Nestin Is a Potential Mediator of Malignancy in Human Neuroblastoma Cells*

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Amplification of the N-myc proto-oncogene signifies aggressive behavior in human neuroblastoma. Likewise, overexpression of the intermediate filament nestin, a neuroectodermal stem cell marker, is linked to increased aggressiveness in several nervous system tumors. We investigated the interaction of these two proteins in human neuroblastoma cells. Neuroblastic cell variants with high levels of N-Myc protein have significantly higher nestin protein levels than non-amplified cell lines, suggesting that the transcription factor N-Myc may regulate nestin expression. Stable transfection of a nestin antisense sequence into neuroblastic, N-myc-amplified, LAI-55n cells results in a 2-fold reduction in nestin protein without altering N-Myc expression. However, cell functions attributed to N-Myc (growth rate, anchorage-independent growth, and motility) all decrease significantly. Transfection studies that modulate N-Myc levels also result in commensurate changes in nestin mRNA and protein amounts as well as in cell proliferation and motility. Thus, nestin appears to be downstream of and regulated by N-Myc. Gel mobility shift assays show that N-Myc binds specifically to E-box sequences in the regulatory second intron of the nestin gene and nuclear run-off studies show that increases in N-Myc protein up-regulate nestin transcription rate. Subcellular fractionation and immunoblot studies indicate that nestin is present in the nucleus as well as in the cytoplasm of neuroblastoma cell lines. Finally, DNA cross-linking experiments show that nestin binds DNA in N-myc-amplified N-type cell lines. Thus, nestin may be one mediator of N-myc-associated tumor aggressiveness of human neuroblastoma.

Neuroblastoma is one of the most common neonatal solid tumors and remains a significant cause of death in young children. The proto-oncogene N-myc is influential in the pathology of this cancer; children with tumors containing single copy N-myc (per haploid genome) often have a favorable prognosis, whereas amplification and/or overexpression is strongly associated with metastasis, rapid disease progression, and a high rate of mortality (1). The N-myc gene encodes a nuclear phosphoprotein containing several distinct domains: a stretch of basic amino acids followed by basic helix-loop-helix-leucine zipper motifs involved in DNA binding and protein dimerization (2). N-Myc protein regulates gene expression at the transcriptional level by dimerizing with itself or other helix-loop-helix proteins and this complex, in turn, binds to DNA E-box sequences (e.g. CACGTG) of regulated genes (3, 4). Microarray analysis has shown that N-Myc regulates genes involved in growth, cell cycle, signaling, and adhesion (5). N-Myc protein expression has also been shown to play a role in cell proliferation and migration, as well as in maintenance of a committed, but less differentiated, cell state (6, 7). The exact mechanism of action of this transcription factor remains to be determined.

A second protein present in embryonic neuroectodermal cells is the intermediate filament (IF) nestin. IFs function in organizing the cytoskeleton (8), but they also have been implicated in cell signaling, organogenesis, and cell metabolism (9). Nestin, a marker of multipotent neuroectodermal precursor cells, is expressed in a cell-cycle-dependent manner and is down-regulated as neuroepithelial stem cells cease division and differentiate along their respective neural or glial lineages (10, 11). Similar to N-Myc, tumor aggressiveness has been associated with elevated nestin levels in some tumors (12). For instance, in primitive neuroectodermal tumors, elevated nestin characterizes the most malignant cell type (13). Also, nestin protein is elevated in the infiltrating parts of highly metastatic human glioblastomas and astrocytomas (13–16) and has been proposed to play a role in tumor invasion in melanomas (17).

Because both N-Myc and nestin have been implicated in the pathogenesis of several tumors of the nervous system, the present study examined the relationship between these two proteins in human neuroblastoma N-type cells. Specifically, we sought to determine whether nestin might be a downstream effector through which N-Myc influences malignancy in human neuroblastoma.

EXPERIMENTAL PROCEDURES

Tissue Culture—The human neuroblastoma cell lines and clones included in this study have been previously described (18–21). Cells were cultured according to published methods (22). Growth rates were determined by previously described methods (21).

Western Blot Analysis—Total cell protein homogenates were prepared by the method of Ikegaki et al. (23). Nuclear and cytoplasmic fractions were isolated as previously described (22). Western blot procedures have been published (22, 24). Primary antibodies included polyclonal antibodies to nestin (331b), homing cell adhesion molecule (HICAM) (Santa Cruz Biotechnology, Santa Cruz, CA), secretogranin II (gift of Dr. Jonathan G. Scammell, Department of South Alabama

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¶ The abbreviations used are: IF, intermediate filament; NF 70, 70-kDa neurofilament protein; NF 160, 160-kDa neurofilament protein; HICAM, homing cell adhesion molecule; iPA, tissue plasminogen activator; ECM, extracellular matrix; G418, Geneticin; BrdUrd, 5-bromo-2’-deoxyuridine.
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College of Medicine, Mobile, AL; tissue plasminogen activator (tPA) (ICN Pharmaceuticals, Inc., Aurora, OH) and vimentin (Chemicon International, Temecula, CA) and monoclonal antibodies to neurofilaments (NFs) 70 and 160, chromogranin A, β-tubulin, and actin (clone C4) (Roche Applied Science). Following incubation with horseradish peroxidase-conjugated secondary antibodies (Roche Applied Science), protein-bound antibody was detected by chemiluminescence (ECL, Amersham Biosciences). Protein amounts were quantified by scanning densitometry and the relative amount compared with actin.

Isolation of mRNA and Northern Blot Analysis—Poly(A) RNA was isolated from cells in exponential growth phase with oligo(dT) cellulose (Collaborative Research, Boston, MA). Northern blots were performed as previously described (22) with 2–4 μg of mRNA and 32P-labeled probe to the first exon of the nestin gene. Expression levels, densitometry, scanning densitometry of resulting autoradiographs, were compared with those of γ-actin or glyceraldehyde-3-phosphate dehydrogenase.

Nestin and N-Myc Transfections—Antisense nestin transfections were performed with a 2.65-kb fragment from the fourth exon of the nestin gene generated by PCR (primers Xba-nestin 5’-CTGCTGAATTCCTCGATTGGAA-3’ and Km-Nestin 3’-CATGCTG AGCGGGTCTCCCTTCCACCGCTATCTT-3’) and inserted into an antisense orientation into the pBactNeo vector (25). Antisense N-Myc transfections

were transfected using LipofectAMINE Plus (Invitrogen) and selected with 400 μg/ml Geneticin (G418). N-Myc non-amplified SH-SYS5 cells were transfected with a pCI vector containing a 2.9-kb N-myc cDNA in the sense direction (also a gift of Dr. Igekaki) and selected with 100 μg/ml of G418. The concentration of G418 was lower than that used for LA1–55n because of the marked sensitivity of SH-SYS5 to antibiotic.

S-Phase Fraction Determination—The percentage of cells in S phase was determined by a modification of the method of Freshney (26). Stably transfected antisense nestin and control LA1–55n clones were seeded into chamber slides and cultured for 6 days; resulting non-amplified SH-SYS5 cell lines (data not shown), thus indicating that higher levels of the gene were not amplified (32), has nestin levels similar to amplified neuroblastoma cell lines (32–35). DNA-protein complexes were resolved by electrophoresis through a 4% nondenaturing polyacrylamide gel. Control experiments included: 1) use of DNA probe in which both E-box sequences were mutated using the primers: 5’-CAGCTGACAGGCACACCTGAGAT-3’ and 5’-GCTGCGCCATATGGGCGGCAACTGCAT-3’, 2) addition of either non-radioactive probe or a nonspecific competitor (i.e. a DNA fragment from the glyceraldehyde-3-phosphate dehydrogenase gene) in molar excess before adding 32P-labeled probe, 3) addition of increasing amounts of protein homogenate (15–90 μg) to reaction mixtures, and 4) precipitation of run-on experiments with antibody specific for N-Myc (sc-142) or p53 (sc-126) (Santa Cruz Biotechnology) and removal of antigen-antibody complexes with protein G-Sepharose (Amersham Biosciences).

DNA-Protein Cross-linking—DNA-protein cross-linking studies were performed following the procedure of Ferraro et al. (29) and Spencer et al. (9). Proteins eluted from the DNA were analyzed by Western blotting. Control experiments utilized cells incubated without cisplatin.

RESULTS

Relationship between N-myc Amplification/Overexpression and Nestin Protein Levels—Increased amounts of both N-Myc and nestin have been correlated with increased aggressiveness and malignancy of neuroectodermal tumors. In human neuroblastoma, N-Myc is overexpressed in tumorigenic N-type cell lines (21). We therefore investigated the coexpression of these two proteins in nine human neuroblastoma N-type cell lines by immunoblot analysis. Cells were collected during logarithmic growth phase as nestin is down-regulated in stationary growth phase (31). As seen in Table I and Fig. 1, nestin protein levels are ~2-fold higher in the cell lines that amplify and/or overexpress N-myc (mean = 23.5 ± 1.0) compared with the group of cell lines that does not (mean = 11.0 ± 0.5). NBL-S, a cell line that has an elevated N-Myc steady-state protein level but does not amplify the gene (32), has nestin levels similar to amplified cell lines (data not shown), thus indicating that higher amounts of nestin correlate specifically with N-Myc protein levels rather than with amplification per se.

Effect of Change in Nestin Expression on Cell Phenotype and Growth Rate—Because elevated nestin levels have been implicated in tumor malignancy, change in nestin expression might affect the malignant behavior of neuroblastoma. To directly address this possibility, stable nestin antisense-transfectant clones were generated from the N-myc-amplified LA1–55n clonal cell line. Western blot analysis confirmed that antisense transfectants have significant, 1.6- or 1.8-fold lower levels of nestin protein compared with vector-transfected cells or non-transfected LA1–55n cells, respectively (Table II).

The reduction in nestin amount has no effect on cell morphology or adhesion to the substrate. Similarly, levels of neu-

TABLE I

| Nestin protein amount in N-myc-amplified and non-amplified human neuroblastoma N-type cells |
|---------------------------------------------------------------|
| **Nestin amounts are expressed in units; each value represents the mean ± S.E. from 5–7 experiments.** |
| **Cell line/clone** | **Amount of nestin protein** |
| LA-N-8 | 9.9 ± 0.6 |
| SMS-LHN | 10.8 ± 0.9 |
| SH-EP15 | 11.1 ± 0.1 |
| SH-SYS5 | 12.1 ± 1.9 |
| Mean | 11.0 ± 0.5 |

N-Myc-amplified
| **BE(2)-M17** | 22.3 ± 3.3 |
| **SK-N-BE(1)n** | 23.7 ± 3.9 |
| **SMS-KAN** | 21.3 ± 0.6 |
| **LA1–55n** | 23.5 ± 2.6 |
| **NBL-Wn** | 26.8 ± 6.1 |
| Mean | 23.5 ± 1.0 |
N-Myc and marker proteins in nestin antisense-transfected and control cells

Each value represents the mean ± S.E. of data from ten vector-transfected control (Ctrl) LA1–55n clonal cell lines or six nestin antisense-transfected (NesAs) LA1–55n clonal cell lines.

| Cell line/clone | Nestin | N-myc | NF 160 | Secretogranin II |
|-----------------|--------|-------|--------|------------------|
| LA1–55n         | 20.9 ± 0.9 | 10.8 ± 1.1 | 6.6 ± 1.7 | 15.2 ± 0.5 |
| Ctrl            | 23.5 ± 2.6 | 9.6 ± 0.3 | 6.7 ± 0.2 | 15.3 ± 0.7 |
| NesAs           | 13.3 ± 0.7* | 10.5 ± 0.5 | 6.2 ± 0.6 | 14.9 ± 0.4 |

*p < 0.001.

Fig. 1. Immunoblot analysis of nestin and actin protein in N-myc-amplified and non-amplified human neuroblastoma N-type cell lines. Lanes and cell lines: 1, SMS-LHN; 2, SH-SY5Y; 3, SH-EP15; 4, LA-N-6; 5, BE(2)c-M17; 6, LA1–55n; 7, NBL-Wn; 8, SMS-KAN. Note that N-myc-amplified lines have significantly more protein than non-amplified lines.

Fig. 2. Cell motility correlates with nestin expression in human neuroblastoma cell lines (A) and transfectants (B). A, LA1–55n (■), other N-myc-amplified cell lines (○) (NBL-Wn, BE(2)c-M17, SMS-KAN), and non-amplified cell lines (□) (SH-SY5Y, LA-N-6, and SMS-LHN). B, parental LA1–55n cells (■), two vector-transfected control clones (○), and two nestin-antisense clones (▲).

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Table II

Amounts of nestin, N-Myc, and marker proteins in nestin antisense-transfected and control cells

As an independent measure of cell growth rate, we determined the percentage of cells in S phase by BrdUrd incorporation. Clones of two nestin antisense transfectants and two vector-transfected controls were cultured for 6 days to reduce incidence of subculture-induced cell synchrony and, while still in exponential growth phase, pulsed with BrdUrd. Stable nestin antisense transfectants had a 1.7-fold lower percentage of cells in S phase (20.6 ± 3.5%) compared with vector-transfected controls (33.7 ± 2.6%) (p < 0.01), numbers consistent with the differences observed for population doubling times. The reduction in growth rate demonstrated by both methods is not a consequence of the action of another N-myc-regulated protein as levels of the oncoprotein do not change.

Nestin Influences Malignant Potential and Metastasis—To assess malignant potential, vector- and nestin antisense-transfected cells were assayed for anchorage-independent growth ability in clonogenic soft agar assays. As shown in Table III, both LA1–55n and the vector-transfected control cells are transformed, with mean plating efficiencies of 12.6% and 12.3%, respectively. By contrast, nestin antisense transfectants show a marked, nearly 5-fold, reduction in colony-forming ability (2.6%). Because N-Myc protein levels are the same in these three groups, changes in nestin appear responsible for changes associated with malignancy.

Cell lines and transfectants were also tested for their ability to digest and migrate through an artificial extracellular matrix (ECM). These assays show that N-type cells with more nestin protein are more migratory (Fig. 2). Thus, N-type cell lines with lower levels of nestin migrate more slowly than their nestin-rich counterparts (Fig. 2A). Similarly, although most vector-transfected and parental LA1–55n cells migrate through non-coated control insert membranes (92 and 100%, respectively), antisense-transfected cells display a greater than 3-fold reduction in migratory ability (Table IV and Fig. 2B). However, cell invasiveness, i.e. the ability to digest and move through Matrigel ECM-coated membrane compared with non-coated inserts, is not altered (Table IV). The ability of cells to digest the ECM was assessed independently by measuring the amount of tissue plasminogen activator protein (tPA), the primary serine protease involved in neuroblastoma metastasis (36). Results showed that tPA levels do not differ among the three groups (Table IV). Therefore, reduction in nestin amount impairs the motility of neuroblasts, but not their ability to digest the ECM.

Effect of Changes in N-Myc Expression on Nestin Expression, Cell Growth, and Malignant Potential—From the above studies, it is clear that several of the cell functions previously attributed to N-Myc, e.g. proliferation and migration, are al-
crease in the antisense transfectants compared with
iment, the N-
5-fold, respectively (Fig. 3

N-stably transfected with an expression vector containing an
expression vector. Nestin mRNA and protein levels in the antisense
were transfected with vector with or without an N-
mic

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crease in both N-Myc and nestin protein and in nestin mRNA

A decrease in the antisense transfectants compared with γ-actin control.

Table V

N-my of mRNA and protein (Fig. 3

N-Myc mRNA and protein compared with parental LA1

Table VI

Modulation of N-myc expression alters cell doubling time,
motility, and invasivity

Each value represents the mean ± S.E. of 2–4 independent experiments
in LA1-55n or SH-SY5Y cell lines, 2–3 vector-transfected controls (Ctrl), or 3 sense- or antisense (As)-N-myc-transfected clones.

A nested ANOVA was used to determine whether N-Myc alters expression of nestin. Similar to the antisense nestin transfectants, antisense N-myc transfectants exhibit longer doubling times (41.6 ± 3.5 h), lower motility, and un

TABLE IV

Nestin influences cell motility but not cell invasiveness

Each value represents the mean ± S.E. of four to six experiments for three LA1-55n cultures, four vector- (Ctrl) and six antisense nestin (NesAs)-transfected clones.

| Cell line/clone | Cell motility | Invasivity | tPA amount |
|-----------------|--------------|------------|-----------|
| LA1-55n         | 46.8 ± 5.6   | 95.5 ± 7.1 | 12.9 ± 1.1|
| Ctrl            | 42.2 ± 6.4   | 96.6 ± 5.2 | 12.0 ± 0.6|
| NesAs           | 14.0 ± 1.6   | 99.0 ± 6.5 | 12.3 ± 0.6|

*p < 0.001.

Table V

N-my of expression and nestin expression and transcription rates in N-myc sense and antisense transfectants

Values represent the mean ± S.E. of protein amount, normalized to actin, or steady-state amount of nestin mRNA, normalized to glyceralde

| Cell line/clone | N-myc construct | N-Myc protein | Nestin Protein | mRNA | Transcription |
|-----------------|-----------------|---------------|---------------|------|--------------|
| LA1-55n         | Vector          | 1.0 ± 0.1     | 1.0 ± 0.1     | ND   |
|                 | Antisense       | 0.4 ± 0.1     | 0.2 ± 0.1     | ND   |
| SH-SY5Y         | Vector          | 1.0 ± 0.1     | 1.0 ± 0.1     | 1.0  |
|                 | Antisense       | 1.8 ± 0.2     | 1.9 ± 0.3     | 1.9  |

*p ND, not determined.

*p < 0.01 determined compared to vector control.

N-Myc alters expression of nestin. Similar to the antisense nestin transfectants, antisense N-myc transfectants exhibit longer doubling times (41.6 ± 3.5 h), lower motility, and unchanged invasivity compared with vector transfectants and LA1-55n cells (Table VI). Conversely, sense N-myc transfectants grow more rapidly and display increased motility compared with parental SH-SY5Y cells or vector transfectants.

N-Myc Binds to E-boxes in the Second Intron of the Nestin Gene and Regulates Its Transcription—As an initial approach to showing that N-Myc might regulate nestin in N-type cells, we investigated whether N-Myc can bind to the nestin gene in vitro in gel mobility shift assays. Studies have shown that N-Myc alters the rates of transcription of specific genes by binding to E-box regions within regulatory elements (37). The second intron of the human nestin gene is regulatory (38) and the 5’ end of this intron contains two E-box elements, one canonical (CACGTG) and one non-canonical (CACGAG). Homogenates from N-Myc-overexpressing LA1-55n cells, when incubated for 10–15 min with a 540-bp DNA probe containing both E-box sequences (Fig. 4, lane 2), form two more slowly migrating complexes whereas homogenates from SH-EP1 cells, which lack N-Myc, do not (data not shown). To confirm that N-Myc protein in the lysate is causing the shift, LA1-55n lysate was preincubated with either anti-N-Myc or anti-p53 antibody and the antibody-antigen complexes removed (see “Experimental Procedures”). Preincubation with N-Myc, but not p53, antibody abrogates the shift (Fig. 4, lane 4 and data not shown). The specificity of N-Myc binding to the
E-boxes in the nestin second intron was assessed by three additional methods. In competition experiments, addition of increasing amounts of unlabeled nestin intronic DNA proportionately decreases the amount of labeled DNA in the complexes (Fig. 4, lane 3); addition of a nonspecific unlabeled glyceraldehyde-3-phosphate dehydrogenase DNA does not (data not shown). Second, in titration experiments, the intensity of the label in the complexes is proportional to the amount of LA155n homogenate added (data not shown). Finally, incubation of LA155n homogenate with a nestin intronic DNA in which both E-boxes were mutated does not result in a mobility shift (Fig. 4, lane 5). These studies strongly suggest that N-Myc regulates nestin expression.

The exact composition of the two DNA-protein complexes, a consistent finding in these experiments, has not been determined.

To directly assess whether N-Myc is affecting nestin transcription rate, nuclear run-offs were performed on N-myc- and vector-transfected SH-SY5Y cells. As shown in Fig. 3B and Table V, there is a nearly 2-fold increase in nestin transcription rate in N-myc-transfected SH-SY5Y cells compared with vector control. Thus, N-Myc directly regulates nestin gene expression.

The Intermediate Filament Protein Nestin Binds Nuclear DNA—As an intermediate filament, nestin is instrumental in organizing the cytoskeleton and thus its altered expression could affect cell proliferation and motility. Less obvious is how nestin amount influences malignancy. In addition to their roles as components of the cytoskeleton, some intermediate filaments are present in the nucleus where they appear to bind and regulate gene expression (9, 39, 40). One possibility is that nestin could function in a similar fashion in human neuroblastoma. Subcellular fractionation and Western blot analyses show that nestin protein is present in both the cytoplasm and the nucleus of N-type neuroblastoma cell lines, LA155n and KCN69n (41; data not shown).

To confirm the presence of nestin in the nucleus, cross-linking experiments were performed using the chemotherapeutic agent cisplatin (29). Cisplatin forms adducts with DNA dinucleotides d(pGpG) and induces intrastrand cross-links; proteins in close proximity to DNA will also be cross-linked. DNA-protein complexes were isolated, and the cross-linked proteins were eluted and analyzed via Western blot analysis. As seen in Fig. 5, nestin proteins in close proximity to DNA only in N-myc-amplified neuroblastoma cell lines; cisplatin does not cross-link nestin to DNA in N-myc-nonamplified cell lines such as CB-JMN and SH-SY5Y. Thus, cell lines that have more N-Myc protein also have higher nestin protein levels, and it is in these cells that nestin is present in the nucleus and appears to interact with DNA.

DISCUSSION

Nestin is expressed in cells that are in a pluripotent, mitotic, and migratory state. The present studies demonstrate a novel role for the intermediate filament nestin in human N-type neuroblastoma cells and strongly suggest that the effects of N-Myc on cell proliferation and motility may be mediated, in part, by nestin. Experimental reduction in nestin protein amount does not alter N-Myc levels, but it dramatically lengthens the population doubling time (by 60%), decreases anchorage-independent growth (by 5-fold), and reduces cell motility (by 3-fold). Experimental reduction in N-myc levels in the same cells has similar results: concomitant reductions in nestin mRNA and protein as well as decreases in growth rate and motility. Conversely, when N-Myc is transfected into an N-myc non-amplified cell line, nestin expression increases, as does invasivity, motility, and growth rate. Together, these observations provide evidence that regulation of nestin expression could be one mechanism by which N-Myc influences neuroblastoma growth, malignancy, and metastasis. The mechanism by which nestin effects these changes is currently under investigation.

Traditionally, IFs are thought to stabilize and support cells and to organize cells into tissues. However, some cytoskeletal proteins have been reported to be involved in nuclear activities as well. It has been shown that IFs are present in the nucleus where they bind specific DNA sequences (42–44) and can influence DNA organization and chromatin structure (40). Some IFs are implicated in inducing a more malignant phenotype; overexpression of vimentin or keratins in melanomas or breast carcinomas, respectively, leads to an augmentation of tumor cell motility and invasiveness (42). Other studies show that the nuclear/cytoplasmic intermediate filament profile is significantly altered in breast carcinoma cells (39, 40).

So it is of considerable interest that, in addition to an extensive nestin cytoplasmic network, Western analysis show that nestin protein is present in both the cytoplasm and nucleus where it appears to bind and regulate gene expression (9, 39, 40). One possibility is that nestin could function in a similar fashion in human neuroblastoma.

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