Elevated *c-myc* Expression in Childhood Medulloblastomas

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ABSTRACT. Medulloblastoma is a rare brain tumor usually occurring in late childhood or early adolescence. Little is known regarding the cell of origin or cellular events leading to its malignant transformation. We have studied the expression of developmentally regulated mRNA in tumor samples by the Northern hybridization assay to determine a relative stage at which a block to further differentiation occurs. In a series of five medulloblastoma tumors analyzed, only one of eight markers for cellular differentiation, the glial fibrillary acidic protein mRNA, was expressed at significant levels. Interestingly, three of six medulloblastoma tumor samples were found to have elevated levels of *c-myc* mRNA. In one of these tumors, we have found evidence of mutation of the *c-myc* protooncogene. We discuss possible mechanisms of *c-myc* activation in medulloblastoma tumors. (*Pediatr Res* 28: 63-68, 1990)

Abbreviations

GFAP, glial fibrillary acidic protein
PCR, polymerase chain reaction

Medulloblastoma brain tumors occur primarily in the pediatric population with 80% of all cases diagnosed before age 15 y (1). The cell of origin remains unknown, although some workers (2) suggest that the tumor arises from external granule cells, which are known to undergo postnatal mitosis and migration before their final differentiation. Although the tumor is statistically rare, it is the single most common brain tumor occurring in childhood (1). Despite significant improvements in multimodality therapy, including aggressive neurosurgical techniques, multiagent chemotherapy, and radiation therapy, only approximately 50% of children with this disease are cured (3). Within this group, there is a high incidence of serious neurologic sequelae.

Recently, molecular events have been described that may contribute to the development of CNS tumors. For example, the serum growth factor PDGF, which is the protein product of the cellular gene *c-sis*, has been found to be secreted by *N*-ethyl-*N*-nitrosourea-induced glial brain tumors (4). Similarly, Liberman *et al.* (5) have reported amplification, enhanced expression, and possible rearrangement of the *c-erbB* gene in glial brain tumors. Of more specific interest to medulloblastomas, tumors of neural origin (2), is the family of *myc* genes. The oncogene *N-myc* was

first described (6) in tumor tissue arising from the peripheral nervous system, *i.e.* neuroblastomas. Evidence presented in this work links overexpression of the related protooncogene *c-myc* to medulloblastomas, tumors that arise from the CNS. Although the precise function of the *c-myc* gene product, a nuclear phosphoprotein (7), has not been defined, expression of the *myc* family may play a role in making cells competent to respond to signals that induce differentiation or proliferation (8). Enhanced *c-myc* gene expression has been found in a variety of tumors and cell lines (9-12), which favors a link between *c-myc* expression and cellular proliferation. Several studies also support a role for *c-myc* in blocking differentiation. Constitutive expression of *c-myc* can block mouse erythroleukemia cell (13) and PC12 pheochromocytoma cell (14) differentiation *in vitro* and maintain the proliferative state of these cells in the presence of differentiation inducers.

In our report, we have assayed for molecular evidence of the extent of differentiation of five medulloblastoma tumors to identify a relative stage at which differentiation is blocked. mRNA isolated from tumor samples was screened with a panel of radiolabeled cDNA probes. These cDNA probes were selected on the basis that they correspond to functions expressed within the CNS at different stages of neuronal or glial development. From our panel of cDNA probes, only that for GFAP, an intermediate filament protein, was expressed at levels detectable by Northern assay in the tumor RNA. In three of six medulloblastoma tumors analyzed for *c-myc* expression, we found elevated levels of *c-myc* mRNA. Moreover, we found that GFAP mRNA expression appeared to be inversely correlated with *c-myc* mRNA levels in six of six informative tumors.

MATERIALS AND METHODS

Tissue samples. After approval by the Institutional Review Board of the New York University Medical Center, tumor samples were collected directly from the operating room and placed immediately in liquid nitrogen.

DNA probes. The following cDNA probes were used in this study: pGmycScrFI, a 72-bp subclone spanning the 3' end of the human *c-myc* 1st exon; pmycX-PstII, a 1.1-kb human *c-myc* subclone that includes 133 bp of the 5' end of the 2nd exon; and pmyc 413-R/C, a 1.3-kb human *c-myc* 3rd exon subclone. The *c-myc* probes were kindly provided by Ethel Cesarman and Riccardo Dalla-Favera (15). A 1-kb *N-myc* subclone, pNb-1, was obtained from the American Type Culture Collections, Rockville, MD (41011). NF68 a 1.2-kb subclone of the murine 68 000 mol wt neurofilament protein, was kindly provided by Lewis and Cowan (16). Plasmid G1, a 2.5-kb mouse subclone of GFAP was also provided by S.A. Lewis and N.J. Cowan (17). pSPT, a 1.8-kb subclone of bovine chromogranin A, was kindly provided by M. L. Grimes (18). Int-1_{ES}, a 520-bp subclone of mouse *int-1*, was kindly provided by D. G. Wilkinson (19). pM1B1, a 1.7-kb human subclone (H.1) for the homeobox protein Hox 2.1, was kindly provided by Craig Hauser (20). Clone 73, which contains

Received June 30, 1989; accepted March 2, 1990.

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Supported by the Children's Cancer Fund no. 64-1-6101 of the Department of Pediatrics, New York University Medical Center; the American Cancer Society Institutional Grant IN-14-30; the Department of Environmental Medicine of the New York University Medical Center (Grant ES00136); and NIH Grant 1K11CA01419-01.

the cDNA for the rat neurofilament protein peripherin, was described by Leonard *et al.* (21). A 580-bp subclone of the murine neural cell adhesion molecule (N-CAM) mRNA, p1.3, was kindly provided by Christo Goridis (22). pHFBA-1, a 2.0-kb subclone of the human β -actin gene, was kindly provided by L. Kedes (23).

Isolation of RNA. Total cellular RNA was extracted by the guanidinium/cesium chloride method (24) from tumor samples frozen in liquid nitrogen. Total cellular RNA from HeLa cells, a cell line that is derived from a human cervical carcinoma and that expresses high levels of *c-myc* mRNA, was a gift from Anna Velcich (Montefiore Hospital, Bronx, NY).

Northern blot analysis. Northern analysis of 20- μ g aliquots of total cellular RNA was carried out as described (25) using fragments from plasmid subclones labeled by the method of Feinberg and Vogelstein (26).

Ribonuclease protection assay. The ribonuclease protection assay was performed by a modification of the procedure described by Winter *et al.* (27). For the synthesis of long RNA transcripts (>1000 bp), cold cytidine 5'-triphosphate was added to the reaction mixture to a final concentration of 10–20 μ M. Unincorporated label was removed by centrifugation through G-50 spin columns. Five $\times 10^5$ cpm of the probe were coprecipitated with 20 μ g of RNA and then resuspended in hybridization buffer.

Isolation of DNA and Southern blot analysis. DNA was isolated (28) from medulloblastoma tumor tissue frozen in liquid nitrogen. Ten μ g of tumor DNA and control DNA isolated from a human T cell line with the normal *c-myc* genomic structure (MOLT-16, a gift from Nino Neri) were digested with restriction enzyme *PvuII*, and processed as described (29).

PCR. The PCR was performed essentially as described (30). Primers included a *c-myc* oligomer of 20 residues homologous with nucleotides 1001 through 1020 (numbering in ref. 31) of the human *c-myc* gene and situated within the 1st exon downstream of the P_2 promoter. This oligomer contained a 5' linker sequence with sites for the restriction enzymes *XhoI*, *SalI*, and *ClaI*, and had the nucleotide sequence: 5'-GACTCGAGTTCGACATCGATCGAAAACCTTTGCCCATAGCA-3'. A second *c-myc* primer was complementary to nucleotides 2859 through 2879 (31) and was situated within the 2nd *c-myc* exon at its 5' boundary. This oligomer contained a linker sequence with sites for the restriction enzymes *EcoRI*, *SmaI*, and *HindIII*, and had the nucleotide sequence: 5'-GAGAATTCCCGGGAAGCTTCCTGTTGGTGAAGCTAACGT-3'. Human β -actin primers included two 20-mers. One primer was homologous with nucleotides 129 through 148 of the human β -actin cDNA (32) and had the nucleotide sequence: 5'-GTCTTCCCCTCCATCGTGGG-3'. The 2nd β -actin primer was complementary to nucleotides 609 through 628 (32) and had the nucleotide sequence: 5'-CGCTCGGTGAGGATCTTCAT-3'.

RESULTS

Three of six medulloblastoma tumors analyzed have elevated *c-myc* expression. Initially, a series of seven pediatric tumors was examined for expression of genes that are indicative of differentiation and that are known to be expressed in cells of neuroectodermal origin. The tumors included five medulloblastomas, one ependymoma, and one neuroblastoma; a 6th medulloblastoma specimen was collected later in the study. Using the selected cDNA probes, Northern analysis from these tumors indicated that only GFAP was expressed at detectable levels (Fig. 1, panel a, lanes 5–8). To assess the potential involvement of the *c-myc* protooncogene, we repeated the Northern blots with *pmyc* 413-R/C, a cDNA that spans the 3rd exon of *c-myc* (Fig. 1, panel b). Two medulloblastomas (M-2 and M-3, Fig. 1, lanes 5 and 6) and the neuroblastoma (lane 10) were found to express high levels of *c-myc* mRNA when compared with adult rat brain and human fetal brain. In each case, the mRNA comigrated with *c-myc* mRNA from HeLa cells, which are wild type in size. A

Fig. 1. Northern blot analysis for GFAP and *c-myc* expression. Twenty μ g of total cellular RNA were collected from the following tissue samples and loaded onto the indicated lanes. Lane 1, adult rat brain; lane 2, fetal human brain; lane 3, HeLa cells. Lanes 4 through 8, RNA from medulloblastoma tumors as follows: lane 4, M-1; lane 5, M-2; lane 6, M-3; lane 7, M-4; lane 8, M-5. Lane 9, ependymoma; lane 10, neuroblastoma. Transfer to Nytran filters (Schleicher & Schuell, Keene, NH) and hybridization are as described in Materials and Methods. The following oligolabeled probes were used: a) G1, a cDNA encoding GFAP, b) *pmyc* 413-R/C, a cDNA representing the entire 3rd exon of *c-myc*. Arrowheads with an O mark the origins. Long arrows identify the 28S and 18S ribosomal markers.

comparison of band intensities in Figure 1, panels a and b, indicates an inverse correlation between GFAP expression and *c-myc* expression in the tumor samples analyzed in Figure 1, lanes 5 through 10.

To control for any possible differences in the quantity of cellular RNA added to each lane, we analyzed by Northern assay the level of β -actin mRNA, which is relatively constant in different samples of similar tissue. The same Northern blot, containing RNA from the original five medulloblastoma tumors plus one additional medulloblastoma, was then stripped and exposed to the radiolabeled human *c-myc* probe, *pmyc* 413-R/C (Fig. 2, panel b). Three of the medulloblastoma tumor samples (M-2, M-3, and M-6) exhibit detectable levels of *c-myc* message; long exposures did not reveal any *c-myc* expression in the remaining three tumor samples (M-1, M-4, and M-5).

To confirm the results of the Northern blot assay and to investigate possible mechanisms of transcriptional deregulation, we examined the tumor *c-myc* mRNA by means of the ribonuclease protection assay. Identical aliquots of total cellular RNA from the six medulloblastomas in Figure 2 were hybridized with a radiolabeled antisense transcript of *mycX-PstI*, which overlaps the junction of the 5' end of the 2nd exon and the 1st intron of human *c-myc* (Fig. 3), digested with nuclease, and analyzed by gel electrophoresis. In Figure 3, protected fragments consistent with elevated *c-myc* mRNA expression in tumors M-2, M-3, and M-6 were observed. The size of these bands was the same as obtained with HeLa *c-myc* mRNA, which is wild type in splicing, indicating that the tumor RNA were correctly spliced at the 2nd exon 5' boundary, and that intron sequences are absent from the mRNA. This observation argues against aberrant initiation