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Differentiation and Apoptosis of Neuroblastoma Cells: Role of N-myc Gene Product

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Abstract To clarify the role and function of the N-myc product in cell differentiation and apoptosis, we used the antisense oligonucleotide technique to inhibit N-myc gene expression in neuroblastoma cells with different phenotypes: intermediate (I) and neuronal (N), or Schwann-glia (S), respectively. The results suggest that N-myc operates along different pathways. Inhibiting N-myc gene expression either results in suppression of cell proliferation or in induction of differentiation and/or apoptosis. J. Cell. Biochem. 73:97–105, 1999. © 1999 Wiley-Liss, Inc.

Key words: N-myc; apoptosis; differentiation; neuroblastoma

The N-myc and c-myc genes, members of a small family of related proteins that function as transcription factors, share stretches of strong sequence homology [Packham and Cleveland, 1995; Rosolen et al., 1990; Zimmerman et al., 1986, Puri et al., 1998]. However, while c-myc is fairly ubiquitous and is expressed in proliferating cells, N-myc expression is limited to brain cells, kidney cells and lymphocytes, particularly during the early stages of differentiation [Negroni et al., 1991; Whitesell et al., 1991]. Deregulated c-myc expression has been implicated in the growth of a large number of tumors. By contrast, aberrant N-myc expression has been observed only in a restricted group of neuroectodermal cancers, such as neuroblastoma (NB), retinoblastoma, and small cell lung carcinoma [Rosolen et al., 1990]. Another difference is the ability of c-myc to induce apoptosis when inappropriately expressed, while little is known about the ability of N-myc to induce programmed cell death [Leonetti et al., 1996; Sakamuro et al., 1995].

Neuroblastoma tumors are good models in which to examine the biological role of N-myc. When established in culture, human NB cell lines are typically heterogeneous cellular subpopulations, including neuroblastic (N), substrate adherent (S), and intermediate (I) cell types. These can be distinguished on the basis of their morphological and biochemical characteristics [Ciccarone et al., 1987; Rettig et al., 1987; Ross et al., 1983].

The role of N-myc in NB cells has been studied by inhibiting its expression in cell cultures either by antisense oligonucleotides targeted against N-myc mRNA or by expression vectors designed to generate N-myc antisense RNA [Negroni et al., 1991; Rosolen et al., 1990; Whitesell et al., 1991].

Negroni et al. [1991] observed that antisense inhibition of N-myc expression decreases the proliferation rate and the induction of neuron differentiation. This agrees with other studies [Hanada et al., 1993; Peverali et al., 1996], which show that Retinoic acid-induced N-myc inhibition anticipates the morphological differentiation of N type cells. However, Whitesell [1991] observed that IMR-32 NB cells, known to differentiate in response to retinoic acid, appeared to be blocked in a non differentiated

Abbreviations used: BrdU, bromodeoxyuridine; HPRT, hypoxanthine guanosine phosphoribosyl transferase; NB, neuroblastoma; ENO, neuron-specific enolase; RA, retinoic acid.

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phenotype when N-myc expression was antisense inhibited.

We decided to investigate the effect of antisense inhibition of N-myc gene expression in NB cells with intermediate, neuron, or Schwann-glia distinct phenotypes, in order to clarify the role of N-myc in differentiation and apoptosis of neuroblastoma cells..

MATERIALS AND METHODS

Cell Cultures

I type SKNBE(2)C human neuroblastoma cells, grown in monolayer, were mantained at 37°C, 5% carbon dioxide in RPMI containing 15% fetal calf serum (FCS), 2 mM L-glutamine, 50 U/ml penicillin, and 100 µg/ml streptomycin. To induce permanent Schwannian cell differentiation (S type cells), the cell cultures were treated with 10 µM BrdU for 13 days [Ross et al., 1991; Hanada et al., 1993]. Neuron cell conversion (N type cells) was obtained by adding 10 µM RA to the culture medium [Ross et al., 1991; Peverali et al., 1996]. The treatment was prolonged for 6 days. Media were replaced every 3–4 days. The onset of differentiation was monitored by morphological analysis and by determination of Ret, neuron-specific enolase (NSE), and fibronectin mRNA levels.

Proliferation Assay

Neuroblastoma cells were seeded at 15,000/flask in 2 ml of medium. N-myc antisense, reverse, or scrambled oligos were added at a final concentration of 20 µM, after 24 h. Cells then were harvested after another 24 h and the viable cells were counted in a hemocytometer by trypan blue exclusion.

Synthesis of Oligodeoxyribonucleotides

Oligodeoxyribonucleotide synthesis was carried out on an automatic DNA synthesiser (model 200A, Beckman Instruments Italia, Milan, Italy) using the β-cyanoethyl phosphoroamidate chemistry, as reported by Iacomino et al. [1994]. The partial phosphorothioated oligonucleotides used to study N-myc gene expression were: antisense 5′-GAT CAT GCC CGG CAT, complementary to the mRNA region encompassing ATG starting codon; reverse 5′-TAC GGC CCG TAC TAG, and scrambled 5′-GAG CAT TCC CAG CGT. These oligos had 11 normal phosphodiester internucleoside linkages, followed by three phosphorothioate linkages, that gave a 3′ terminal phosporothioated section.

Sequences for the human N-myc, β-actin, HPRT, ENO, Ret, fibronectin, Bcl-2, Bax, Bak, and NAIP mRNAs from GeneBank (DNASTAR, Madison, WI) were used to design the primer pairs for the RT-PCR experiments (OLIGO 4.05 software, W. Rychlik ©1992). Primers were 20/22 nucleotides long and contained 50–60% G/C.

RNA Extraction and RT-PCR

Total RNA was extracted from cell cultures using the RNAzol reagent (Biotec Laboratories, San Diego, CA) according to the manufacturer's protocol. The mRNA levels of the genes under analysis were measured by reverse transcription-polymerase chain reaction (RT-PCR) amplification, as reported [Galderisi et al., 1996].

The RT-PCR product sizes were 224 bp for N-myc, 581 bp for β-actin, 369 bp for HPRT, 154 bp for ENO, 444 bp for Ret, 450 for fibronectin, 389 bp for Bcl-2, 154 bp for Bax, 465 bp for Bak, and 433 bp for NAIP.

Appropriate regions of HPRT and/or β-actin cDNAs were used as controls. Amplifications, carried out for 28–30 cycles, were as follows: 94°C for 1′, 57°C for 1′ and 72°C for 1′. Each RT-PCR experiment was repeated at least three times. Amplification products were electrophoresed on 2% agarose gel in 1× TAE buffer. Semiquantitative analysis of mRNA levels was carried out by the GEL DOC 1,000-UV fluorescent gel documentation system (BioRad, Hercules, CA).

TUNEL Assays and Determination of Apoptic Index

The cells for TUNEL assays were grown on glass coverslips. After oligo treatment, the cells were fixed with 4% paraformaldehyde for 15′, and the TUNEL reaction was performed according to manufacturer's instructions (Boehringer Mannheim). The cells were then counterstained with Giemsa dye and observed under a light optical microscope. The apoptic index was calculated by the number of positive TUNEL cells out of 1,000 cells in 4/5 different microscope fields.

RESULTS

The effects of N-myc antisense, reverse, and scrambled oligomers were evaluated first on N-myc gene expression in SKNBE(2)C cell cultures. Oligonucleotides were added exogenously

at a final concentration of 1 µM, 5 µM, 10 µM, and 20 µM to NB cultured cells. Effects on target mRNAs were monitored at 6 and 20 h after oligo addition by semiquantitative RT-PCR amplification of N-myc and HPRT mRNA, respectively. After a 6-h incubation, antisense oligos produced a threefold reduction of N-myc mRNA level at most as compared with reverse and scrambled oligos (Fig. 1A). After a 20-h incubation, only the highest concentrations (10 and 20 µM) maintained a good level of gene inhibition (Fig. 1B). These results verified that the antisense effect is dose dependent and sequence specific. Further antisense treatment of NB cultures always was done with oligomers at 20-µM concentration.

I type is the predominant subpopulation of the SKNBE(2)C cell line [Piacentini et al., 1992]. This line can be induced to differentiate permanently to S phenotype by treating with BrdU [Ross et al., 1991] or to N phenotype by RA treatment [Ross et al., 1991; Peverali et al., 1996].

N type cells with a small, rounded, loosely adherent cell body and numerous neuritic processes contain noradrenergic biosynthetic enzymes and express N-myc, ENO, and Ret protooncogene, an early marker of neuronal differentiation. S type cells, resembling epithelial or fibroblast cells and committed to glial phenotype, have a high expression of fibronectin. In addition, the N-myc levels are lower than in N type cells. I type cells show intermediate characteristics between N and S phenotypes. Several studies have demonstrated that both N and S type cells are capable of spontaneous interconversion (transdifferentiation) to the other cell type. I type cells could represent cellular intermediates or, alternatively, stem cells from which both N and S cells arise [Ciccarone et al., 1987; Whitesell et al., 1991; Bunone et al., 1995].

To obtain NB cells with a distinct intermediate, neuronal, or Schwann-glia phenotypes, SKNBE(2)C cultures were treated for 13 days with BrdU, or for 6 days with RA. Differentiation onsets were monitored by morphological and biochemical analyses. As expected, RA-treated cells exhibited neurite extensions, reduction in size and small loosely adherent cell bodies along with a great increased early neuronal differentiation markers, such as ENO and Ret. Conversely, a reduction of fibronectin gene expression, a marker of S cell phenotype, was observed (Fig. 2). BrdU-treated cells became larger than I type and showed abundant strongly adherent cytoplasm and no processes. A substantial increase of fibronectin expression and a remarkable reduction of ENO and Ret levels was observed at the same time (Fig. 2). N-myc mRNA targeted antisense oligonucleotides were then added to cultures showing I, S, and N phenotypes, respectively, and a number of different biological effects were evaluated.

Antisense-treated I type cells showed a significant reduction of cell proliferation. It was noted that RA and BrdU induced SKNBE(2)C cells, already grew at a slower rate than I type cells. However, when these SKNBE(2)C induced cultures were treated with N-myc antisense molecules they had an even slower rate of proliferation (Table I).

The number of viable cells determined by tripan blue exclusion was greater than 93% in all cultures treated with control oligomers. I

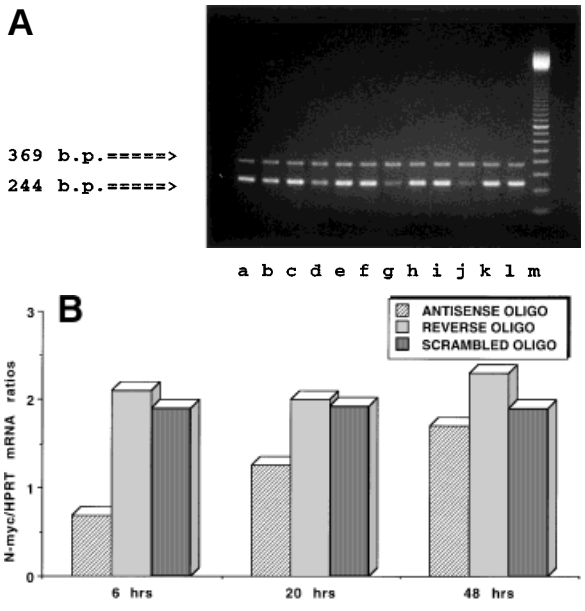

Fig. 1. **A:** Agarose gel electrophoresis analysis of reverse transcription-polymerase chain reaction (RT-PCR) products of N-myc and HPRT mRNAs. Arrows, relative position of the DNA fragments (369 bp for HPRT and 244 bp for N-myc, respectively). Cells were treated with partial phosphorothioate oligos for 6 h. **Lanes a, d, g, j,** antisense oligo at 1, 5, 10, and 20 µM concentration, respectively; **lanes b, e, h, k,** reverse oligo at 1, 5, 10, and 20 µM concentration, respectively; **lanes, f, i, l,** scrambled oligo at 1, 5, 10, and 20 µM concentration, respectively; **lane m,** 100-bp ladder as molecular-weight marker. **B:** N-myc/HPRT mRNA ratios measured by RT-PCR in SKNBE(2)C cell cultures treated with 20 µM partial phosphorothioate oligos for 6, 20, and 48 h, respectively. Each value is the mean of three different experiments.

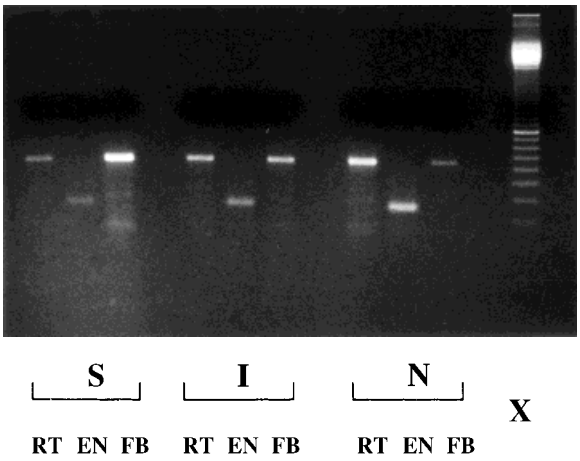

Fig. 2. Agarose gel electrophoresis analysis of ENO, Ret, fibronectin, and HPRT reverse transcription-polymerase chain reaction (RT-PCR) products in untreated SKNBE(2)C cells (I type) treated with RA for 6 days (N type cells) and with BrdU for 13 days (S-type cells). RT, Ret; EN, ENO; FB, fibronectin; X, 100-bp ladder as molecular-weight marker.

TABLE I. Cell Proliferation Assay[a]

	S type cells	I type cells	N type cells
Antisense	17,000	24,000	22,000
N-Myc	(±2,100)	(±2,000)	(±3,100)
Reverse	27,000	43,000	31,000
N-Myc	(±2,300)	(±3,000)	(±2,500)
Scrambled	29,000	39,000	33,000
N-Myc	(±2,900)	(±3,100)	(±3,000)

[a]Neuroblastoma cells seeded at 15,000/flask in 2 ml of medium. After 24 h N-myc antisense, reverse or scrambled oligos were added to a final concentration of 20 µM. After a further 24 h of incubation, cells were harvested, and the number of viable cells was determined.

type cell cultures and NB cells, RA induced to N phenotype, showed the same level of viable cells when treated with antisense molecules. Conversely, cultures BrdU induced to S phenotype had a decline in cell viability to an 80% level after 24 h of treatment with antisense oligos.

To determine whether antisense-mediated N-myc inhibition can induce NB cell differentiation, ENO, Ret, and fibronectin mRNA levels were measured by semiquantitative RT-PCR. At 6 h after adding antisense molecules, SKNBE(2)C cells (I type) showed no change in ENO, Ret, and fibronectin mRNA levels, compared with cells treated with reverse or scrambled oligos (Fig. 3, Table II). The expression level of these genes did not change when antisense treatment was prolonged to 20 and 48 h (Table II). NB cells committed to be neurons (N type) seemed to be induced further toward a more differentiated phenotype when treated with antisense molecules. In fact, a great increased ENO and Ret gene expression along with reduction of fibronectin levels was observed in RA treated neuroblastoma cells (Fig. 3, Table II). Antisense oligo treatment of cells with S phenotype further increased the fibronectin level. At the same time ENO and Ret mRNAs were barely detectable (Fig. 3, Table II).

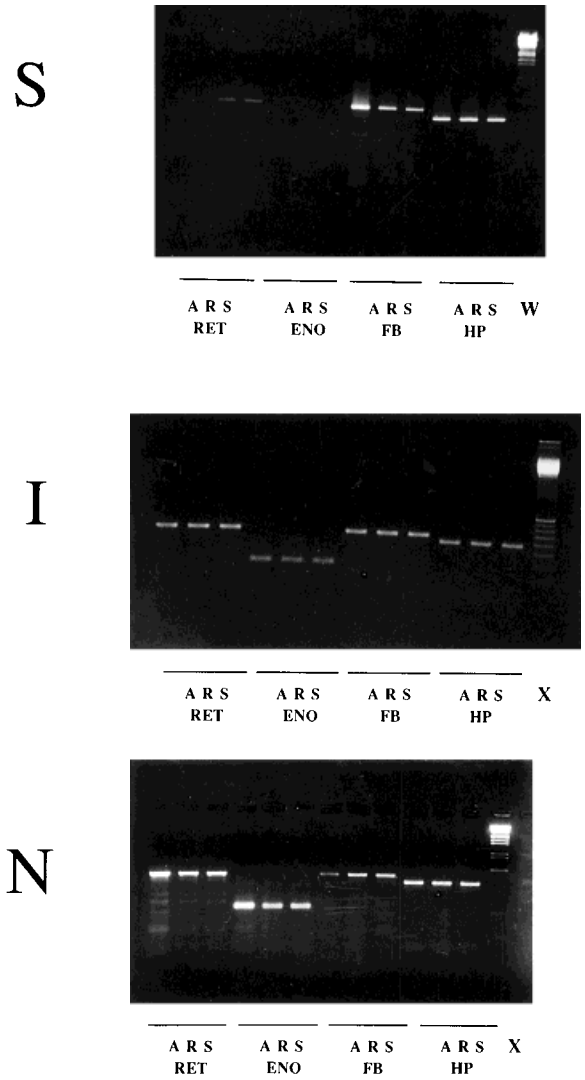

Fig. 3. Agarose gel electrophoresis analysis of Ret, ENO, fibronectin, and HPRT reverse transcription-polymerase chain reaction (RT-PCR) products in neuroblastoma cell treated for 6 h with 20 µM antisense (A), reverse (R), and scrambled (S) oligos, respectively. Left, S, S type; I, I type; N, N-type cells. RET, Ret; ENO, ENO; FB, fibronectin; HP, HPRT; X, 100-bp ladder as molecular weight marker; W, molecular-weight marker III (Boehringer Mannheim).

TABLE II. Gene Expression Determined by RT-PCR[a]

	S type cells			I type cells			N type cells		
	6 h	20 h	48 h	6 h	20 h	48 h	6 h	20 h	48 h
RET	21*	15*	16*	109	107	105	232*	247*	254*
ENO	0*	0*	0*	99	105	102	189*	198*	215*
Fibron	256*	360*	398*	96	95	104	35*	30*	12*
β-Actin	104	98	109	96	104	95	103	98	100
HPRT	94	96	103	99	105	100	103	97	97
Bcl-2	36*	30*	24*	104	106	103	110	106	107
Bax	202*	213*	228*	100	97	101	97	98	95
Bak	168*	190*	203*	99	96	98	93	94	93
NAIP	106	103	104	99	102	105	108	106	103
β-Actin	96	98	106	103	102	107	104	95	102
HPRT	100	96	104	98	105	102	101	95	97

[a]The values reported are calculated considering the mRNA levels of the above indicated genes in the cells treated with control oligos at 100. The mRNA levels were measured after 6, 20, and 48 h of oligo treatment. Each value is the mean of at least three different experiments. β-actin and HPRT mRNA levels were used as reverse transcription-polymerase chain reaction (RT-PCR) control.

*Significant level variations ($p < 0.01$).

To assess the role of N-myc in programmed cell death, we analyzed the effect of its down-regulation on the expression of some genes belonging to the Bcl-2 family. This gene family comprises death-inducing and death-inhibitory members which differ in tissue and activation expression patterns. Among these genes, Bcl-2, possessing an antiapoptic activity, and Bax or Bak, possessing proapoptic features, play a central role in almost all cell types. The ratio of death agonist to death antagonist genes can determine cell fate [Kroemer, 1997; Reed, 1997]. Another gene that encodes a neuronal apoptosis inhibitor protein (NAIP) has been isolated recently. This gene seems to play a fundamental role in nervous system development. In fact, it is deleted partly in individuals with neurodegenerative disorders [Liston et al., 1996].

The expression of these Bcl-2, Bax, Bak, and NAIP genes was then evaluated in NB cell cultures treated either with antisense or with control oligos. Neither I type NB cells nor those induced toward N phenotype showed any modification of Bcl-2, Bax, Bak, and NAIP mRNA levels when treated with anti N-myc oligonucleotide (Fig. 4, Table II). On the other hand, the anti N-myc oligonucleotide treatment of NB cell with S phenotype produced a clear upregulation of the proapoptic Bax and Bak gene expressions along with a drastic decrease in the antiapoptic Bcl-2 mRNA level. No change in NAIP gene expression was noted (Fig. 4, Table II).

To confirm the activation of the apoptosis program in N-myc antisense-treated cells, showing S type morphology, we evaluated the number of apoptic cell by in situ cell death detection technique (TUNEL reaction). Several cells with apoptic features were identified after only 6 h of antisense treatment (Fig. 5A,B). A threefold increase of the apoptic index was observed in cells treated with antisense molecules for 48 h compared with reverse and scrambled oligo-treated cells (Fig. 6).

DISCUSSION

Myc family proto-oncogenes act as transcription factor to regulate gene expression. While the role of the c-myc gene in the regulation of the cell cycle, cell proliferation, and apoptosis is clearly understood, the biological function of N-myc is not as clearly understood. The antisense inhibition of N-myc gene expression in different Neuroblastoma cell types provides us with an opportunity to examine the role that N-myc plays in the nervous system.

The identification of a 15-mer oligodeoxynucleotide that reduces human N-myc expression in neuroblastoma cell lines [Rosolen et al., 1990; Negroni et al., 1991] presents some problems. The authors used a high concentration of normal phosphodiester antisense oligonucleotides and only sense oligo as a control. Instead, we used partial phosphorothioate oligos that show an increased lifespan and are known to be effective at low concentration. To claim a spe-

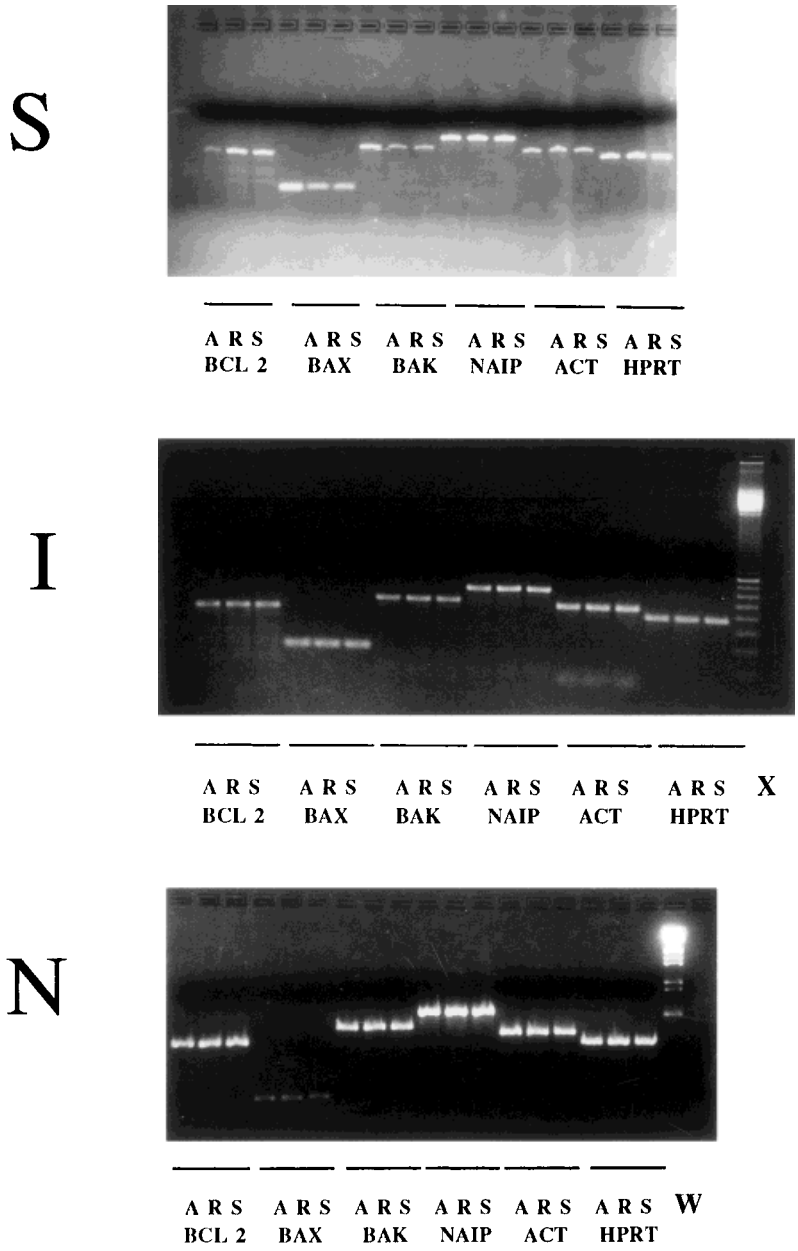

Fig. 4. Agarose gel electrophoresis analysis of Bcl-2, Bax, Bak, NAIP, β-actin, and HPRT reverse transcription-polymerase chain reaction (RT-PCR) products in neuroblastoma cell treated for 6 h with 20 μM antisense (A), reverse (R), and scrambled (S) oligos, respectively. Left, S, S type; I, I type; N, N-type cells. BCL-2, Bcl-2; BAX, Bax; BAK, Bak; NAIP, NAIP; ACT, β-actin; HPRT, HPRT; X, 100-bp ladder as molecular-weight marker; W, molecular weight marker III (Boehringer Mannheim).

cific effect for our antisense molecules, we used two control oligos containing dG/dC stretches that are also present in the antisense oligo sequence. These oligos are more suitable than sense oligos for investigating the specificity of target mRNA inhibition. Several investigators have admitted problems in interpreting data derived from in vitro and in vivo use of antisense oligos which present stretches of dG and dC in their sequence [Yaswen et al., 1993]. The anti N-myc oligos that we used clearly produced a dose-dependent and sequence-specific inhibition of N-myc gene expression, and were particularly useful for studying cellular gene function.

Recognizing that NB sublines exhibit a variety of phenotypic and functional characteristics, we used an antisense oligo to inhibit N-myc expression in NB cells having I type morphology, before and after their induction toward an overt neuronal or Schwann-glia phenotype. Exposure of SKNBE(2)C cells (I type) to anti N-myc molecules resulted in no modification of ENO, Ret, and fibronectin mRNA levels, chosen as hallmark indicators of N type and S type differentiation, respectively. By contrast,

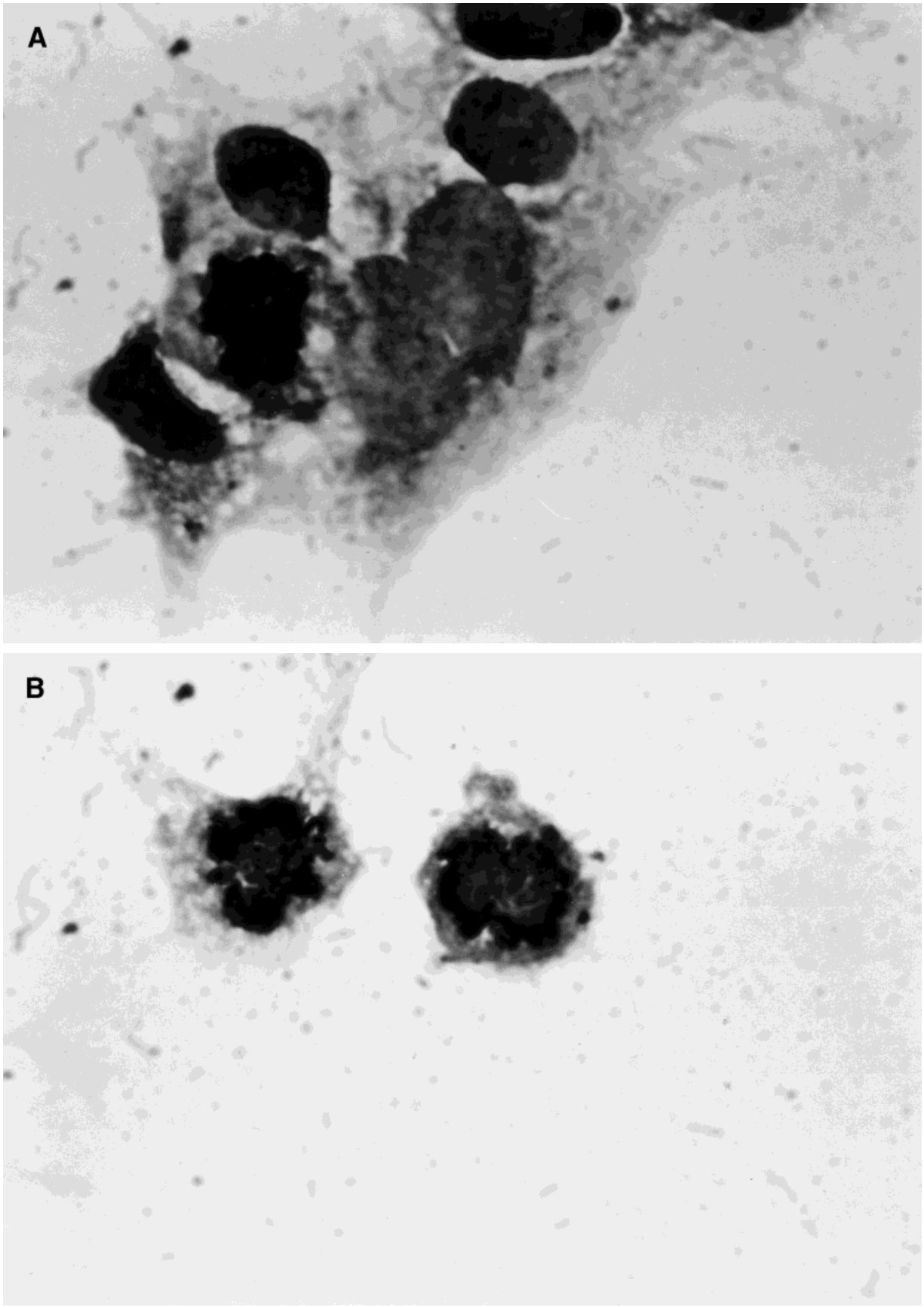

Fig. 5. **A,B:** Tunel reaction perfomed on neuroblastoma S-type cells grown on glass coverslips. The apoptic cells, showing condensed chromatin mainly at nuclear margins, were stained with Tunel dye. The other cells were counterstained with Giemsa. ×1,200.

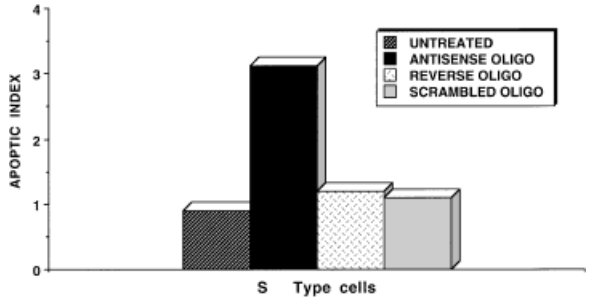

Fig. 6. Apoptic index of neuroblastoma S-type cells grown for 48 h in the presence or absence of 20 μM partial phosphorothioate oligos (upper box).

NB cells committed to be either neurons or Schwann-glia cells seemed to show a further induction toward a more differentiated phenotype when treated with antisense molecules. These effects did not revert after the decline of antisense-mediated inhibition of N-myc expression. In addition, SKNBE(2)C cells (I, S, and N type), treated with N-myc antisense oligomers, showed a significant reduction in cell proliferation compared with cells treated with control oligos. I and N type cell cultures showed a 93% cell viability when treated with antisense molecules. Conversely, S cell cultures showed an 80% cell viability level 24 h after treatment with antisense oligos.

Taken together, these results indicate that in the intermediate cells, N-myc expression appears to have a direct effect on cellular proliferation, independent of differentiation. By contrast, inhibition of proliferation, in the N and S type cell seems to be because of differentiation induced by N-myc antisense molecules. In addition, antisense treatment determined a loss of cell viability in the Schwannian-glia cells (S type) along with induced differentiation. Both phenomena contributed to a decline in cell proliferation.

The apoptosis could also account for the reduced S cell viability. Anti N-myc oligo treatment of S cells resulted in a clear increase of the proapoptic Bax and Bak gene expression, along with a drastic decrease in the level of antiapoptic Bcl-2 mRNA. Furthermore, a threefold increase of the apoptic index is observed in these cells after treatment with antisense molecules for 48 h. Because NB cells with I and N phenotypes did not show any modification of Bcl-2, Bax, Bak, and NAIP mRNA levels when treated with anti N-myc oligos, we believe it is reasonable to exclude triggering of any apoptic process.

The data do not allow us to determine whether downregulation of N-myc expression has a direct or an indirect effect on apoptosis in S type cells. Hanada et al. [1993] reported that Bromodeoxyuridine stimulation of I type cells induced differentiation toward a Schwannian phenotype. This type of induction of differentiation was accompanied by a substantial reduction in the anti-apoptic Bcl-2 gene expression, leading to an increase in apoptic death. Thus, the apoptic process observed in S type cell lines treated with N-myc antisense molecules could be a side effect of differentiation toward a Schwannian-glia phenotype. However, no increased expression of the proapoptic genes Bax and Bak in neuroblastoma cells induced toward S type differentiation has been reported.

The differentiation and apoptosis that followed antisense treatment of neuroblastoma cells persisted after the end of N-myc gene inhibition, indicating that a lasting N-myc downregulation is not required to induce these processes. These studies suggest that N-myc gene inhibition in neuroblastoma cells can result in one of the three pathways: (1) suppressed cell proliferation; (2) induced differentiation, and/or (3) apoptosis. Thus, it seems that N-myc operates through different pathways that involve activation of different genes. The chosen pathwaywill depend on the different cell types.

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REFERENCES

Bunone G, Borrello MG, Picetti R, Bongarzone I, Peverali FA, De Franciscis V, Della Valle G, Pierotti MA. 1995. Induction of RET proto-oncogene expression in neuroblastoma cells precedes neuronal differentiation and is not mediated by protein synthesis. Exp Cell Res 217:92–99.

Ciccarone V, Spengler BA, Meyers MB, Biedler JL, Ross RA. 1989. Phenotypic diversification in human neuroblastoma cells: Expression of distinct neural crest lineages. Cancer Res 49:219–225.

Galderisi U, Cipollaro M, Melone MAB, Iacomino G, Di Bernardo G, Galano G, Zappia V, Cascino A. 1996. Myotonic dystrophy: Antisense oligonucleotide inhibition of DMPK gene expression in vitro. Biochem Biophys Res Commun 221:750–754.

Hanada M, Krajewski S, Tanaka S, Cazals-Hatem D, Spengler B, Ross RA, Biedler JL, Reed JC. 1993. Regulation of Bcl-2 oncoprotein levels with differentiation of human neuroblastoma cell. Cancer Res 53:4978–4986.

Iacomino G, Galderisi U, Cipollaro M, Di Biase S, La Colla P, Marongiu ME, De Rienzo A, De Falco S, Galano G, Cascino A. 1994. Phosphorothioated antisense oligonucleotides: Prospects for AIDS therapy. Life Sci Adv 13:69–74.

Kroemer G. 1997. The proto-oncogene Bcl-2 and its role in regulating apoptosis. Nature Med 3:614–619.

Leonetti C, D'Agnano I, Lozupone F, Valentini A, Geiser T, Zon G, Calabretta B, Citro G, Zupi G. 1996. Antitumor effect of c-myc antisense phosphorothioate oligodeoxynucleotides on human melanoma cells in vitro and and in mice. J Natl Cancer Inst 88:419–429.

Liston P, Roy N, Tamai K, Lefevbre C, Baird S, Cherton-Hovart G, Farahani R, McLean Ikeda JE, MacKenzie A, Korneluk RG. 1996. Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. Nature 379:349–353.

Negroni A, Scarpa S, Romeo A, Ferrari S, Modesti A, Raschellà G. 1991. Decrease of proliferation rate and induction of differentiation by MYCN antisense DNA oligomer in a human neuroblastoma cell line. Cell Growth Diff 2:511–518.

Packham G, Cleveland JL. 1995. c-myc and apoptosis. Biochim Biophys Acta 1242:11–28.

Peverali FA, Orioli D, Tonon L, Ciana P, Bunone G, Negri M, Della Valle G. 1996. Retinoic acid-induced growth arrest and differentiation of neuroblastoma cells are counteracted by N-myc and enhanced by max overexpressions. Oncogene 12:457–462.

Piacentini M, Annicchiaro-Petruzzelli M, Oliviero S, Piredda L, Biedler JL, Melino G. 1992. Phenotype-specific tissue transglutaminase regulation in human neuroblastoma cells in response to retinoic acid: Correlation with cell death by apoptosis. Int J Cancer 52:271–278.

Puri PL, Maclachan M, Levrero M, Giordano A. 1998. The intrinsic cell cycle: From yeast to mammals. In: Stein GS, Baserga R, Giordano A, Denhardt D, editors. The molecular basis of cell cycle and growth control. p 15–79.

Reed JC. 1997. Double identity for proteins of the Bcl-2 family. Nature 387:773–776.

Rettig WJ, Spengler BA, Chesa PG, Old LJ, Biedler JL. 1987. Coordinate changes in neuronal phenotype and surface antigen expression in human neuroblastoma cell variants. Cancer Res 47:1383–1389.

Rosolen A, Whitesell L, Ikegaki N, Kennet RH, Neckers M. 1990. Antisense inhibition of a single copy N-myc expression results in decreased cell growth without reduction of c-myc protein in a neuroepithelioma cell line. Cancer Res 50:6316–6322.

Ross RA, Spengler BA, Biedler JL. 1983. Coordinate morphological and biochemical interconversion of human neuroblastoma cells. J Natl Cancer Inst 71:741–747.

Sakamuro D, Eviner V, Elliott KJ, Showe L, White E, Prendergast GC. 1995. c-Myc induces apoptosis in epithelial cells by both p53-dependent and p53-independent mechanisms. Oncogene 11:2411–2418.

Whitesell L, Rosolen A, Neckers LM. 1991. Episome-generated N-myc antisense RNA restricts the differentiation potential of primitive neuroectodermal cell lines. Mol Cell Biol 11:1360–1371.

Yaswen P, Stampfer MR, Ghosh K, Cohen JS. 1993. Effects of sequence of thioated oligonucleotides on cultured human mammary epithelial cells. Antisense Res Dev 3: 67–77.

Zimmerman KA, Yancopoulos GD, Collum RG, Smith RK, Kohl NE, Denis KA, Nau MM, Witte ON, Toran-Alleran D, Gee CE, Minna JD, Alt FW. 1986. Differential expression of myc family genes during murine development. Nature 319:780–783.