Decrease of Proliferation Rate and Induction of Differentiation by a MYCN Antisense DNA Oligomer in a Human Neuroblastoma Cell Line

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Abstract
The effects of an antisense oligodeoxynucleotide to codons 2–7 of the oncogene MYCN on the human neuroblastoma cell line LAN-5 were studied. Treated cells showed a decreased MYCN protein expression and synthesis by immunoperoxidase staining and immunoprecipitation. At the same time, the replication rate was inhibited, and the phenotype was modified toward a more differentiated type. Our data suggest the involvement of oncogene MYCN in both proliferative and differentiative processes.

Introduction
A recent approach to the study of the function and regulation of known genes is the use of antisense DNA and RNA molecules either in cell-free systems or in cell cultures (1). Antisense DNA molecules are, in general, short synthetic oligodeoxynucleotides complementary to target mRNAs and able to block their translation. Recently, it has been suggested that RNase H plays a role in this mechanism by cleaving the RNA moiety of the duplex formed between the mRNA and the oligodeoxynucleotide, thus acting as an amplifier of translation inhibition (2). A similar approach is represented by antisense RNAs (1, 3). These molecules are generated by cloning DNA sequences in antisense orientation in transcription vectors used to transfect cell lines. antisense RNA molecules can also be microinjected directly into cells (4). They may act by forming a hybrid with the target mRNA, thereby blocking RNA processing, transport out of the nucleus, and translation (3). Very recently, an alternative to antisense molecules became available with the characterization of ribozymes. Ribozymes are RNAs with enzymatic activity capable of cleaving specific target RNAs (5–7). Finally, homologous recombination must be mentioned as a method to inactivate a specific gene (8).

A recent paper (9) reports the germ line transmission of an inactive MYCN allele generated by homologous recombination in mouse embryonic stem cells. This can be a powerful tool to understand the role of MYCN oncogene during embryonic life.

To date, antisense DNA oligomers represent the most commonly used approach, offering many advantages for experimentation, although they are often poorly taken up by cells, are sensitive to cellular nucleases; and may contribute to cellular toxicity. Proliferation induced by Rous sarcoma virus (10) and human T-cell lymphophitropic virus III virus (11) has been inhibited by antisense oligomers. Block of fibroblast growth factor mRNA by the same technique was shown to inhibit proliferation in a human melanoma cell line (12). By a similar approach, oncogenes c-myc and c-abl were shown to interfere with normal hemopoiesis (13, 14). The effect of c-myc oncone gene has been extensively studied by means of antisense DNA molecules. Available data are in agreement with a direct involvement of c-myc in the proliferation state of the cell (15, 16).

MYCN is a member of the myc family. MYCN shares stretches of strong sequence homology specifically localized in the second and third exon (17, 18) with c-myc and is also a nuclear protein (19). Nevertheless, differences exist between these genes. In fact, whereas c-myc is almost ubiquitous in proliferating tissues (20, 21), MYCN is more restricted to neuroectodermal and mesodermal cells, particularly, at early stages of differentiation (22). In tumors, MYCN was associated with more aggressive tumor presentation when amplified in neuroblastomas (23, 24), and its overexpression was also found in retinoblastomas (25) and Wilms' tumors (26). Although the amino acid sequence of the MYCN product strongly suggests a DNA-binding protein (19), the biological role of this oncoprotein is still unknown. Nevertheless, a large body of data indicates that MYCN is involved in differentiative processes at various stages of embryonic and fetal life (22, 27, 28). A possible function of MYCN in maintaining an undifferentiated or a poorly differentiated state preceding overt differentiation has been proposed since its down-regulation induced by RA1 in neuroblastoma cell lines preceded their morphological differentiation (29). Less is known about the involvement of MYCN in cellular proliferation. Recently, it was shown that the B-cell compartment of transgenic mice harboring a human MYCN gene, under control of an immunoglob-
MYCN Antisense on Neuroblastoma Cell Line

Results

Effects on Cell Proliferation. After 24, 40, and 90 h of treatment in the presence of 20 μM MYCN antisense oligomer, nuclear labeling was carried out, as described in “Materials and Methods.” At each time point, cell viability was checked by trypan blue exclusion. More than 70% viable cells were always found in treated cells and controls. For each point, at least 300 cells were scored for nuclear labeling (Fig. 2A), and LI were calculated (Table 1). At 24 h, no significant differences in LI in the antisense and sense oligomer-treated and untreated cells were found (data not shown).

At 40 h, a LI varying from 30 to 40% was found in antisense-treated cells, whereas sense-treated and untreated cells had LI varying from 38 to 50% and from 39 to 48%, respectively. The mean reduction of LI in antisense-treated cells was 22% with respect to controls. This difference was statistically significant (P < 0.05).

At 90 h, LI in the antisense-treated cells ranged from 26 to 38% compared to the LI in the sense-treated and untreated cells, in which it ranged from 38 to 48% and from 40 to 51%, respectively. A statistically significant (P < 0.05) LI mean reduction of 32% was found in antisense-treated cells compared to controls.

A decrease in S and G2 cells in cultures treated for 90 h with antisense oligomer was also confirmed by flow cytometric analysis (Fig. 2B). In this experiment, 35% of S and 7% of G2 cells were found in antisense-treated cells as compared to 45 and 8% in sense-treated cells and 45 and 11% in untreated cells. Low percentages of S and G2 cells, 9 and 2%, respectively, were found in LAN-5 cells treated for 1 week in the presence of 5 μM retinoic acid, which has already been described as causing an accumulation of cells in G1 phase (Ref. 29; Fig. 2B). The values found in antisense- and sense-treated cells and in controls are in the same range as those obtained in LI experiments.

Effects on Cell Phenotype. Morphological changes were not evident in cells treated with antisense or sense oligomers. A certain degree of spontaneous differentiation was detectable in our experiments, with some cells extending neurites (Fig. 2A). However, this phenomenon was not found to be substantially different in treated cells and controls. To determine whether the use of antisense oligomers could modulate the phenotypic expression of some antigens, we stained LAN-5 cells that were untreated and treated for 24, 40, and 90 h with antisense and sense oligomers by immunofluorescence. First of all, two components of the cytoskeleton, vimentin and neurofilaments, were tested; these are typical markers of differentiation in neuroblastoma cells (31, 32); a third intermediate filament, GFAP, which has been described in human brain tumors (33, 34), was also tested. Then, the expression of HLA I and II class, which are normally down-regulated in neuroblastoma (35) and related to MYCN amplification (36–38), was analyzed. MBP, a specific marker for neural tissue sometimes expressed by neuroblastoma cells, was also tested. Finally, all of the major constituents of the extracellular matrix were investigated: laminin, fibronectin, and collagen types I, III, IV, and V, which are produced by neuroblastoma cells (39) and can be modulated during the differentiation process, reflecting the specific differentiation pathway of the immature neuroblasts (neural, Schwannian, and melanocytic) (40); laminin, in particular, controls the migration of the neural crest and neurite formation (41).

Fig. 1. Sense and antisense oligomer uptake and stability in LAN-5. A, uptake kinetics at different times, as described in “Materials and Methods.” B, autoradiogram from a 20% polyacrylamide gel where aliquots of cell extracts after incubation for 24, 40, and 90 h with 32P-labeled antisense oligomer were run. 18b, 18-base band.

uln promoter, underwent anomalous proliferation, and there was a frequent occurrence of B-lymphomas (30).

The experiments described in this paper had the aim of testing the effects of MYCN translational block on differentiation and proliferation in a neuroblastoma cell line (LAN-5) by means of an oligodeoxynucleotide complementary to the mRNA of this gene. The expression of several markers usually involved in differentiation was analyzed together with the proliferative state of the cells. The effects of MYCN translational block were also compared to those induced when retinoic acid was used as a differentiation agent.

Kinetics of Oligomer Uptake. In order to assess the kinetics of oligomer uptake in the LAN-5 cell line, 10⁶ exponentially growing cells were cultured in the presence of 10⁶ cpm/ml 32P-labeled antisense and sense oligodeoxynucleotides, as described in “Materials and Methods.” After 7, 24, 40, 90, 120, and 168 h, radioactivity in medium and in cells was measured, and uptake was calculated (12). In Fig. 1A, oligomer uptake at various times is shown. Sense and antisense deoxynucleotide uptake had very similar kinetics. Oligomer uptake reached a plateau after 90 h and started to decline after 120 h. Cell lysates at various times were then utilized to run a 20% polyacrylamide gel to check oligomer integrity. An autoradiogram derived from this experiment (Fig. 1B) shows an 18-base band even 90 h after the addition of the oligomer. It must be pointed out that the batch of serum used in this test is the one chosen for the following experiments.
At 24 h, there was no detectable difference in the expression of all tested antigens in antisense-treated LAN-5 cells, compared with controls. Among the intermediate filaments, neurofilament and GFAP were negative, and vimentin was positive; MBP was also positive. Both HLA-A, B, C and HLA-DR were negative; among the extracellular matrix proteins, laminin and interstitial type I and III collagens were positive, and fibronectin and type IV and V collagens were barely visible.

At 40 h, neurofilament and vimentin expression showed modifications in opposite directions: neurofilament, which was negative in untreated and sense-treated cells (not shown), became positive (Fig. 3B), and, at the same time, vimentin, highly positive in the controls (Fig. 3A), decreased in staining after antisense treatment (Fig. 3C). The expression of the other intermediate filament, GFAP, did not show any detectable change, nor did any of the other tested markers, MBP, HLA I and II, and all extracellular matrix components: none was modified by the treatment (Table 2). This modified phenotype was maintained after 90 h of treatment.

To compare the modifications in expression of these two intermediate filaments, caused by antisense oligomer treatment, with those obtained during the morphological differentiation induced by the biological modifier retinoic acid, we stained LAN-5 cells by immunofluorescence,

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**Fig. 2.** A, thymidine incorporation in LAN-5 cells after 90 h of treatment with antisense (a) and sense oligomers (b); c, untreated control. × 200. Exposure time, 14 days. B, FCM histograms of LAN-5 cells treated with antisense (a) and sense oligomers (b); c, untreated control; d, cells treated with retinoic acid (5 μM) for 1 week. a and d show a decrease in S and G2 cells as compared to b and c.
after 3, 6, and 9 days of RA treatment, with anti-neurofilament and anti-vimentin antibodies. A change in expression of these two intermediate filaments was evident, comparable to the inversion observed before in the cells treated with antisense oligomers: neurofilament staining showed a progressive increase starting at 3 days up to its maximum at 6 and 9 days of RA treatment, and vimentin expression had a simultaneous decrease (Table 2).

By Northern blot analysis, we also checked the mRNA levels for MYCN (Fig. 4A) and 160 kDa neurofilament (Fig. 4B) during the differentiation induced by retinoic acid. As expected, MYCN mRNA was already decreased to very low levels after 2 days of RA treatment, whereas neurofilament mRNA reached a peak after 1 week.

**Effects on MYCN Protein Expression and Synthesis.** To control the effect of MYCN oligodeoxynucleotides on MYCN protein expression, we stained, by indirect immunoperoxidase, LAN-5 cells that were untreated and treated with sense and antisense oligomers for 90 h with an anti-MYCN-specific monoclonal antibody. Both untreated and sense-treated cells showed a strong dark nuclear staining, and the cytoplasm was negative in every cell; the intensity of the nuclear staining was partially heterogeneous, and a few nuclei were fainter (Fig. 5, A, B, and F). All cells treated with the antisense oligomer showed only a pale nuclear staining homogeneously distributed in the majority of the cells (Fig. 5, C and F); decreased intensity, compared to the controls, was evident. A similar decrease in nuclear staining was visible in LAN-5 cells treated with RA for 48 h (Fig. 5D). The specificity of the antibody was checked by a totally negative nuclear and cellular staining of normal human fibroblasts (data not shown).

In order to determine whether the synthesis of MYCN protein was also affected by the treatment with antisense oligomer, LAN-5 cells that were untreated and treated with sense and antisense oligomers were metabolically radiolabeled with [35S]methionine, and the cell extracts were immunoprecipitated using the MYCN-specific monoclonal antibody (Fig. 6). From the cell extracts of both untreated and sense-treated LAN-5 cells, the MYCN antibody immunoprecipitated a thick band, perhaps corresponding to a doublet migrating just below the 69 kDa bovine serum albumin band (Fig. 6, Lanes 2 and 3) and resembling the 60–63 kDa doublet described by Ikekagi et al. (42); no protein bands were immunoprecipitated by the antibody from the antisense-treated LAN-5 cells (Fig. 6, Lane 4) or by normal mouse IgG from untreated LAN-5 cells (Fig. 6, Lane 5).

**Discussion**
LAN-5 cells were treated with antisense oligomer for up to 90 h because of the following considerations: (a) oligomer uptake in LAN-5 cells is a slow process (see Fig. 1A); and (b) LAN-5 cells have a doubling time of approximately 40 h under our experimental conditions. We thought that, in order to see a possible proliferative and/or differentiative effect on LAN-5 cells, a treatment of at
least one cell cycle would be required. In keeping with this hypothesis, an oligomer to c-myc oncogene needs a rather long time to elicit its effect on myeloid cells.\(^*\) The antisense oligomer, complementary to codons 2–7 of the human MYCN gene, was chosen for the following reasons: (a) the majority of the antisense oligodeoxynucleotides used to block RNA translation are complementary to the region near the initial AUG codon (13, 15, 16); and (b) no stable internal secondary structures, in the chosen oligomer, could compete with the RNA-DNA duplex formation. The 20 \(\mu\)M concentration used in the experiments is in the range of the most frequently used (13, 15, 16). MYCN nuclear protein immunoperoxidase staining and immunoprecipitation clearly demonstrated that the treatment with antisense oligodeoxynucleotide decreased MYCN protein expression and synthesis, which might be the primary cause of the effects on cell phenotype and proliferation. It is evident that neurofilament and vimentin can be modulated by the use of an antisense oligomer complementary to MYCN, in a specific way, since all of the other markers remained unchanged after treatment, and no change was detectable after 24 h of treatment, but change became evident after only 40 h and constant after 90 h. Similar results were obtained by treatment of LAN-5 cells with the biological modifier retinoic acid, which induces a morphological neural differentiation, determined by the extension of neoformed neuritic processes and by the increase of neurofilament expression, which is the major constituent of neuronal axons and dendrites, and usually by a decrease in the amount of vimentin, which is normally the predominant intermediate filament type in undifferentiated neuroblastoma cells (31). Retinoic acid treatment also induces a down-regulation of MYCN, but this decrease has been demonstrated to precede the morphological neural differentiation of many neuroblastoma cell lines (29). It has been stated that one gene whose expression seems to be affected by MYCN levels is neurofilament (28). A neuroblastoma cell line transfected with a plasmid containing MYCN gene under the influence of the mouse mammary tumor virus promoter becomes unresponsive to retinoic acid-induced differentiation, and the levels of neurofilament mRNA fail to increase, compared to the levels seen in differentiated control cells (28). Thus, the high levels of MYCN expression might prevent differentiation of neuroblastoma by affecting the expression of genes associated with the maturation of neuroblasts. Our data agree with this statement and suggest that alterations in the regulation of MYCN expression can modulate, at least in part, the differentiation process of neuroblastoma cells.

The translational block imposed by the MYCN antisense oligomer also has an effect on cell proliferation, which, as for the phenotypic modifications, becomes evident after 40 h of treatment, increasing at 90 h. There are three possible explanations of this finding: (a) inhibition of proliferation is due to the differentiation process induced by the block of MYCN translation; (b) MYCN inhibition can cause an extension of the cell cycle time; and (c) MYCN, like c-myc, has a direct effect on proliferation which can be parallel to but independent from that on differentiation (16).

### Materials and Methods

#### Synthesis and Purification of Oligomers

Unmodified 18-base oligodeoxynucleotides were synthesized using an Applied Biosystem (Foster City, CA) 380B DNA synthesizer by means of \(\beta\)-cyanoethylphosphoramidite chemistry. The oligomers were then purified by multiple ethanol precipitations and washes in 70% ethanol. After lyophilization and resuspension, they were checked for purity on 20% polyacrylamide gels. The antisense sequence was: 5’ CTTGCAAGATCATGCCCGG 3’ complementary to codons 2–7 of the human MYCN gene. The sense sequence was: 5’ CCGGGCATGATCTGCAAG 3’ identical to codons 2–7 of the human MYCN gene.

Serum nuclease activity was assayed by checking the stability of 5’ end-labeled oligomers after 48 h of incubation in RPMI 1640 medium (Sigma, St. Louis, MO),

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\(*S. Ferrari, personal communication.*
supplemented by 15% fetal bovine serum (Sigma) at 37°C in 5% CO₂. Molecular sizes were then checked on 20% polyacrylamide gels.

**Cell Line.** Neuroblastoma cell line LAN-5 (43) was maintained in RPMI 1640 medium (Sigma) supplemented with 15% heat-inactivated fetal bovine serum (Sigma) in 5% CO₂.

The cells were negative for *Mycoplasma* by standard techniques. Experiments with oligodeoxynucleotides were carried out with a single lot of fetal bovine serum. For immunofluorescence and proliferation experiments, cells were plated in Labtek slides at a density of 10⁴ cells/cm². After 3 days, medium was changed, and antisense and sense oligomers were added at a final concentration of 20 μM.

**Oligomer Uptake and Stability.** To test oligomer uptake by LAN-5 cells, oligomers were 5’ end-labeled using the 5’ end-labeling kit (Boehringer) according to the manufacturer’s specifications. Labeled oligomer in the supernatant and in the cells was detected essentially as described by Becker et al. (12). Briefly, 10⁶ cpm of 5’ end ³²P-labeled oligomer were added to 10⁵ cells in 5.0 ml of medium with 15% fetal bovine serum. After 7, 24, 40, 90, 120, and 168 h of incubation, medium was removed and saved, and cells were trypsinized and sedimented. Pellets were washed in phosphate-buffered saline. Aliquots of medium, cell washes, and cell suspension were analyzed by liquid scintillation counting, and the percentage of oligomer uptake by cells was calculated. Cells were also lysed in Tris-buffer-saline containing 1% SDS. Cell lysates were extracted in phenol-SCC, lyophilized, resuspended in water, and run on a 20% polyacrylamide gel to determine integrity.

**Labeling Index.** After 24, 40, and 90 h of incubation in the presence of oligodeoxynucleotides, cells were incubated with [³H]thymidine (specific activity, 5 Ci/mm; final concentration, 5 μCi/ml) for 2 h and then fixed in methanol-acetic acid according to Freshney (44), coated with photographic emulsion (Ilford K2), and developed after 15 days. x² test was used to check the actual statistical significance of the LI decrease in antisense-treated cells and in controls.

**Flow Cytometric Analysis.** Five × 10⁶ cells, after 90 h of treatment with antisense and sense oligomers, were fixed in 30% acetone-45% ethanol-25% H₂O for 1 h at 4°C. Cells were then centrifuged, resuspended at a concentration of 1 × 10⁶ cells/ml, and treated for 30 min
with RNase A (Sigma) at a concentration of 40 μg/ml. Propidium iodide (Fluka, Buchs, Switzerland) was then added at a final concentration of 50 μg/ml. FCCM measurements were carried out using a Becton Dickinson (Mountain View, CA) FACS Star Plus cytometer equipped with an argon laser emitting at 514 nm with a power output of 100 mW. Percentages of cells in G1, S, and G2 phases were calculated by a computer program described by Ubezie (45).

**Cell Differentiation.** The cells were treated with all-trans-retinoic acid (Sigma). Cells were plated at a density of 10⁴ cells/cm², and RA was added at 3-day intervals beginning on the third day after plating, for three total doses at a final concentration of 5 μM. RA was dissolved in absolute ethanol at 5 mM concentration.

**Immunocytochemistry.** Cells grown in Labtek chamber slides and treated for 24, 40, and 90 h with antisense and sense oligomers together with untreated cells were stained by immunocytochemistry for the anti-myelin protein (Zymed), anti-glial fibrillary acidic protein (Zymed); anti-myelin basic protein (Sanbio); anti-HLA-A,B,C and anti-HLA-DR (Sorin, Saluggia, Italy). The following rabbit antisera were used: anti-fibroactin and anti-laminin (BRL, Bethesda, MD); anti-type I and III collagens (Pel-Freez, Rogers, AR; Miles, Cavenago, Italy); anti-type IV and V collagens (39). For immunoperoxidase, the cells were rinsed with PBS and fixed with 2% paraformaldehyde in PBS for 10 min at RT. They were rinsed again with PBS and incubated with the previously described MYCN-specific monoclonal antibody NMC II-100 diluted 1:100 (42) (Oncogene Science, Manhasset, NY) for 1 h at 4°C, and then stained using the Histostain-SP kit (Zymed) based on the streptavidin-biotin system without counterstaining. Human normal fibroblasts were also stained as a negative control.

**Immunoprecipitation.** LAN-5 cells treated with sense and antisense oligomers for 90 h were metabolically radiolabeled with 100 μCi/ml of [35S]methionine (800 Ci/ mmol; Amersham, Braunschweig, Germany) for 18 h, as described elsewhere (46). Radiolabeled cell layers were washed twice with PBS, and cells were pelleted at a concentration of 1 x 10⁷ cells/ml in 50 ml Tris-0.2% SDS-20% NaCl-1% Nonidet P-40-2 ml EDTA-1 mM phenylmethylsulfon fluoride-1% aprotinin and extracted at 4°C for 5 min. They were then vortexed for 1 min and finally centrifuged for 10 min at 4°C in a microcentrifuge. The supernatant was collected, and the incorporation of [35S]methionine was quantified in a beta counter after precipitation of small amounts with 10% cold trichloroacetic acid. A volume of supernatant equivalent to 10⁴ trichloroacetic acid-precipitable cpm of each sample was immunoprecipitated using the anti-MYCNSpecific monoclonal antibody NMC II-100 following the previously described technique (46). The immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis using 5% stacking gel and 10% separating gel under reducing conditions. A mixture of [14C]-labeled proteins was used as molecular weight markers (Amersham): phosphorlase b, 92.5 kDa; bovine serum albumin, 69 kDa; ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa.

**Northern Blot Analysis.** Total RNA from undifferentiated and differentiated cells was extracted according to Chomczynski and Sacchi (47). RNAs were then size fractioned on 1% denaturing formaldehyde-agarose gel (48), transferred to Z-probe membranes (Bio-Rad) by a vacuum blot apparatus (Hoefer), and hybridized to different probes [32P] labeled by random priming extension according to the manufacturer’s instructions (Boehringer) to a specific activity of 4 x 10⁸ cpm/μg. High stringency hybridization conditions were: 50% formamide (BRL)-7% SDS-0.25 M phosphate buffer-0.25 M NaCl-1 mM EDTA. Washings were carried out in 2x SSC-0.1% SDS, 0.5x SSC-0.1% SDS, and 0.1x SSC-0.1% SDS. The first two washes were at room temperature, and the last was at 65°C, each for 30 min. For rehybridization, the bound probe was stripped off by washing filters two times in 0.1x SSC-0.5% SDS at 95°C for 20 min.

**Probes.** Clone Nb 19-21 containing a 2-kilobase region spanning the second exon of the human MYCN gene was provided by Dr. F. Alt (Columbia University, New York, NY). Clone pNF 36, including sequences from exon III of the human 160 kDa protein-encoding gene, was obtained from the American Type Culture Collection (no. 57470).

**Acknowledgments**

We gratefully thank Dr. Carol J. Thiele for helpful discussion, Dr. Claudio Caporali for statistical analysis, Dr. Filippo Moretti for FCCM measurements, and Adelma Di Stefano for technical assistance.

**References**