Augmented MYCN Expression Advances the Malignant Phenotype of Human Neuroblastoma Cells: Evidence for Induction of Autocrine Growth Factor Activity

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ABSTRACT

Amplification and enhanced expression of the MYCN oncogene are thought to contribute to the development and progression of human neuroblastomas. Here, we have transfected human neuroblastoma cells that harbor a single MYCN gene copy with the human MYCN gene driven by a viral enhancer/promoter, and we have compared the properties of the parental and the transfected cells. The transfected cells show an enhanced expression of the exogenous MYCN gene. Unlike the parental cells, they have acquired an increased proliferative potential, induce tumors in nude mice, grow in soft agar, and require low amounts of exogenous growth factors in order to proliferate. The MYCN-transfected, but not the parental, cells can synthesize and utilize autocrine growth factor activity. These results demonstrate that enhanced MYCN expression contributes to malignant progression of human neuroblastoma cells, conceivably by stimulating the expression of autocrine growth factor activity.

INTRODUCTION

The cellular oncogene MYCN was initially demonstrated in neuroblastomas and derived cell lines (1). It was discovered due to its amplification and limited sequence homology with the MYC oncogene (1–3). MYCN has a restricted pattern of tissue expression with preference in neuroblastomas and some other pediatric tumors (1–8). In contrast, MYC is distributed in a wide variety of cells and tissues and appears to play a general role in proliferative events (reviewed in Ref. 9).

MYCN expression has been found in embryonal, but not in adult, tissues (6, 7), indicating that MYCN plays an important role in embryonal development and differentiation. MYCN expression declines during experimentally induced differentiation of human teratocarcinoma or neuroblastoma cells (7, 8), suggesting that differentiation is contingent on a decline of MYCN expression. One could speculate that enhanced MYCN expression consequent to amplification inhibits cell differentiation and thereby arrests the cells at an early stage of differentiation. This process, in concert with other mutational events, may eventually lead to tumor development. In an experimental setting, the transfection of embryonal cells with a MYCN expression vector rescues the host cells from senescence (10) and assists mutationally activated HRAS in malignant transformation (11).

Constitutively enhanced MYCN expression may also support tumor progression (12). Enhanced MYCN expression due to MYCN gene amplification is found in human neuroblastomas at advanced stages of malignancy and indicates poor clinical prognosis (13, 14). Thus, enhanced MYCN expression may exacerbate the malignant phenotype of human neuroblastomas.

The nature of the biological and biochemical events resulting from enhanced MYCN expression and the mechanisms by which they might contribute to malignant cell transformation are unknown. In order to shed some light on the consequences of enhanced MYCN expression, we have transfected human neuroblastoma cells that express MYCN at low levels with an MYCN expression vector, and we have then examined the biological and biochemical changes induced by enhanced MYCN expression.

MATERIALS AND METHODS

Materials. Ten- or 15-cm tissue culture dishes and 850-cm2 roller bottles were from Falcon (Oxnard, CA). Thirty-five-mm tissue culture dishes were obtained from Nunc (Roskilde, Denmark); Bacto-agar, from Difco (Detroit, MI); and human transferrin, from Sigma (St. Louis, MO). bFGF and HDL were prepared from bovine pituitaries and human plasma, respectively, as previously described (15, 16). SII neuroblastoma cells (17) were obtained from Dr. June Biedler.

Protein. Determination of protein was by a modification of the method of Bradford (15, 16).

Molecular Clones. For transfection, we used the MYCN clone pMP-34.18 (encompassing MYCN exons 2 and 3 and a 3’-region of exon 1) and the frame-shift mutation pMP-34.1axho which served as a control (11). These constructs were cloned into plasmid pAVCV/007 containing a selectable marker gene that confers resistance to the cytotoxic antibiotic G-418.

For determination of total MYCN RNA we used the Xho/BamHI fragment (Nb-1/XB) of exon 2 (Fig. 14) of the MYCN gene (18) cloned into pGEM3Z (Promega, Madison, WI). To discriminate between endogenous and exogenous (pMP-34.1p) MYCN transcripts, we used a Smal fragment (NMYCEN1, encompassing nucleotides 64-417 of the MYCN gene) that had been cloned into the Smal site of pGEM3Z, thereby generating placid pNMYCEN1 (Fig. 14; a gift of Dr. Mark A. Israel).

Cell Culture and Transfection. Stock cultures of neuroblastoma cells were grown in 10-cm tissue culture dishes containing RPMI-1640 medium supplemented with fetal calf serum (10%), streptomycin (100 μg/mL), and amphotericin B (0.25 μg/mL). Transfections were done by using calcium phosphate coprecipitation. Transfected cells were selected in the presence of G-418 (200 μg per ml of medium) (11, 19).

Isolation of DNA and RNA. Cells were lysed in guanidinium thiocyanate, and the lysates were centrifuged through a solution of 5 M CsCl as previously described (20). DNA was recovered from the viscous interphase, dialyzed against 10 mm Tris:1 mm EDTA (pH 7.0) containing 100 mm NaCl, exposed to proteinase, extracted with phenol, and precipitated with ethanol (20). Total RNA was recovered from the bottom of the tube, dissolved in 10 mm Tris: 1 mm EDTA (pH 7.0), and precipitated with ethanol.

Analysis of DNA and RNA. To demonstrate MYCN DNA, cellular DNA was digested with restriction endonuclease EcoRI, fractionated...
on 1% agarose gels, and subjected to Southern blot analysis using a
\( ^32 \)P-labeled insert of plasmid pNb-1 for hybridization (1, 11).

To detect total MYCN RNA or to discriminate between endogenous and exogenous MYCN RNA, we performed RNase protection assays
using in vitro-synthesized \( ^32 \)P-labeled probes representing antisense RNA to either the Nb-1/XB sequence (Fig. I4) or to NMYCE1
spanning nucleotides 64-417 of MYCN exon 1. Ten \( \mu \)g of total RNA
were annealed to either of the labeled probes, and single stranded RNA
was then completely digested with RNase T, and A. The remaining
RNA-RNA hybrids were analyzed by denaturing electrophoresis on 5%
polyacrylamide gels (18).

Radiolabeled DNA or RNA was visualized by autoradiography.

Soft Agar Assay. Cells were suspended in 3 ml of RPMI-1640 medium/10%
FCS containing 0.35% agar and layered above a 3-ml base layer of 0.5%
agar in the same medium in 5-cm tissue culture dishes. Cells were fed once a week with 0.5% agar in medium, and visible colonies (>0.2 mm) were counted after various times.

Nude Mouse Assay. Athymic (nu/nu) mice were kept in a sterile
atmosphere at the German Cancer Research Center animal facilities. Mice received a single s.c. injection with neuroblastoma cells (1 x 10^6).
Tumor development was checked at regular intervals.

Preparation of Conditioned Medium and Cell Extracts. Cells were
grown to near confluence in 15-cm tissue culture dishes or in 850-cm^2
roller bottles rotated at 20 cycles/h. To prepare conditioned medium,
cells were washed for 12 h with 50 ml of serum-free medium per roller
bottle and then incubated for 48 h with 20 ml of serum-free medium
per roller bottle. The latter fraction is referred to as “conditioned
medium.” To prepare cell extracts, cells were trypsinized, washed twice
with ice-cold PBS, and resuspended in PBS. Cells were then sonicated
for 2 min at 4°C, the resulting suspensions were centrifuged (20 min at
50,000 x g and 4°C), and the supernatants, referred to as “crude
extracts,” were sterilized by filtration through 0.45-\mu m filters (21).

Cell Proliferation Assays. Stock plates of neuroblastoma cells were
washed once with PBS and then exposed to a solution containing
trypsin (21). When cells rounded up, they were suspended in serum-
supplemented RPMI-1640 medium. An aliquot of the cell suspension
was counted in a Coulter Counter, and cells were distributed in 2-ml
aliquots into 35-mm dishes at the initial cell density indicated in the
figure legends. Dishes received growth factors or samples to be tested
or no additions. After various time intervals, cultures were trypsinized,
and cell densities were determined with a Coulter particle counter (21).
Experiments were performed at least 3 times (SD < 10%).

RESULTS

Establishment of Neuroblastoma Cells with High Expression
of MYCN. The aim of the present study was to examine the

When NMYCE1 is used as the probe to detect transcripts of endogenous MYCN, RNA species of various sizes are protected due to different transcriptional start
sites. The 130-base-pair (bp) fragment (c) is specific for transcripts derived from
exogenous MYCN, and larger fragments (a, b) are specific for transcripts of
endogenous MYCN. Transcripts a, b, and c correspond to transcripts a, b, and c
of Fig. 1D. Restriction endonuclease key: B, BamHI; C, CiaI; H, HindIII; K,
KpnI; R, EcoRI; S, SalI; X, Xhol. In B, 10 \mu g of DNA derived from human
neuroblastoma cell lines SK-N-SH, SH-EP, and SH-EPII clones WAC1, WAC2,
and WAC3, and Kelly were digested, fractionated on agarose gels, and then analyzed by Southern blot using a \( ^32 \)P-labeled MYCN
antisense RNA to a region represented by the clone NMYCE1 as described in “Materials and Methods.” Arrow a indicate a 2.0-
kilobase pair (kbp) MYCN signal. High-molecular-weight MYCN DNA in SH-
EPII cells and soft agar clones is due to partial MYCN DNA digesta. In C, 10 \mu g of
RNA derived from neuroblastoma cell lines SH-EP or SH-EPII were analyzed
by RNase protection assay using a \( ^32 \)P-labeled MYCN antisense RNA as probe as described in “Materials and Methods.” Arrow a indicate a 2.0-
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consequences of enhanced MYCN expression in human neuroblastomas by comparing the properties of neuroblastoma cells with low MYCN expression before and after transfection and high expression of the MYCN gene. Cells of the catecholamine-producing human neuroblastoma line SK-N-SH (17) appeared suitable for the proposed experiments, since they contain a single MYCN gene copy. However, since the SK-N-SH line is composed of a heterogeneous population of cells we rather selected SH-EP (17), a homogeneous SK-N-SH-derived subline. Using transfection assays, we introduced the expression vector pMP-34.1k (Fig. A) or the mutant vector pMP-34.1kΔXho into SH-EP cells. The mutant vector carries a MYCN frame-shift mutation encoding a functionally inactive protein. After transfection, the cells were passaged 3 times a week at a 1:2 ratio in medium containing the antibiotic G-418. Colonies of G-418-resistant cells appeared after about 2 wk. They were trypsinized and combined to yield mass cultures of SH-EPII or control (SH-EPIIc) cell lines.

Copy Number and Expression of MYCN. To determine the number of MYCN gene copies, cellular DNA was subjected to Southern blot analysis using a MYCN-specific radiolabeled cDNA probe (Fig. 1B). A weak 2.0-kilobase signal of MYCN is seen in SH-EP cells. Its intensity is comparable to that in the human SK-N-SH neuroblastoma cell line which is known to contain a single MYCN gene copy (1, 3, 12). In contrast, SH-EPII or SH-EPIIc cells (not shown) gave considerably more intense signals of MYCN DNA, corresponding to about 5 MYCN gene copies. Somewhat higher signals were found in various soft agar clones derived from SH-EPII cells (named WAC1, WAC2, and WAC3, respectively). For comparison, the signal of the human neuroblastoma line Kelly carrying 100-fold amplification of MYCN (1, 3, 12) is also shown.

We next examined MYCN expression using an RNase protection assay and molecular probes complementary to various regions of the MYCN gene. Using the Nb-1/XB probe, we detected a MYCN signal of weak intensity in SH-EP cells, but of considerably stronger intensity in SH-EPII (Fig. 1C) and SH-EPIIc cells (not shown). These results clearly demonstrate enhanced MYCN expression in the MYCN-transfected neuroblastoma cells.

Expression of Exogenous MYCN. In order to determine if enhanced MYCN expression is due to the transfected exogenous MYCN, we used a molecular probe (NMYCE1) complementary to nucleotides 64-417 of the first exon of the MYCN gene (see also Fig. 1A). Using this probe, we detected a short MYCN transcript corresponding to exogenous MYCN in the MYCN-transfected SH-EPII cells and derived soft agar clones WAC1, WAC2, and WAC3 (Fig. 1D, Arrow c). No such transcripts were detectable in the nontransfected SH-EP cells or in SK-N-SH cells. Two larger MYCN transcripts (Fig. 1D, Arrows a and b) which correspond to endogenous MYCN were detected in the Kelly cell line. In the control cell lines SK-N-SH and SH-EP, endogenous MYCN RNA was undetectable (Fig. 1D). These results demonstrate stable integration, elevated copy number, and enhanced expression of exogenous MYCN in SH-EPII neuroblastoma cells.

Biological Properties. When seeded into plastic culture dishes, SH-EP (Fig. 2A) or SH-EPIIc cells (not shown) attached poorly to the substratum. After several days in culture, they displayed an elongated, spindle-like morphology and grew in a monolayer formation. In contrast, the MYCN-transfected (SH-EPII) cells readily attached to the dishes, displayed a polygonal morphology, grew to high densities and, after having reached confluency, grew on top of each other (Fig. 2B). SH-EP cells (Fig. 2C) or SH-EPII control cells (not shown) were unable to grow in soft agar. In contrast, MYCN-transfected (SH-EPII) cells readily grew in soft agar and formed large colonies (Fig. 2D). Three colonies were expanded and gave rise to soft agar clones WAC1, WAC2, and WAC3.

Tumorigenic Capacity. To examine the effect of enhanced MYCN expression on the tumorigenic capacity of the neuroblastoma cells, four nude mice were given injections (11, 18) of 1 x 10^6 WAC2-cells, a soft agar clone of SH-EPII cells with high MYCN expression (Fig. 1C, Lane 5). Another four nude mice served as controls and were given injections of SH-EP cells. Upon injection of the MYCN-transfected (SH-EPII/WAC2) cells large, visible tumors developed within 10 wk in three of four mice; one of the mice died before tumor development was overt. In contrast, none of the mice that received injections of the nontransfected (SH-EP) cells developed a tumor, even after prolonged times (7 mo) of observation. Likewise, no tumor growth was observed when cells transfected with the control vector were injected into the mice. These results indicate that enhanced MYCN expression contributes in vivo to the tumorigenic conversion of human neuroblastoma cells.

Proliferation and Growth Factor Requirements. Fig. 3 shows the proliferation of SH-EP or SH-EPII cells, respectively, in the presence of decreasing concentrations of FCS and as a function of time. In the presence of optimal (10%) concentrations of FCS, cultures of both cell lines exhibited a high initial rate of proliferation which plateaued after 4 days. However, the final cell density of SH-EPII cell cultures exceeded that of SH-EP cell cultures by a factor of up to 5 (Fig. 3). Remarkably,
Cell densities were determined at the times indicated by medium containing 10% (○, ♦), 1% (●, □), 0.5% (○, △), or 0% (●, □) FCS. SH-EP (A) or SH-EPII (B) cells were seeded at densities of 3 x 10^4 into 35-mm dishes containing RPMI-1640 medium supplemented with 10% FCS and antibiotics. After 10 h, the number of attached cells was determined with a Coulter Counter (Day 0). The medium in the remaining dishes was replaced by medium containing 10% (○, ♦), 1% (●, □), 0.5% (○, △), or 0% (●, □) FCS. Cell densities were determined at the times indicated.

Cultures of SH-EPII cells grown in the presence of only 0.5% FCS displayed the same rate of proliferation as cultures of SH-EP cells grown in the presence of 10% FCS (Fig. 3). These results demonstrate that enhanced MYCN expression in neuroblastoma cells results in their ability to proliferate in the presence of only minute amounts of exogenous growth factors.

SH-EPII Cells Express and Can Utilize MYCN-induced Growth Factor Activity. To assess the possible presence of self-stimulating (autocrine) growth factors in SH-EP or SH-EPII cells we prepared extracts of both cell lines and examined the effects of increasing concentrations of these extracts on the proliferation of either cell line. When extracts of SH-EP or SH-EPII cells were added to SH-EP cells, neither extract could stimulate cell proliferation significantly (Fig. 5A). Likewise, extracts of SH-EPII cells did not stimulate proliferation of SH-EP cells (data not shown). Extracts of SH-EPII cells appeared to inhibit their own proliferation (Fig. 5A) and that of SH-EPII cells (Fig. 5B). In contrast, extracts of SH-EPII cells stimulated the proliferation of their own species, and 100 µg of extract per ml of medium induced a more than 2.5-fold increase in the number of SH-EPII cells (Fig. 5B). This effect was similar to that obtained with optimal concentrations (10%) of FCS.

We also examined the effect of medium conditioned by SH-EP or SH-EPII cells on their own proliferation. For a comparison, we also tested medium conditioned by Kelly, a neuroblastoma line with high MYCN expression (see Fig. 1 and Refs. 1, 3, and 12). Medium conditioned by SH-EP cells had essentially no effect on SH-EP cell proliferation, while low amounts of medium conditioned by SH-EPII or Kelly cells stimulated the proliferation of SH-EP cells modestly (Fig. 6A). In contrast, medium conditioned by SH-EPII or Kelly cells stimulated proliferation of SH-EPII cells to a considerable extent, while medium conditioned by SH-EP cells had only a minimal effect on the proliferation of SH-EPII cells (Fig. 6B).

In conclusion, these results demonstrate the existence of autocrine growth factor activity and the corresponding effector

**Fig. 3.** Time course of cell proliferation in the presence of various concentrations of FCS. SH-EP (A) or SH-EPII (B) cells were seeded at densities of 3 x 10^4 into 35-mm dishes containing RPMI-1640 medium supplemented with 10% FCS and antibiotics. After 10 h, the number of attached cells was determined with a Coulter Counter (Day 0). The medium in the remaining dishes was replaced by medium containing 10% (○, ♦), 1% (●, □), 0.5% (○, △), or 0% (●, □) FCS. Cell densities were determined at the times indicated.

**Fig. 4.** Growth factor requirements. SH-EP (A) or SH-EPII (B) cells were seeded at a density of 2 x 10^4 into 35-mm dishes containing RPMI-1640 medium supplemented with 10% FCS. After 6 h, the medium was replaced by serum-free medium supplemented with transferrin (10 µg/ml). The indicated concentrations of HDL or, for comparison, FCS (10%) were then added once. SH-EP (C) or SH-EPII (D) cells were seeded as described above. After 6 h, the medium was replaced by serum-free RPMI-1640 medium supplemented with transferrin (10 µg/ml) and HDL (500 µg/ml). Every other day, the cells received the indicated concentrations of bFGF. Controls received one addition of FCS (10%). Cells of Experiments A to D were counted after 7 days.

**Fig. 5.** Autocrine growth factor activity in cell extracts. SH-EP (A) or SH-EPII (B) cells were prepared from 3 x 10^4 into 35-mm dishes containing RPMI-1640 medium supplemented with 10% FCS. After 6 h, the medium was replaced by the same medium supplemented with 0.5% FCS or, in the case of controls, with 10% FCS. Every other day, the indicated concentrations of extracts prepared from SH-EP (○) or SH-EPII (●) cells were then added in equal (up to 20 µl) volumes, and cells were counted after 4 days. Similar results were obtained in three further experiments.
cells were counted after 5 days.

or SH-EPII (•) cells were seeded as described in the legend to Fig. 5. The MYCN, acquire an enhanced malignant phenotype (11).

of only minute amounts of exogenous growth factors (Figs. 3 and 4). (b) MYCN-transfected cells seeded into extracellular matrix-coated (26) dishes, unlike the nontransfected cells, can actively proliferate in the absence of any exogenous growth factors (data not shown). (c) Autocrine growth factor activity is demonstrable in extracts and in conditioned medium of transplanted, but not nontransfected, cells and also in a cell line (Kelly) with known MYCN amplification. The growth factor activity can stimulate the proliferation of MYCN-transfected cells to a considerable extent, while no significant effect is seen in the untransfected cells. This suggests the existence of a corresponding effector system in the transfected cells which appears to be either nonexistent or expressed at low levels in the untransfected cells. Thus, it is possible that enhanced MYCN expression contributes to tumorigenic conversion of human neuroblastoma cells by augmented expression and maintenance of an autocrine growth factor system.

Native (SH-EP) neuroblastoma cells appear to contain a cell-associated activity able to inhibit proliferation of the MYCN-transfected and, to a lesser extent, that of the nontransfected cells (Fig. 5). This is not a simple cytotoxic effect, since addition of cell extract, even at the highest concentrations, never resulted in fewer cells than were initially present. At present, the significance of the growth-inhibitory effect is unclear.

Tumorigenesis is a complex multistage process that requires the cooperation of several transforming signals (27). Previous studies in rodent cells have shown that enhanced MYCN expression may be an important factor in malignant tumor progression (11). Here, we have shown that introduction and constitutive expression of exogenous MYCN in human tumor cells results in their autonomous growth, their progression to an advanced malignant phenotype, and their ability to induce tumors in nude mice. We have also presented evidence that autonomous growth may be due to the constitutive expression of autocrine growth factor activity. Studies to further characterize this activity are in progress. It is tempting to speculate that neutralization of this activity might provide a future approach for the treatment of human neuroblastomas.

DISCUSSION

Enhanced expression of MYCN has been found in human neuroblastomas of advanced, but not initial, stages of tumor development (12–14), suggesting that enhanced MYCN expression contributes to the development and/or progression of human neuroblastomas. This hypothesis is supported by the demonstration that cultured rodent cells, upon introduction of MYCN, acquire an enhanced malignant phenotype (11).

Human SH-EP neuroblastoma cells express MYCN at low levels. Transfection of SH-EP cells with the human MYCN gene driven by a viral enhancer/promoter resulted in a cell line (SH-EPII) with stable integration and enhanced expression of exogenous MYCN. The transfected cells, in contrast to their nontransfected counterparts or cells transfected with a control vector, were able to grow in soft agar and had acquired a strong proliferative potential. In addition, the transfected, but not the parental, cells were able to induce tumors in nude mice. These properties indicate an MYCN-induced progression of the neuroblastoma cells to an advanced malignant phenotype.

Earlier studies (23, 24) have indicated that cells, in order to grow in soft agar, must be able to synthesize their own (auto- or autocrine) growth factors. The ability of the MYCN-transfected, but not the nontransfected, cells to grow in soft agar therefore suggested an MYCN-induced synthesis of self-stimulating (autocrine) growth factors. Autocrine growth factors are synthesized by many normal cells. They either remain cell associated or are released (24) and interact with specific cell surface receptors, eventually stimulating a limited growth of the normal cells (24, 25). Inappropriate expression of such factors, for example, as a result of an activated oncogene, might disrupt normal cell proliferation and could thereby contribute to tumorigenic conversion of the normal cells or to the malignant progression of tumor cells (24, 25).

Based on these observations, it appeared possible that enhanced MYCN expression might stimulate the expression of autocrine growth factors. Three lines of evidence support this hypothesis. (a) MYCN-transfected neuroblastoma cells, unlike their nontransfected counterparts, can proliferate in the presence of only minute amounts of exogenous growth factors (Figs. 3 and 4). (b) MYCN-transfected cells seeded into extracellular matrix-coated (26) dishes, unlike the nontransfected cells, can actively proliferate in the absence of any exogenous growth factors (data not shown). (c) Autocrine growth factor activity is demonstrable in extracts and in conditioned medium of transplanted, but not nontransfected, cells and also in a cell line (Kelly) with known MYCN amplification. The growth factor activity can stimulate the proliferation of MYCN-transfected cells to a considerable extent, while no significant effect is seen in the untransfected cells. This suggests the existence of a corresponding effector system in the transfected cells which appears to be either nonexistent or expressed at low levels in the untransfected cells. Thus, it is possible that enhanced MYCN expression contributes to tumorigenic conversion of human neuroblastoma cells by augmented expression and maintenance of an autocrine growth factor system.

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