNFα the percentage of dying cells was higher compared to anti-APO-1 or TNFα alone, regardless of *MYCN* expression (data not shown). Thus, IFNγ and MycN cooperate by increasing CD95 cell surface expression and enhancing the susceptibility for the CD95 death signal.

Induction of genes in response to IFNγ and MycN

Several IFNγ and Myc activated genes play a role in the induction and/or execution of apoptosis including the IFNγ immediate early gene *IRF1*, which encodes the interferon regulatory transcription factor (IRF)-1 (Tanaka *et al*., 1994; Tamura *et al*., 1995) and the Myc induced *ornithine decarboxylase* gene (Packham and Cleveland, 1994). We previously showed induction of *ornithine decarboxylase* expression by MycN in neuroblastoma cells (Lutz *et al*., 1996). Here, we used quantitative RT–PCR to show that the expression level of *IRF1* in cells exposed to IFNγ was elevated sixfold in of $MycN^+$ and 20-fold in $MycN^-$ cells (Figure 4). The *MYCN* mRNA level, which was determined as a control, was increased about 20-fold 72 h after induction of *MYCN* (Figure 4), which is in agreement with our previous data (Lutz *et al*., 1996).

ISGF3γ, which encodes the DNA binding subunit of the heteromeric transcription factor ISGF3 is another interferon inducible gene (Levy *et al*., 1990). Murine *ISGF3γ* was recently shown to be induced not only by IFNγ but also by c-Myc (Weihua *et al*., 1997). RT–PCR revealed that *ISGF3γ* mRNA was increased threefold in response to IFNγ (Figure 4). *ISGF3γ* expression did not change in response to MycN (Figure 4). The expression of *APAF1*, the recently cloned mammalian homologue of the *C. elegans ced*-4 cell death gene (Zou *et al*., 1997), and of *PITSLRE* β1 (also named *CDC2L1*), which encodes a protein kinase involved in apoptosis of Chinese hamster ovary cells (Lahti *et al*., 1994, 1995) remained unchanged in response to either IFNγ or MycN or both (Figure 4). The possible role of IRF-1 and ISGF3γ in the IFNγ and MycN triggered apoptosis of neuroblastoma cells should be determined further in future experiments.

IFNγ and MYCN *regulate pro-apoptotic members of the* BCL2 *family*

Bcl-2 and related proteins constitute an apoptosis regulatory network upstream of the caspases (Chinnaiyan *et al*., 1996; for a review see Kroemer, 1997). To test whether cooperation between MycN and IFNγ would affect the status of the Bcl-2 network, we

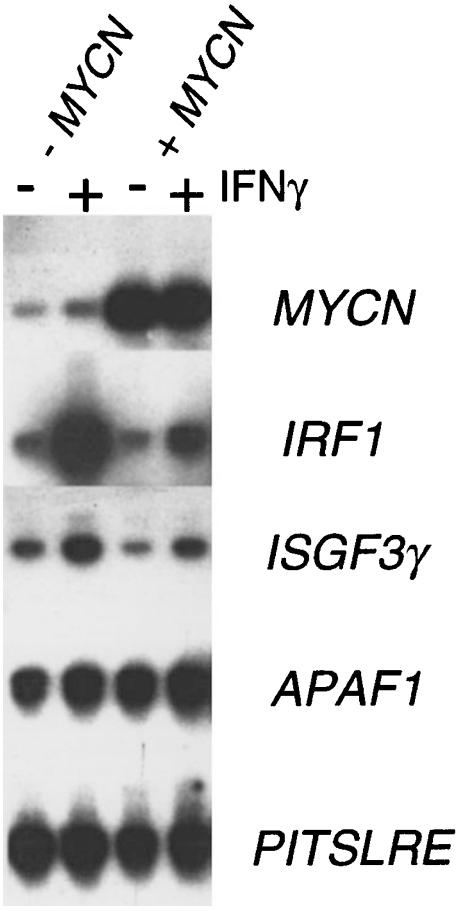


Figure 4 Gene expression in response to IFNγ and/or MycN. Quantitative RT–PCR was done employing total RNA (see Materials and methods) from cells treated with 1000 U/ml IFNγ and/or expressing *MYCN* for 72 h. PCR products after 18–21 cycles of amplification were fractionated through an agarose gel, blotted onto nylon membranes and hybridized with ^{32}P-labeled probes. After autoradiography the signals were quantitated with a phosphorimager and normalized against a co-amplified control gene (not shown)

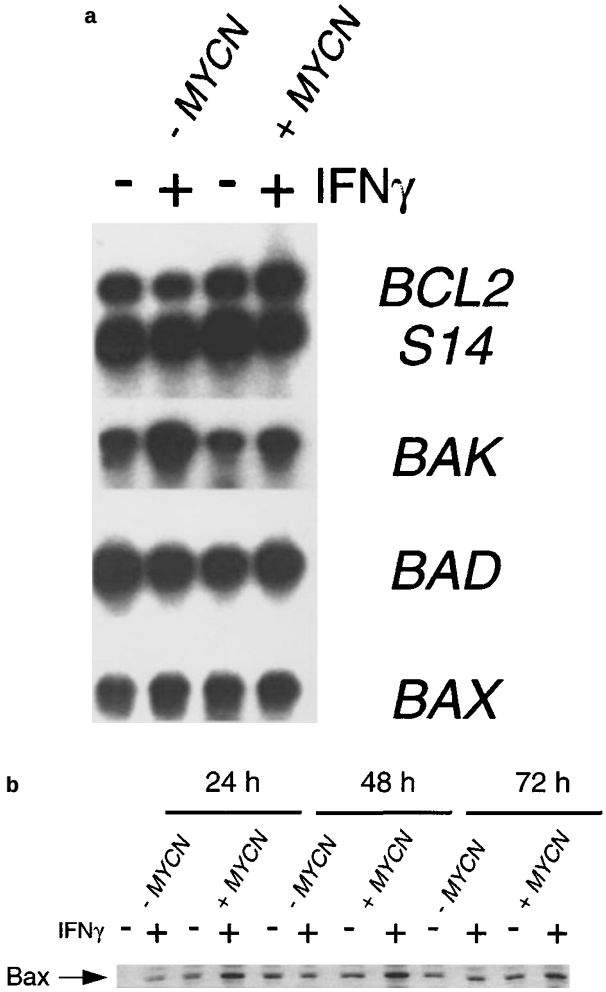


Figure 5 Expression of members of the *BCL2* family in response to IFNγ and/or *MYCN* expression. (**a**) Quantitative RT–PCR of *BCL2*, *BAK*, *BAD*, and *BAX*. (**b**) Detection of Bax protein in cell lysates from cells treated for various times with 1000 U/ml IFNγ and/or expressing *MYCN* by Western blotting with an anti-Bax polyclonal antibody (a band will appear in the first lane upon longer exposure). Proteins were carefully quantitated by triplicate analyses with the Protein Assay Kit from BioRad and samples were adjusted to equal protein concentrations

determined the expression of the anti-apoptotic *BCL2* and of the three pro-apoptotic *BCL2* family members *BAK*, *BAD* and *BAX-alpha*. The mRNA levels of *BCL2*, *BAD* and *BAX-alpha* remained unchanged after 72 h of treatment with IFNγ and/or expression of *MYCN* (Figure 5a). *BAK* mRNA level was increased threefold by IFNγ (Figure 5a). Neither MycN nor IFNγ individually changed the level of Bax protein (Figure 5b). However, in combination MycN and IFNγ led to an increase of Bax protein suggesting that they cooperate to regulate Bax at a posttranscriptional level (Figure 5b). Together, MycN and IFNγ appear to shift the balance of Bcl-2 related pro- and anti-apoptotic proteins towards death promoting proteins like Bak and Bax thereby increasing the propensity of a cell to undergo cell death in response to apoptotic signals.

Discussion

We have analysed the potential of MycN and IFNγ, alone or in combination, to influence the cell death decision of neuroblastoma cells. We show that conditional expression of *MYCN* or treatment with IFNγ barely influence the survival of neuroblastoma cells when acting alone. In contrast, we observed massive cell death when MycN and IFNγ act in concert suggesting a strong cooperation between the cytokine and the transcription factor. MycN and IFNγ triggered cell death showed major hallmarks of apoptosis as it was accompanied by DNA fragmentation and required caspase activity. Our finding of pro-caspase-3 activation in the course of MycN and IFNγ triggered apoptosis provides a potential explanation for the recently reported observation of higher levels of pro-caspase-3 in favorable neuroblastomas (Nakagawara *et al*., 1997).

Our failure to observe substantial cell death following conditional expression of *MYCN* in growth factor deprived cells does not necessarily distinguish *MYCN* from the closely related *MYC* whose over-expression in several systems has been observed to drive cells into S-phase in the absence of growth signals

and to trigger apoptosis at the same time (reviewed in Henriksson and Lüscher, 1996). *MYC* expression has been found to implement cells with programs for both proliferation and cell death. Myc triggered apoptosis has been interpreted as a safeguard mechanism that allows the elimination of cells sensing two conflicting signals, expression of the proliferation stimulating *MYC* and at the same time the absence of growth signals in the cellular environment (Evan and Littlewood, 1993). We have shown previously that ectopic expression of *MYCN* is not sufficient to drive growth factor deprived neuroblastoma cells into S-phase (Lutz *et al*., 1996). Consequently, a conflict of signals is not generated, which would explain the lack of apoptosis.

Participants in MYCN/IFNγ apoptosis of neuroblastoma cells

The cooperation between IFNγ and MycN appears complex. It included both a modulation of death signaling through the death receptor CD95 as well as an increase of Bcl-2 related proteins promoting apoptosis. Cooperation at the level of CD95 involved as one element the IFNγ mediated increase of cell surface expression of CD95, which has previously also been seen in other cell types (Friesen *et al*., 1996; Sato *et al*., 1997; Ossina *et al*., 1997). The other element was the MycN mediated increase of cellular susceptibility towards the death signal that is initiated from CD95. As shown very recently, c-Myc sensitizes rat fibroblasts to CD95 mediated apoptosis (Hueber *et al*., 1997).

Cooperation through the apoptosis-regulatory network of Bcl-2 related proteins is best demonstrated by the fact that IFNγ only in concert with MycN increased the amount of Bax protein. IFNγ alone induced expression of *BAK* while MycN alone did not transcriptionally activate any of the *BCL2* related genes tested including *BAX*, in spite of the presence of several potential binding sites for Myc proteins in the *BAX* promoter (Miyashita and Reed, 1995). The increased Bax level therefore must result from a posttranscriptional regulatory mechanism. MycN in combination with the anti-cancer drug doxorubicin also increases the amount of Bax in neuroblastoma cells (S Fulda and K-M Debatin, unpublished observations). The role of Bax as a positive regulator of apoptosis is well established (Oltvai *et al*., 1993). It is therefore reasonable to assume that the enhanced level of Bax as a consequence of *MYCN* expression and simultaneous treatment with IFNγ is a major factor in triggering apoptosis. The induction of *BAK* expression by IFNγ may further increase the propensity of a cell to undergo apoptosis. Overexpression of *BAX-alpha* has been found to restore the sensitivity to different apoptotic stimuli including serum starvation and CD95-L triggered apoptosis (Bargou *et al*., 1996). Thus, the increased Bax level in response to IFNγ and MycN may be responsible for the enhanced susceptibility towards anti-APO-1 triggered death.

MYCN mediates susceptibility for apoptosis

MycN not only cooperated with IFNγ and increased the cellular response to CD95 mediated death signals, but also enhanced the cellular susceptibility towards various additional death signals including the death ligands TRAIL and TNFα (Wiley *et al*., 1995; Pan *et al*., 1997; Sheridan *et al*., 1997). Overexpression of *MYC* has been found to increase the cytotoxicity of TNFα in rat fibroblasts (Klefstrom *et al*., 1994; Jänicke *et al*., 1994). It appears that Myc proteins can increase the cellular response towards various death signals, much in the same way they potentiate the effect of multiple growth factors. The death signals provided by TNFα, anti-APO-1/CD95-L and TRAIL, respectively, are transmitted into the cell by death receptors that use overlapping signaling pathways (reviewed by Fraser and Evan, 1996). This could explain why all of these death signals are modulated by MycN. In contrast, signaling by the IFNγ receptor proceeds through a completely different pathway (for a review see Bach *et al*., 1997). It therefore would seem unlikely that MycN targets specific components of these diverse signal transduction pathways to regulate the responses to IFNγ or other death signals. MycN may rather regulate the overall propensity of a cell to initiate cell death regardless of the death signal involved. Since the transcription factor IRF-1 is required for apoptosis in several cell types (Tanaka *et al*., 1994; Tamura *et al*., 1995), it may also mediate the IFNγ death signal in neuroblastoma cells. The weaker induction of *IRF1* (and also *BAK* and *ISGF3γ*) by IFNγ in cells expressing *MYCN* compared to cells without *MYCN* expression remains unexplained. 'Technical knock out' has identified several genes that beside *IRF1* are required for IFNγ triggered apoptosis (Deiss and Kimchi, 1991; Levy-Strumpf *et al*., 1997).

Clinical perspectives?

The observations made in this study entail several considerations of clinical relevance. The CD95 system is involved in drug-induced apoptosis in neuroblastoma cells, and Bak, at least in lymphoid cells, accelerates chemotherapy-induced apoptosis (Fulda *et al*., 1997; Simonian *et al*., 1997). By up-regulating both *CD95* and *BAK* IFNγ may increase the susceptibility of neuroblastoma cells to death triggered by anti-cancer drugs, providing a rationale for employing IFNγ in the treatment of neuroblastomas. The changes in gene expression described here may constitute part of a general cellular response to IFNγ as up-regulation of *CD95* and *BAK* expression by IFNγ was recently found in the human colon adenocarcinoma cell line HAT-29 (Ossina *et al*., 1997). Since IFNγ cooperates with MycN to trigger apoptosis those patients with tumors showing strong *MYCN* expression as a result of gene amplification may benefit particularily from IFNγ based therapeutical regimens.

Materials and methods

Cell culture and treatment with cytokines, antibodies and caspase inhibitor

The generation, characterization and culture of the *MYCN* inducible cell line SH-EP Tet-21/N has been described (Lutz *et al*., 1996). Induction of *MYCN* expression by removal of tetracycline from the growth medium and addition of recombinant human IFNγ (Boehringer, Mannheim) at 1000 U/ml or recombinant human TNFα at 1 ng/ml (BIOTREND, Köln, Germany) were carried out

shortly before cultures reached confluence as apoptosis was most pronounced in confluent cultures. The broad range caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp(*O*-methyl-)fluoromethylketone, zVAD-fmk (Enzyme Systems Products, Dublin, CA), was used at a concentration of 60 μM. Anti-APO-1 was added at 1 μg/ml (Trauth *et al*., 1989) and recombinant TRAIL purified from *Pichia pastoris* at 10 μg/ml (Jeremias J, Herr I, Böhler T, Debatin K-M. TRAIL-induced apoptosis in T-cells, submitted).

DNA fragmentation assays

For visualization of DNA fragmentation by gel electrophoresis both adherent and non-adherent cells were collected from a 10 cm cell culture dish and lysed with 200 μl lysis buffer (20 mM Tris pH 7.4/1 mM EDTA/0.4% Triton X-100) for 5 min on ice. After clearing the lysate by centrifugation nucleic acids were extracted with phenol/chloroform, precipitated with ethanol, resuspended in TE, treated with 300 μg/ml RNase A, fractionated on a 1.5% agarose gel and stained with ethidium bromide.

For determination of the fraction of cells with DNA fragmentation nuclei prepared from cells grown in 24-well microtiter plates were stained with propidium iodide and subsequently analysed by flow cytometry (Nicolleti *et al*., 1991). Ten thousand events were acquired for each sample using a FACScan (Becton Dickinson, Heidelberg, Germany) and CELLQuest software. The extent of cell death is given as the percentage of nuclei with a subdiploid amount of DNA as a consequence of DNA-fragmentation.

Western blotting

Preparation of cell lysates and determination of protein concentration were performed as described (Lutz *et al*., 1996). For Western-blotting 15 μg of protein were separated on an SDS-polyacrylamide gel and blotted onto nitrocellulose membranes (Schleicher & Schuell). Proteins were visualized using the ECL detection system (Amersham). The following antibodies were used: anti MycN monoclonal antibody (Wenzel *et al*., 1991), anti Bax polyclonal antibody Ab-1 (Calbiochem), anti-PARP polyclonal antibody (Boehringer, Mannheim), anti CPP-32 monoclonal antibody (Signal Transduction Laboratories, Lexington, KY).

FACS quantification of CD95 cell surface expression

10^6 cells were harvested, washed in PBS/1% BSA and incubated successively with 1 μg/ml anti-APO-1 (CD95) monoclonal antibody (Trauth *et al*., 1989) and with an phycoerythrin coupled goat anti-mouse secondary antibody (Immunotech, Hamburg, Germany) for 45 min at 4°C each. FII23 antibody (Trauth *et al*., 1989) was used as an isotype matched non-binding antibody to control for unspecific binding.

Gene expression analysis by RT–PCR

PCR primers (Table 1) were designed based on sequences retrieved from the EMBL data base. Total RNA was isolated from cells using guanidinium thiocyanate (Chomczynski and Sacchi, 1987), treated with DNase I, and extracted with phenol/chloroform. All subsequent enzymatic reactions were performed with all samples at the same time using master mixes. 1 μg of RNA was used for cDNA first strand synthesis in a 20 μl reaction (1st strand cDNA synthesis kit for RT–PCR; Boehringer, Mannheim). 1 μl of this reaction (corresponding to 50 ng of reverse transcribed RNA) was used as template in the polymerase chain reaction which was prepared on ice and contained in a volume of 50 μl 1.5 mM $MgCl_2$, 200 μM of each dNTP, 50 pmol of each primer, 5–25 pmol of primers for the co-amplification of the genes encoding ribosomal protein S14, glycerolaldehyde-3-phosphate dehydrogenase (*GAPDH*) or dihydrofolate reductase (*DHFR*), which served as an internal control and 1 U of Taq DNA Polymerase (Promega). For amplification of the *MYCN* PCR fragment with a GC content of 75% the Advantage-GC cDNA PCR kit (Clontech Laboratories) was used. PCR conditions were 95°C for 3 min followed by 95°C, 62°C, and 74°C for 1 min each in a Biometra thermal cycler. For the initial testing of primers and primer combinations 30 cycles were performed. For quantitative PCR the reaction was terminated after 18 or 21 cycles before non linear conditions were reached. 10 μl of each reaction was seperated on a 1.5% agarose gel, blotted onto Hybond N^+ (Amersham), and sequentially hybridized to ^{32}P-labeled probes. Radioactive signals were quantitated using a model 400 phosphorimager (Molecular Dynamics) and normalized to the signal of the co-amplified gene.

Table 1 Primers used for RT–PCR

Gene	*co-amplified gene*	*Sequence of primers 5′→3′*	*Length of PCR product*
MYCN	–	TCCAGGACTGCATGTGGAGC	513
		CCACAGTGACCACGTCGATTTC	
IRF1	S14	CATCCCAGTGGAAGTTGTGCC	475
		CAGAAGTCCAGCTTCTCTGCACC	
ISGF3γ	DHFR	AGGACTTCCGGGAGGACCAG	766
		GGTGCATTCCAGGAGATGGG	
BCL2	S14	TGTGGCCCAGATAGGCACCCAG	370
		ACTTCGCCGAGATGTCCAGCCAG	
BAX	DHFR	AGCAGATCATGAAGACAGGGGC	519
		TCTTCCAGATGGTGAGCGAGG	
BAD	GAPDH	TTAAGAAGGGACTTCCTCGCCC	423
		AAACCTGGCTCGCGACTTAGC	
BAK	S14	GATTCTGGGCTTGGGGTGTG	438
		TAGACAGGTGAGGAGCATGGAGG	
APAF1	–	AAGGTGGCTGATTGCAGAGGAC	487
		TGTCCAATTGCCAATTCCATACC	
PITSLRE β1	S14	TCAGAAGCCTCTGTTCCCCG	441
		GTGGTGGTAAGGTGGAAGCCC	
SI4		GGCAGACCGAGATGAATCCTCA	144
		CAGGTCCAGGGGTCTTGGTCC	
DHFR		AGACCTGGTTCTCCATTCCTGA	175
		GTCTTGCATGATCCTTGTCACA	
GAPDH		TCGGAGTCAACGGATTTGGTCGTA	480
		ATGGACTGTGGTCATGAGTCCTTC	

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