Expression of Oligodendrocyte-associated Genes in Cell Lines Derived from Human Gliomas and Neuroblastomas

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ABSTRACT

Two putative human oligodendroglioma cell lines were examined for the expression of the oligodendrocyte-associated genes, 2",3"-cyclic nucleotide 3'-phosphodiesterase, myelin basic protein, myelin proteolipid proteins, and myelin-associated glycoprotein. The expression of these genes was also examined in control astrocytoma and neuroblastoma cell lines. In addition, the expression of the oligodendrocyte-associated genes, glial fibrillary acidic protein (GFAP), neuron-specific enolase and neurofilaments (NF) NF-L and NF-M also were examined. All the cell lines expressed 2",3"-cyclic nucleotide 3'-phosphodiesterase, neuron-specific enolase, and vimentin, and none expressed myelin-associated glycoprotein. The "oligodendrocyte-specific" myelin proteolipid protein mRNAs and the "neuron-specific" NF-L mRNA were expressed in the two astrocytoma cell lines, which also expressed GFAP. Expression of intermediate filament protein genes was more restricted. The astrocytoma, neuroblastoma, and oligodendroglioma cell lines expressed only GFAP, NF-M, and cytokeratin K7, respectively. These results: (a) provide molecular data confirming the classification of the two cell lines as oligodendrogliomas and suggest that their molecular profiles are indicative of immature oligodendrocytes; (b) demonstrate the expression of cytokeratins in oligodendroglioma cell lines and suggest that apparent GFAP expression in oligodendrogliomas detected by immunocytochemical methods may be due to cross-reactivity with cytokeratins, with which they share common polypeptide sequence; and (c) indicate that astrocytoma cell lines can exhibit a "mixed" phenotype, expressing genes associated with fully differentiated oligodendrocytes and neurons.

INTRODUCTION

The identification of oligodendrogliomas has been based largely upon morphological and immunocytochemical criteria (1), although relatively few tumors have been studied and immunohistochemical data have not always been available. Interestingly, astrocytomas may show focal regions of oligodendroglialomatous differentiation (2), although the significance of this is unclear. This association of the two types of differentiation has led to speculation that oligodendrogliomas and astrocytomas might be derived from the transformation of a common glial precursor or that neoplastic oligodendrocytes might transform into astrocytomas giving rise to mixed gliomas (3, 4).

The oligodendrocyte is the myelin-forming cell in the central nervous system and it expresses the genes coding for the myelin structural proteins in a cell-specific and temporally regulated fashion (5). However, characterization of oligodendrogliomas with markers of mature oligodendrocyte gene expression has met with mixed success, since tumors may be derived from immature or precursor oligodendrocytes or by the time of examination the tumor cells may have lost the phenotype of the mature cell.

Biochemical markers (protein, nucleic acid, and lipid) of immature and precursor stages of oligodendrocyte differentiation have become readily available, providing a battery of reagents with which to examine oligodendrogliomas at the molecular level (5, 6). The use of these markers for in vivo and in vitro studies has resulted in a schema by which oligodendrocytes can be subclassified by differentiation state based upon immunocytochemical and molecular biological criteria (6). Until now, expression of oligodendrocyte (stage)-specific genes has not been applied extensively to oligodendrogliomas or cell lines derived from them.

The purpose of this work was to examine two putative human oligodendroglioma cell lines to determine the extent to which these cell lines expressed oligodendrocyte-specific genes. We also compared the expression of these genes in the oligodendroglioma-derived cell lines to their expression in cell lines derived from other neural cell types. In addition to supporting the assignment of these cell lines to the oligodendrocyte lineage through their expression of both normal and novel markers of oligodendrocyte derivation, we also found that astrocytoma- and, to some extent, neuroblastoma-derived cell lines, expressed "oligodendrocyte-specific" genes.

MATERIALS AND METHODS

Cell Culture. Tumor cell lines were grown in Iscove's modified Dulbecco's medium (Irvine Scientific, Santa Ana, CA) supplemented with 10% fetal calf serum (Sigma, St. Louis, MO), 2 mm L-glutamine, and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin, and 250 μg/ml Fungizone; Irvine Scientific) at 37°C in 5% CO2. The human brain tumor cell lines used were two oligodendroglioma cell lines, HOG (7) and TCG620 (8), two astrocytoma cell lines, U251MG and U373MG (9), and one neuroblastoma cell line, SK-N-SH (10). The original TCG620 cells were supplied by Dr. L. Manuellides (Yale University, New Haven, CT) and subcloned by Dr. J. Merrill (11). U251MG cells were supplied by Dr. B. Westerman (University of Uppsala, Uppsala, Sweden). U373MG and SK-N-SH cell lines were purchased from the American Type Culture Collection (Rockville, MD).

The characteristics of the TCG620 cell lines used in this study have been published previously (8, 11). Under our growth conditions the cells had a doubling time of 24 h, somewhat shorter than the astrocytoma lines. They exhibited a bipolar, epithelioid-like morphology with thick, short cytoplasmic processes; and a high nucleus/cytoplasm ratio with the nuclei generally containing a single nucleolus. The cells have been reported to express surface galactocerebroside, thereby placing them in the oligodendrocytic lineage (11). Their expression of surface components, determined by immunocytochemical analysis, is characteristic of immature oligodendrocytes (11).

Some characteristics of the HOG oligodendroglioma cell line have been published recently (7). These cells grew well under our growth conditions, exhibiting a doubling time of 18 h. Morphologically, these cells were also epithelioid-like with a bipolar and in some cases triangular form. Occasionally, the cells exhibited long, thin processes, particularly when grown at low density. The nucleus/cytoplasm ratio in these cells was also very high. These cells have been reported to express the oligodendrocytic markers galactocerebroside, galactocerebroside sulfate, and CNPase.3

Northern Blot Analysis. Total RNA was isolated by the method of Chomczynski and Sacchi (12) from the cortex of a 4–5-month-old human and confluent human tumor cells. Total RNA was selected for polyadenylated RNA

3 The abbreviations used are: CNPase, 2",3"-cyclic nucleotide 3'-phosphodiesterase; cDNA, complementary DNA; SDS, sodium dodecyl sulfate; SSPE, saline-sodium phosphate-EDTA; GFAP, glial fibrillary acidic protein; MAG, myelin-associated glycoprotein; MBP, myelin basic protein; NF, neurofilament protein; NSE, neuron specific enolase; PLP, myelin proteolipid protein; IF, intermediate filament.
by chromatography on oligodeoxynucleotidylcellulose (Collaborative Biomedical Products, Bedford, MA) (13). Polyadenylated RNA (7 μg/lane) was electrophoresed in denaturing agarose gels containing 2.2 M formaldehyde and transferred to nylon membrane filters (Nytran; Schleicher & Schuell, Inc., Keene, NH) with 10× standard saline-citrate (1× concentration is 150 mm sodium chloride-15 mm sodium citrate) (14). The RNA was immobilized onto filters by UV-cross-linking at 160 J/m² after drying them at 80°C. cDNA probes were labeled to a specific activity of 1–3 × 10⁶ dpm/μg with [α-3²P]-dCTP (3000 Ci/mmol; DuPont, NEN, Boston, MA) by random priming (15). The blots were hybridized at 42°C in 50% formamide, 5% SDS, 25 mm sodium phosphate (pH 7.2), 1 mm EDTA, and 10 μg/ml denatured low molecular weight DNA for at least 15 h and washed once in 2X SSPE/0.1% SDS at room temperature for 15 min, twice in 1X SSPE/0.1% SDS at 37°C for 15 min, and twice in 0.1× SSPE/0.1% SDS at 55°C for 15 min. The blots were exposed to X-ray film, XAR-5 (Kodak, Rochester, NY) with an intensifying screen at −80°C (14).

Western Blot Analysis. Cell pellets and frozen tissues were homogenized in 10% SDS buffer (50 mg tissue/ml) and protein concentrations were determined by the modified method of Lowry et al. (16) (Sigma; protein assay). Fifty μg of protein of each sample were electrophoresed in SDS-polyacrylamide gels (17). Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell) by the method of Towbin et al. (18). Specific antibodies and membranes were incubated in phosphate-buffered saline containing 0.5% blocking reagent (Boehringer Mannheim, Indianapolis, IN) at room temperature for at least 10 h. Bound antibodies were detected with 15–20 μCi/blot 125I-labeled protein (10–15 μCi/mg protein) (DuPont, NEN), washed three times with phosphate-buffered saline containing 0.1% Triton X-100, and exposed to X-ray film at −80°C.

Probes and Antibodies. cDNA probes were obtained from the following sources: rat CNPase (19) and rat MAG cDNAs (20) were obtained from Dr. David Colman; human MBP (21) and PLP (22) cDNAs were isolated in our own laboratory; rat NSC-23 (23) was obtained from Dr. Gregor Sutcliffe; a full-length mouse GFAP cDNA was isolated by us from a mouse brain library with a clone provided to us by Dr. Nicholas Cowan (24); the human GFAP cDNA was obtained from Dr. Steven Reeves (25); a human vimentin cDNA was purchased from the American Type Culture Collection (26); and the anti-CNPase polyclonal antibodies were the gift of Dr. David Colman; anti-GFAP monoclonal antibodies were purchased from Immunoblot, Inc.; anti-vimentin monoclonal antibody (clone 3B4) and anti-cytokeratin K7 monoclonal antibody (clone CK 7) were purchased from Boehringer-Mannheim; and anti-NSE polyclonal antisera were purchased from Polyclones, Inc.

RESULTS

In these studies several markers of cells in the oligodendrocyte lineage were examined. Markers of mature oligodendrocyte gene expression included MBP, PLP, and the MAG. Markers of earlier stages of oligodendrocyte gene expression were CNPase and the 2.6- and 5-kilobase MBP gene-related mRNAs (28, 29). Markers of other neural cell types included GFAP for astrocytes as well as NSE and NF-L and NF-M for neurons. Vimentin expression also was examined because of its relationship to GFAP, a member of the same intermediate filament protein subclass. In addition to the HOG and TC620 oligodendroglioma cell lines we examined the expression of these markers in the astrocytoma-derived cell lines, U251MG and U373MG, and the neuroblastoma-derived cell line, SK-N-SH.

Northern Blot Analysis of the Human Oligodendrogloma-derived Cell Lines, HOG and TC620

Expression of Oligodendrocyte-specific mRNAs. The rat CNPase cDNA probe hybridized to the 2.6-kilobase CNPase mRNA in all the human neurogenic tumor cell lines (Fig. 1). The human MBP cDNA hybridized to the expected 2.2-kilobase mRNA, the product of the major MBP gene promoter, in RNA isolated from human cortex (Fig. 1, Lane 1), but it did not hybridize to a 2.2-kilobase RNA in the cell lines. Instead, the probe hybridized to two MBP gene-related 2.6- and 5.5-kilobase mRNAs in some of the cell lines. The HOG cell line (Fig. 1, Lane 3) expressed both the 2.6-kilobase MBP mRNA and the 5.5-kilobase MBP-related mRNA. The TC-620 cell line (Fig. 1, Lane 4) expressed only the 5.5-kilobase MBP-related mRNA. In one of the astrocytoma cell lines (U251MG; Fig. 1, Lane 5) the 2.6-kilobase mRNA appeared to be expressed, while in the other (U373MG; Fig. 1, Lane 6), both MBP gene-related mRNAs were expressed very faintly. There was no detectable expression of these mRNAs in the neuroblastoma cell line (Fig. 1, Lane 7). The expression of these two mRNAs occurs earlier in the developing mouse brain in vivo (30) and in culture (31) than the 2.2-kilobase MBP mRNAs transcribed from the major promoter of the MBP gene.

Like the 2.2-kilobase MBP mRNA, PLP is expressed later in development, in the mature oligodendrocyte. Although PLP mRNA was not detected in the Northern blots of the two oligodendroglioma (Fig. 1, Lanes 3 and 4) and the neuroblastoma (Fig. 1, Lane 7) cell lines, surprisingly, PLP/D20 mRNA of the appropriate size (3.2 kilobases) was expressed at low levels in the astrocytoma cell lines, U251MG and U373MG (Fig. 1, Lanes 5 and 6). We could not detect the expression of MAG mRNAs in any of the tumor cell lines (data not shown).

Expression of Neuronal-specific mRNAs. Two 2.8- and 1.8-kilobase bands were evident in the Northern blots of all the cell lines probed with the NSE cDNA (Fig. 2). The 2.8-kilobase band is the size of authentic NSE mRNA and the 1.8-kilobase band is likely to represent non-neuronal enolase mRNA based upon its size and cross-hybridization characteristics (32). Although all the cell lines expressed both enolase mRNAs, the non-neuronally derived cell lines expressed a higher proportion of the non-neuronal enolase mRNA. In the neuroblastoma cell line the proportion of NSE of non-neuronal enolase mRNA was much higher than in the other lines.

We also examined the expression of the NF-L and NF-M mRNAs in the cell lines using human NF-L and NF-M cDNA probes (Fig. 2). The 3.5-kilobase NF-M mRNA was most abundant in the neuroblastoma (Fig. 2, Lane 7) and was expressed to a lesser extent in the human cortex (Fig. 2, Lane 1). It was not expressed in the cell lines of glioma origin. Surprisingly, the 2.2- and 4.0-kilobase NF-L transcripts were detected not only in the neuroblastoma (Fig. 2, Lane 7) and human cortex (Fig. 2, Lane 1) but also in the astrocytoma cell lines (Fig. 2, Lanes 5 and 6). There was no expression of either neurofilament mRNA in the oligodendrogloma-derived cell lines (Fig. 2, Lanes 2 and 3).

Expression of the Astrocyte-specific GFAP mRNA and the Related Intermediate Filament Gene, Vimentin. Upon short exposure (3 h) of the Northern blots to a mouse cDNA probe, a 3.5-kilobase human GFAP mRNA was evident only in the human cortex and the two astrocytoma cell lines (Fig. 3, Lanes 1, 5, and 6, respectively). After longer exposures (18 h), however, two other bands of 2.0 and 1.7 kilobases were evident in the HOG and TC620 oligodendroglioma cell lines (Fig. 3, Lanes 3 and 4). The 2.0-kilobase band also could be seen in the neuroblastoma cell line upon longer exposure (Fig. 3, Lane 7).

Although the 1.7 kilobase and 2.0 kilobase RNA transcripts were expressed in the HOG and TC620 oligodendroglioma cell lines, we could not determine if it also was expressed in the astrocytoma cell lines because of the spreading of the GFAP band with increased exposure time. Furthermore, because vimentin, an intermediate filament protein, belongs to the same subclass as GFAP and shares a 66% homology at the nucleotide level with GFAP mRNA in their coding
regions (25, 26), we wondered if the 2.0-kilobase band could be vimentin mRNA.

We prepared cDNA libraries of the two oligodendroglioma cell lines, screened them with the mouse GFAP cDNA, and isolated several distinct cDNA clones. The clones were sequenced and the sequences were compared with others filed in the GENBANK and EMBL databases. Such an analysis revealed that one of the clones corresponded to a full-length human vimentin mRNA and hybridized specifically to the 2.0-kilobase band in the Northern blot shown in Fig. 3. The vimentin mRNA appeared to be expressed in all the tumor cell lines examined.

We also isolated a cDNA clone (GRG-6) which, upon analysis, indicated that it encoded human cytokeratin K7 (32). We then subcloned a portion of the GRG-6 clone that was specific to cytokeratin K7 mRNA and probed Northern blots with it (Fig. 3). The results indicated that cytokeratin K7 mRNA expression was restricted to the oligodendrogliaoma-derived cell lines (Fig. 3, Lanes 3 and 4), and it was not expressed in the astrocytoma or neuroblastoma-derived cell lines (Fig. 3, Lanes 5–7).

Western Blot Analysis of the Human Glioma-derived Cell Lines, HOG and TC620

Expression of Oligodendrocyte-specific RNAs. The presence of two CNPase polypeptide isoforms with molecular weights of 46,000 and 48,000 was detected in samples of human cortex by Western blot analysis, with the M, 46,000 isoform predominating, although this is not evident in Fig. 4 (bottom, Lane 1) because the film was overexposed in order to detect the CNPase bands in the cell lines. The M, 46,000 CNPase isoform was expressed in the TC620 (Fig. 4, Lane 3), U251MG (very faintly; Fig. 4, Lane 4), U373MG (Fig. 4, Lane 5) and the neuroblastoma (Fig. 4, Lane 6) cell lines. The M, 48,000 isoform of CNPase was not detected in the HOG or TC620 cell lines, and some lower molecular weight immunoreactive products appeared to be restricted to the two astrocytoma cell lines (Fig. 5, top, Lanes 4–6) after an extended exposure of 72 h. Right: A 3.2-kilobase band corresponding to the PLP/DM20 mRNA was detected in the human cortex (Lane 1) and the two astrocytoma cell lines (Lanes 5 and 6).
DISCUSSION

We undertook this study, initially, to determine the extent to which transformed cell lines isolated from oligodendrogliomas might serve as models with which to study stage-specific oligodendrocyte gene expression. We thought that oligodendroglioma-derived cell lines might be useful models of cells in the oligodendrocyte lineage "arrested" at specific developmental stages, much as cells derived from lymphomas and leukemias have served as useful models to further our understanding of normal B- and T-cell ontogeny (31, 33). Unfortunately, few oligodendroglioma cell lines have been analyzed with respect to their expression of oligodendrocyte stage-specific genes; therefore we undertook such an analysis of two such cell lines.

In order to examine the specificity of gene expression in the HOG and TC620 oligodendroglioma lines we compared their patterns of gene expression with two established astrocytoma- and one established neuroblastoma-derived cell lines. The pattern of gene expression in the two oligodendroglioma cell lines generally reflected what might be expected of cells in the oligodendrocyte lineage. However, our analyses of the cell lines, in general, revealed some interesting and unanticipated results.

The presence of authentic M, 51,000 GFAP, even after extended exposure, in the oligodendroglioma or neuroblastoma-derived cell lines (data not shown). Expression of the related intermediate filament protein, vimentin (M, 58,000), was observed in all the tumor cell lines, along with the presence of lower molecular weight immunoreactive products in the astroglia cell lines (Fig. 5, bottom, Lanes 2–6).

Additional evidence for the expression of cytokeratin K7 in the HOG and TC620 cells was provided through Western blot analysis of the cell lines with a cytokeratin K7-specific monoclonal antibody (Fig. 6, Lanes 2 and 3). The M, 55,000 cytokeratin K7 protein was clearly expressed in the two oligodendroglioma cell lines, consistent with the expression of the cytokeratin K7 mRNA, but its expression could not be detected in the other cell lines examined.

Similarly, we detected the neuron-specific enolase M, 46,000 polypeptide in human cortex and in all of the tumor cell lines (Fig. 4, top, Lanes 1–6), consistent with the presence of the NSE mRNA observed on the Northern blots (Fig. 2).

Tables 1 and 2 summarize the Northern and Western blot data in order to permit easy comparison of the gene expression patterns of the tumor cell lines.

**Fig. 3.** Northern blot analysis of mRNA from the cell lines with IF-related gene cDNAs (GFAP, keratin K7, and vimentin). Both a long (18-h) and short (3-h) exposure of the GFAP blot is shown. Lane 1, human cortex; Lane 2, blank; Lane 3, HOG; Lane 4, TC620; Lane 5, U251MG; Lane 6, U373MG; Lane 7, SK-N-SH. GFAP panel: The 3.5-kilobase (kb) GFAP mRNA was detected in human cortex (Lane 1) and the two astrocytoma cell lines (Lanes 5 and 6) at short exposure times. At longer exposures, additional 2.0- and 1.7-kilobase mRNAs became evident in the other cell lines (e.g., Lanes 3, 4, and 7). Keratin panel: Cytokeratin K7 mRNA was detected exclusively in the oligodendroglioma cell lines (Lanes 3 and 4) when the blots were hybridized to a K7-specific probe. Vimentin panel: The 2.0-kilobase vimentin mRNA was expressed in all the cell lines.

**Fig. 4.** Western blot analysis of the cell lines for expression of neuron-specific enolase and CNPase proteins. Fifty ug of total protein per lane were electrophoresed in SDS-polyacrylamide gels and electrotransferred to nitrocellulose membranes. The proteins were detected by specific polyclonal and monoclonal antibodies, and the antigen-antibody complexes were visualized by [125I]labeled protein A and autoradiography (1 week). Lane 1, human cortex; Lane 2, HOG; Lane 3, TC620; Lane 4, U251MG; Lane 5, U373MG; Lane 6, SK-N-SH. Top: The M, 46,000 NSE polypeptide was expressed by all the cell lines. Bottom: The two CNPase M, 46,000 and M, 48,000 isoforms were merged into one large band in human cortex because the gel was overexposed to reveal hands in the cell lines. The M, 46,000 CNPase isoform was expressed in the TC620 (Lane 3), U251MG (very faintly, Lane 4), U373MG (Lane 5) and the neuroblastoma (Lane 6) cell lines. The M, 48,000 isoform of CNPase was expressed in the neuroblastoma (Lane 6) and, possibly, the TC620 (Lane 3) cell lines. K, thousands.

**Fig. 5.** Western blot analysis of the cell lines for the several intermediate filament proteins. Each lane contained 50 ug of total protein. Proteins were detected by anti-GFAP polyclonal antibody and anti-vimentin monoclonal antibody (panel B) with exposure times of 18 h. Lane 1, human cortex; Lane 2, HOG; Lane 3, TC620; Lane 4, U251MG; Lane 5, U373MG; Lane 6, SK-N-SH. Top: The M, 51,000 GFAP and several lower molecular weight cross-reactive polypeptides were evident in the two astrocytoma cell lines (Lanes 4 and 5) and in the human cortex (Lane 1). Bottom: The M, 58,000 vimentin polypeptide was detected in all the cell lines along with the presence of lower molecular weight cross-reactive products in the astrocytoma cell lines.

**Fig. 6.** Western blot analysis of the cell lines for cytokeratin K7. Each lane contained 50 ug of total protein. Proteins were detected by anti-cytokeratin K7 monoclonal antibody with an exposure time of 18 h. Lane 1, human cortex; Lane 2, HOG; Lane 3, TC620; Lane 4, U251MG; Lane 5, U373MG; Lane 6, SK-N-SH.
The patterns of gene expression, summarized in Tables 1 and 2, suggested that certain neural genes were expressed in all the transformed cell lines regardless of origin. These included CNPase, vimentin, and GFAP. Neuron-specific enolase is considered to be a neuronal marker (34) and CNPase is generally considered to be an oligodendrocyte specific, yet in this study all the cell lines expressed these genes (at least at the mRNA level), suggesting that their expression may be less specific than previously thought. In this regard, NSE has been reported to be expressed in rat C6 glioma cell lines (23) and CNPase mRNA has been reported to be expressed in the astrocytoma cell line U251MG (35), in agreement with the findings in this study.

Other genes appeared to be expressed in the tumor-derived lines that are not generally associated with the cell type presumed to give rise to the tumor. This was particularly true for the astrocytoma lines, which appeared to present a "mixed" phenotype. For example, two mRNAs of a MBP-related gene (29) were expressed principally in the two oligodendroglialoma cell lines but also to a lesser extent in the astrocytoma cell line U251MG (35), in agreement with the findings in this study.

The finding that cytokeratin K7 is expressed in the oligodendroglialoma cell lines. raises a question about the molecular identity of the GFAP immunoreactivity observed in immature oligodendrocytes and oligodendroglias. In view of the potential for cross-immunoreactivity among members of the IF family, it is possible that the antigens being recognized might be vimentin, cytokeratin, or some other member of the IF protein family. It underscores the need to use additional molecular criteria beyond the immunocytochemical identification of antigens in such situations.

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