Contrib Oncol, vol 39, pp 86–93 (Karger, Basel, 1990)

Molecular Events During Tumor Progression in Neuroblastoma

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The N-myc oncogene was fist identified as an amplified DNA element with homology to c-*myc* in human neuroblastoma [1, 2]. Since this initial observation, amplification of N-*myc* has also been observed in other types of human cancer, predominantly in retinoblastoma and small cell lung cancer [3, 4]. The apparent role of the N-*myc* oncogene in neuroblastoma oncogenesis is unusual in that amplification of N-*myc* is hardy ever observed in primary non-metastatic neuoblastoma. Rather, amplification of N-*myc* has been found predominantly in advanced, widely metastatic neuroblastoma [5, 6]. This close association between N-*myc* amplification and metastatic ability suggests that a relationship exists between these two phenomena, namely that N-*myc* over-expression is responsible for the increased metastatic ability of the N-*myc*-amplified tumor cells. The role of N-*myc* in neuroblastoma thus appears to be quite different from its role in other types of tumors, in which N-*myc* expression is thought to be an early event in the process of cell transformation. For instance, N-*myc* has been found to be frequently activated by proviral insertion in murine T-cell lymphomas [7], suggesting that transcriptional deregulation of N-*myc* is an early event in the genesis of T-cell lymphomas. Consistent with this latter notion, in vitro experiments have indicated thath the N-*myc* gene can immortalize primary rat embryo fibroblasts in tissue culture. Taken together, these data suggest that N-*myc* plays a role early in the process of oncogenic transformation, causing the transformation of a cell with limited in vitro lifespan into one that is immortal [8].

Our research has focused on understanding the mechanism by which the N-*myc* gene contributes to the increased metastatic ability of N-*myc*-amplified neuroblastomas, the results of which are discussed below.

N-myc as a Regulator of Cellular Gene Expression

The N-*myc* oncogene has been shown to encode a nuclear phosphoprotein that has the ability to bind to DNA [9]. These properties of the N-*myc* protein suggest that it might exert its oncogenic effect by deregulating the expression of certain key cellular genes, the altered expression of which is responsible for the N-*myc*-induced changes in cellular behavior. Thus, N-*myc* over-expression in neuroblastoma may cause an alteration in the expression of cellular genes which leads to increased metastatic ability. We have attempted to identify cellular genes whose expression is affected by N-*myc* in neuroblastoma in an attempt to find an explanation at the molecular level for the increased metastatic ability of N-*myc*-amplified neuroblastoma tumor cells.

To identify genes whose expression is altered in neuroblastoma by N-*myc* we followed up on an observation made earlier regarding the mechanism of adenovirus-mediated transformation. In this system, we found that the E1A oncogene of the highly oncogenic adenovirus type 12 was able to suppress the expression of the major histocompatibility compley (MHC) class I antigens in transformed cells [10]. Since MHC class I antigens are required for the recognition of foreign antigens by cytotoxic T-lymphocytes [11], their absence from the surface of adenovirus 12-transformed cells explained why these cells could induce tumors in immunocompetent hosts.

To investigate whether the N-*myc* oncogene shared the ability of the adenovirus E1A oncogene to suppress the expression of the MHC class I antigens, we screened a number of human neuroblastoma tumor cell lines for their expression of both MHC class I antigens and N-*myc*. We found that in all cell lines tested the expression of N-*myc* was inversely correlated with the expression of MHC class I antigens [121]. Although these experiments suggested that there might be a relationship between the expression of N-*myc* and MHC class I in neuroblastoma, these initial observations did not prove that a causal relationship existed between the expression patterns of these two genes.

To further study the possible relationship between the expression of N-*myc* and MHC class I, we used a rat neuroblastoma cell line named B104. These rat cells resembled human neuroblastoma which did not carry an

amplified N-*myc* gene in that they grew slowly but progressively in immunocompetent rats without progressing to form metastases. By transfecting an N-*myc* expression vector into B104 cells, we obtained a series of N-*myc* expressing derivatives of this parental cell line. The N-*myc*-transfected derivatives were found to have undergone several major changes in phenotype. First, the N-*myc*-transfected derivatives of the B104 cell line were strikingly more malignant: following injection in immunocompetent syngeneic rats, N-*myc*-transfected B104 cells grew rapidly and formed widespread metastases. In this respect, the N-*myc*-transfected B104 cells seemed to resemble advanced childhood neuroblastoma in which over-expression of N-*myc* is also associated with increased metastatic ability [5, 6]. Significantly, N-*myc*-transfected B104 cells were also found to have a substantial decrease in the expression of MHC class I antigens, indicating that N-*myc* was indeed responsible for the low MHC class I phenotype of neuroblastomas [12].

Mechanism of Transcription Regulation by N-myc

The experiments described above supported the long-standing hypothesis that *myc* oncogenes could act to deregulate cellular gene expression. It therefore appeared of considerable interest to us to investigate in detail the mechanism by which the N-*myc* gene product could modulate the expression of MHC class I antigens.

To do this, we first investigated whether N-*myc* acted to suppress the transcription of MHC class I antigens in neuroblastoma cells. We found that the murine MHC class I H-2K^b promoter was approximatelly 10- to 15-fold less active in B104 N-*myc*-transfected cells as compared to the parental B104 cells, indicating that N-*myc* acted to suppress MHC class I at the level of transcription initiation [13]. By analyzing the activity of a series of deletion mutants from this murine H-2K^b promoter construct in both B104 and B104 N-*myc*-transfected cells, we found that one of the major sites on which N-*myc* acted to suppress MHC class I antigen expression was an enhancer element centered around nucleotide-166 of the murine H-2K^b promoter [13]. This enhancer had previously been shown to stimulate the activity of the MHC class I gene promoter [14].

Furthermore, it had been shown that this enhancer element stimulated transcription through the binding of a nuclear factor, named H2TF1 [14]. These results suggested the possibility that N-*myc* suppressed the activity of

this enhancer element by interfering with the binding of this nuclear factor to the MHC class I gene enhancer.

To investigate this, we prepared nuclear extracts from B104 neuroblastoma cells and its N-*myc*-transfected derivatives and tested these in a gel electrophoresis DNA-binding assay with an oligonucleotide corresponding to the MHC class I gene enhancer as a probe. The results of this experiment, shown in figure 1a, indicate that B104 neuroblastoma cells contain a nuclear protein that binds to the wild-type MHC class I gene enhancer (lane B104-wt), but not to a mutant version of this element (lane B104-M). Significantly however, B104 N-*myc*-transfected cells were found to contain much lower levels of this nuclear factor. Reduced binding of nuclear factor to the MHC class I gene enhancer in the N-*myc*-transfected cells did not appear to be caused by proteolytic degradation of the nuclear extract, since the same extract contained high levels of nuclear factor binding to the immunoglobulin $\varkappa$E2 or μE3 binding sites (fig. 1b). Since this initial observation, we have also shown that N-*myc* markedly abrogates the binding of nuclear factors to the MHC class I gene enhancer in human neuroblastoma [13]. We conclude from these experiments that N-*myc* suppresses the expression of MHC class I genes by decreasing the binding of a nuclear factor to MHC class I gene enhancer, thereby functionally inactivating this element of the MHC class I gene promoter. The further study of the interaction between the N-*myc* oncoprotein and the H2TF1 transcription factor could yield important insights into the mechanism by which N-*myc* acts to deregulate cellular gene expression.

Neural Cell Adhesion Molecule Expression in Neuroblastoma Cells

The experiments described above established the N-*myc* gene product as a critical regulator of cellular gene expression. However, it appeared unlikely to us that in neuroblastoma N-*myc* would only affect the expression of MHC class I antigens. We therefore investigated the pattern of expression of several other genes, whose altered expression could potentially contribute to the increased metastatic ability of N-*myc* amplified neuroblastomas. One of the genes that we explored was the gene encoding neural cell adhesion molecule (NCAM), a family of related cell surface glycoproteins involved in cell-cell recognition and cell-cell adhesion.

Evidence that reduced NCAM expression could be relevant to the induction of metastatic ability comes from the study of NCAM expression

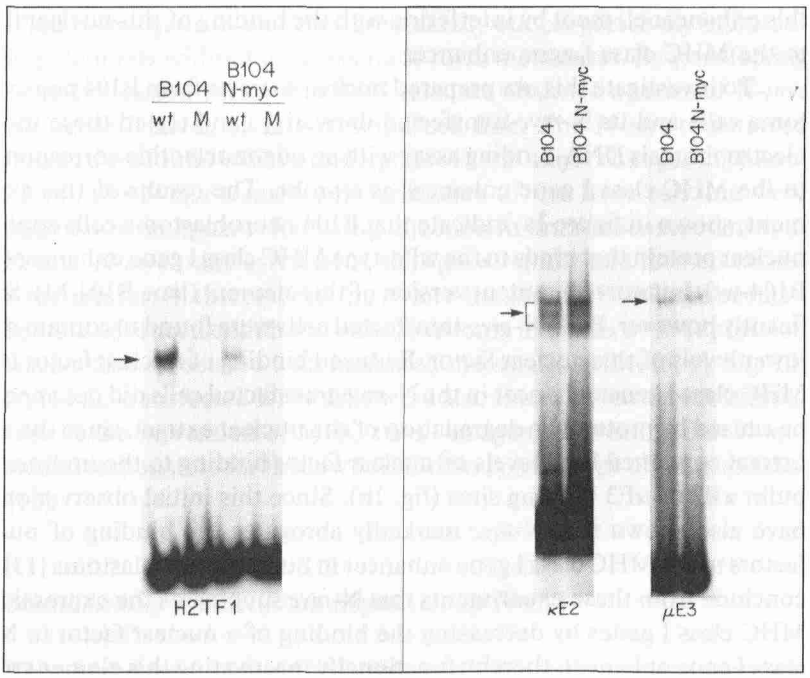

Fig. 1. Abundance of nuclear factors in neuroblastoma cells. Nuclear extracts were prepared from B104 neuroblastoma cells and N-*myc*-transfected B104 cells. Binding of nuclear factors was detected using a gel electrophoresis DNA-binding assay. *a.* 4000 cpm of ^{32}P-labeled oligonucleotide specifying either the consensus H2TF1-binding sequence (lanes indicated wt) or mutant H2TF1 sequence (lanes indicated M) was incubated with a nuclear cell extract from either B104 cells or B104 N-*myc*-transfected cells and separated on 4 % polyacrylamide gel. *b.* 4000 cpm of a DNA fragment specifying the binding site for either nuclear factor NF-*κE2 or NF-μ*E3 [18] was incubated with a nuclear extract as indicated and separated on a 4 % polyacrylamide gel.

during embryogenesis. Here it was found that premigratory neural crest cells, which are clustered adjacent to the dorsal neural tube, express readily detectable levels of NCAM polypeptides. However, as the cells leave this site to migrate towards the periphery as single cells, they do not express immunologically detectable NCAM. Upon arriving at their final anatomic location in the embryo they re-express NCAM [15]. Thus, migratory potential and NCAM expression are inversely related in neural crest cells. Since neural crest cells and neuroblastoma tumor cells are both derived from the neuro-ectodermal lineage, we felt it was possible that alterations in cell

adhesion also contribute to the migratory potential of neuroblastoma tumor cells.

To investigate this, we measured NCAM levels in both B104 neuroblastoma cells and B104 N-*myc*-transfected cells. We found that several independently derived transfectants of the B104 neuroblastoma cell line that expressed N-*myc* at a high level had lost to a significant extent the expression of NCAM polypeptides and mRNA. A number of other neural components were not specifically suppressed by N-*myc*. Our data further indicate that revertants of the high N-*myc* phenotype that had lost most of their N-*myc* expression had regained significant levels of NCAM polypeptides, indicating that the continued expresion of N-*myc* was required to maintain the low levels of NCAM expression [16].

Conclusions

We have used a rat model system to study the effects of N-*myc* amplification on the malignancy of neuroblastoma tumor cells. Our data indicate that over-expression of N-*myc* in neuroblastoma tumor cells has at least three major effects. First, the rate at which N-*myc*-amplified tumor cells grow *in vivo* is greatly accelerated. In our rat B104 tumor system, we found that N-*myc* expressing neuroblastoma cells grew within 14 days to yield a tumor that was 300-fold larger in size than the tumor induced by the parental neuroblastoma cells. Second, we found that expression of N-*myc* leads to a dramatic reduction in the expression of MHC class I antigens, rendering the N-*myc*-amplified tumor cells resistant to T-cell-mediated immune destruction. Third, our data indicate that N-*myc* has the ability to suppress the expression of neural cell adhesion molecule. Since NCAM allows neuronal cells to adhere to each other, one would expect that neuroblastoma tumor cells that express reduced levels of these adhesion molecules have less of a tendency to adhere to the primary tumor mass than their non N-*myc*-amplified counterparts. N-*myc*-amplified neuroblastoma tumor cells may therefore break away from the primary site of tumor growth more readily than the non N-*myc*-amplified tumor cells, thus contributing to the increased metastatic ability of N-*myc*-amplified tumor cells. In support of this view is the recent finding that a highly metastatic subclone from a mouse melanoma cell line expressed much lower levels of NCAM than the parental low metastatic melanoma [17]. During their journey from the primary site of tumor growth to the secondary site, N-*myc*-amplified tumor cells

should be protected from attack by cytotoxic T-lymphocytes due to the paucity of MHC class I antigens on these cells. Finally, the dramatically accelerated growth rate should help the N-*myc*-amplified tumor cells to proliferate at the secondary site to form a metastasis.

There is no doubt that the scenario described above represents an oversimplification of the events that take place during tumor cell metastasis. For instance, we have ignored the need for tumor cells to invade barriers such as the basement membrane and blood vessel walls. Nevertheless we feel that the observations made in the rat model system may very well begin to explain the differences in metastatic ability between non N-*myc*-amplified neuroblastomas and their N-*myc*-amplified counterparts. Molecular biology provides us with the tools to experimentally modulate the expression of MHC class I antigens and NCAM polypeptides in neuroblastoma cells. This should allow us to investigate the contribution of the altered expression of these cellular genes to the metastatic phenotype of neuroblastoma tumor cells in more detail.

Acknowledgments

The work described here was supported in part by grants from the Edward J. Mallinckrodt Foundation and the Searle Scholarship Foundation.

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