Neoplastic Transformation of Newborn Rat Oligodendrocytes in Culture

Joseph P. Bressler, Ruth Cole, and Jean de Vellis

ABSTRACT

We have developed a model to study the neoplastic transformation of rat oligodendrocytes in culture. This procedure utilizes a technique previously developed by McCarthy and de Vellis which allows the preparation of 99% pure astrocyte and oligodendrocyte populations from 1- to 2-day-old rat cerebral cortices. Pregnant rats on the 19th day of gestation were given injections with either ethyl nitrosoare (10 μg/g body weight) in phosphate-buffered saline or phosphate-buffered saline, and oligodendrocyte cultures were prepared. Oligodendrocytes appear to be unstable in culture since transformation was observed with cells derived from either pups from pregnant rats either treated with nitrosoare or phosphate-buffered saline. Transformation required 78 to 108 days and 3 to 9 passages, at which time a marked increase in cellular proliferation was observed. The possibility that the transformed cells were derived from a nonoligodendroglial cell was excluded by the following evidence. Light and scanning electron micrographs of the transformed cells revealed cytological features essentially similar to those of primary oligodendroglial cultures. Furthermore, 2 biochemical oligodendroglial markers, the induction of lactate dehydrogenase by N6,O6-dibutyryl cyclic adenosine 3':5'-monophosphate and the presence of 2',3'-cyclic nucleotide 3' phosphohydrolase, were also retained. Conversely, another oligodendroglial marker, the hydrocortisone induction of glycerol phosphate dehydrogenase, was not found in any of the cell lines. These transformed cells grew as tumors when injected intracranially into 21-day-old rats. Histologically, these tumors did not appear as classical oligodendrogliomas, but their oligodendroglial origin was confirmed since the tumor tissue contained 2':3'-cyclic nucleotide 3'-phosphohydrolase activity, and the cells which grew from tumor explant cultures morphologically appeared similar to the parent cell line.

The transformed cells were also characterized for in vitro properties which correlate with the expression of tumorigenicity. The transformed cells exhibited anchorage-independent growth and were agglutinated by concanavalin A treatment. Changes in fibrinolytic activity were not an exclusive property of transformed glial cells. This model should now allow us to study various mechanisms involved in the neoplastic transformation of oligodendrocytes.

INTRODUCTION

Human glial tumors are morphologically heterogenous, suggesting that more than one type of glial cell is capable of undergoing neoplastic transformation. To understand the mechanisms responsible for the neoplastic transformation of these cells, a system had to be developed where we could transform pure cell populations and where the microenvironment of the cells could be manipulated. Such a system would enable us to discover specific growth requirements as well as to discern the loss or gain of specific properties. Recently, an in vivo-in vitro glial cell transformation model was reported in which fetal rat brain cells, obtained from pregnant rats treated with ENU, transformed in culture after several months and a few passages (6, 18). This type of study allows the investigator the opportunity to study glial transformation in vivo, although it does not define the particular type of glial cell actually involved in the transformation process.

Our laboratory has previously established a method to obtain from neonatal rat brains populations of the 2 main types of glial cells, oligodendrocytes and astrocytes, which are 99% pure (13). Both cell types in culture have a distinct morphology. Oligodendrocytes appear as phase-dark cells with a small cell body and several fine extended processes, while astrocytes appear as large phase-light cells lacking noticeable processes. Upon ultrastructural analysis, the oligodendrocytes in primary culture exhibited properties which are characteristic for these cells in vivo and distinguish them from astrocytes. These properties include microtubules, a well developed Golgi apparatus, dilated cisternae of rough endoplasmic reticulum, finely clumped chromatin, and an eccentric nucleus (16). In addition, primary oligodendrocyte cultures express biochemical markers which distinguish them from astrocytes or neurons. These include CNPase (EC 3.1.4.37) activity, hydrocortisone induction of GDPH (EC 1.1.1.18), and Bt2cAMP induction of LDH (EC 1.1.1.27) (13).

In this study, we have combined the in vivo-in vitro transformation model and the cell culture purification step in order to study oligodendrocyte transformation. We now report that these cells can undergo neoplastic transformation in culture.

We ruled out the possibility that these cells were derived from a nonoligodendroglial population since the transformed cells retained some specific oligodendroglial morphological and biochemical properties. Additionally, the transformed cells displayed various in vitro properties which correlated with tumorigenicity.

MATERIALS AND METHODS

Treatment of Animals. Pregnant Wistar rats were injected on the 19th day of gestation with either 10 μg ENU per g (Tridom, Hauppauge, New York) or phosphate-buffered saline. We ruled out the possibility that these cells were derived from a nonoligodendroglial population since the transformed cells retained some specific oligodendroglial morphological and biochemical properties. Additionally, the transformed cells displayed various in vitro properties which correlated with tumorigenicity.

The abbreviations used are: ENU, ethylnitrosourea; CNPase, 2',3'-cyclic nucleotide 3'-phosphohydrolase; GDPH, glyceral phosphate dehydrogenase; Bt2cAMP, N6',O6'-dibutyryl cyclic adenosine 3':5'-monophosphate; LDH, lactate dehydrogenase; PBS, Dulbecco's phosphate-buffered saline without calcium and magnesium; BME, Eagle's basal medium; FCS, fetal calf serum; DMEM, Dulbecco's Modified essential medium; i.c., intracranially; FHS, fetal horse serum; Con A, concanavalin A.

Received January 4, 1982; accepted November 5, 1982.

FEBRUARY 1983 709
N. Y.) body weight dissolved in PBS, pH 6.0 (and used within 30 min), or with PBS through the femoral vein under anesthesia.

Preparation of Cultures. Oligodendrocyte cultures were prepared essentially as described (13). Briefly, cerebral hemispheres from 1- to 2-day newborn Wistar rats were freed from meningeal tissues, mechanically dissociated, and plated at a density of 1 x 10^6 cells per 25-

Transmission Electron Microscopy. Cell cultures were rinsed 3 times with 0.1 M cacodylate buffer (pH 7.4) containing 0.05% calcium chloride and then fixed with a solution containing 2% formaldehyde (freshly prepared from paraformaldehyde) and 2% glutaraldehyde in the same buffer. This was followed by a postfixation with osmium tetroxide in the same buffer. The cultures were dehydrated in a graded series of alcohol and embedded in araldite. Ultra-thin sections were cut and stained with uranyl acetate and lead citrate. A Siemens’ 1A electron microscope was used to view and photograph the specimens.

Enzyme Induction. Bt-cAMP was dissolved in culture medium, sterilized by filtration, and used immediately. The cells were either treated with a final concentration of 1 nM Ca^2+ or with fresh medium 24 hr before harvesting the culture. Hydrocortisone was dissolved in absolute ethanol at 1 mg/ml. The cells were either incubated with a concentration of 1.38 #M hydrocortisone or with an equivalent amount of solvent 24 or 48 hr prior to harvesting the cells.

Enzyme Assays. The preparation of samples and the assays for GPDH, CNPase, and LDH activities have been described previously (13). For all enzymes, the assays were done in triplicate. One unit of activity was defined as that amount which catalyzes the conversion of 1 nmol of substrate per min at 30°. Specific activity is expressed as enzyme units per mg protein. Protein was determined by the method of Lowry et al. (10) using bovine serum albumin as the standard.

Tumorigenic Growth. A sample of 1 x 10^5 cells in 50 #l of medium, without sera, was injected i.c. into 25-day-old animals. Animals showing neurological stress were sacrificed, and the tumors were processed for histology. After 12 months, all animals were sacrificed.

Rate of Proliferation. A sample of 25 x 10^3 cells from each transformed cell line was plated on 35-mm dishes. On Days 1, 3, 5, and 7, the cells were removed with trypsin and counted. The generation time of the control cell line was determined by counting the number of cells in 1 sq cm and multiplying this value by the surface area of a 35-

RESULTS

Transformation of Primary Oligodendrocyte Cultures. One pregnant rat was injected with 10 #g ENU per g body weight in PBS, and another with PBS on the 19th day of gestation. Brain cell cultures were then prepared from the cerebral hemispheres of the entire litter no later than 24 hr after birth. Oligodendrocytes were purified from 7-day-old cultures and plated in plastic flasks. Subsequently, the cells from each flask were detached at confluency by vigorous shaking and then plated in a new flask without splitting or pooling the flasks. The shaking procedure further ensured the selection of the oligodendrocyte population since there was always a small (51%) nonoligodendroglial contaminant. Cells from rats given injections of buffer were cultured with BME supplemented with 15% FCS or with DMEM:F-12 (1:1) supplemented with 7% FCS and 3% FHS, while cells from ENU-treated rats were fed with BME supplemented with FCS media.

Control and ENU-derived cells looked alike until the 79th to 95th day of culture, at which time a marked increase in oligodendrocyte proliferation was observed in cultures from carcinogen-treated animals. After 98 to 108 days, a marked increase in oligodendrocyte proliferation was also observed in flasks from buffer-treated animals fed with the medium containing FHS (Table 1). The transformed cells from each flask were then cloned by limiting dilution, and one clone from each line was grown and passed at a 1:10 ratio at confluency. These lines were used between the third and sixth passage.

In each of our experiments, transformation was always observed in those cultures derived from ENU-treated pregnant
rats or in those cultures from PBS-treated animals maintained on DMEM:F-12 supplemented with FCS and FHS. On the other hand, oligodendrocytes from control-treated animals, cultured with BME supplemented with FCS, usually did not survive, although in one experiment a nontransformed oligodendroglial cell line was established (Table 1). Primary cultures of oligodendrocytes can be maintained for at least 2 months without growth if they are not passed. For the first 3 months of culture, these nontransformed cells were passed by shaking the culture to further ensure the selection of the oligodendrocyte population. After 3 months, we found that the presence of the nonoligodendroglial population helped the survival and growth of the oligodendroglial population. Therefore, we cultured both cell types as one cell line. It was necessary, though, to isolate these control oligodendroglial cells by shaking to measure the biochemical and physiological parameters described below.

### Morphological Characterization
All 4 of the transformed cell lines morphologically appeared similar to primary cultures of oligodendrocytes. Shown in Fig. 1 are light micrographs of a primary culture of oligodendrocytes and a culture of the transformed cell line 12. The transformed cells can be seen to grow to a much higher saturation density, and they tend to aggregate after prolonged culture. The primary oligodendrocyte cultures remain at this density even after 2 months of culture.

**Scanning Electron Microscopy**. The shape and processes of both cell types can be seen more clearly by scanning electron microscopy (Fig. 2). The cell bodies in both cultures are small and spherical with fine extended processes. We were unable to detect microvilli protruding from the cells.

**Transmission Electron Microscopy**. The ultrastructure of the transformed oligodendrocytes was studied and compared to primary cultures of oligodendrocytes. Shown in Fig. 3 are light micrographs of a primary culture of oligodendrocytes and a culture of the transformed cell line 12. The transformed cells have round cell bodies and long extended processes. We were unable to detect microvilli protruding from the cells.

### CNPase Activity
Although the cell lines appear morphologically similar to oligodendrocytes, it is also necessary to identify these cells with biochemical criteria. Therefore, the cells were assayed for CNPase activity. As seen in Table 2, significant amounts of activity were present in each of the cell lines.

**LDH Induction**. Another biochemical marker, typical of oligodendrocytes, is the induction of LDH by Bt2cAMP or by a β-adrenergic agonist (13). After a 24-hr treatment with Bt2cAMP, each of the cell lines exhibited inducible LDH levels (Table 3). Three of the cell lines, lines 3, 4, and 12, exhibited an increase in CNPase activity, while line 13 exhibited a 1.5-fold induction.

**GPDH Induction**. The ability of hydrocortisone to induce GPDH levels was the final oligodendrocyte characteristic studied. After a 24- or 48-hr incubation with hydrocortisone, we were unable to detect any induction in GPDH activity in any of the cell lines (Table 3). Previous work from our laboratory demonstrated that the regulation of GPDH synthesis is under glucocorticoid and cAMP regulation (13). The cyclic nucleotide was found to augment the rate of GPDH synthesis in glucocorticoid-treated C6 cells grown in log phase, as well as in primary brain cell cultures. Cultures from each cell line were incubated with both Bt2cAMP and hydrocortisone; however, an increase in GPDH levels was still not detected (data not shown). We also could not detect GPDH induction in the oligodendroglial cell population of the nontransformed cell line.

**Tumorigenicity**. We injected intracranially 10⁵ cells into 25-day-old rats to determine whether the transformed oligodendroglial-derived cell lines were tumorigenic. After 40 to 60 days, the animals exhibited neurological stress, and tumors were observed after sacrificing the animals. The tumor was also lethal to the host if allowed to grow.

**Characterization of the Tumor**. A section from the growing tumor can be seen in Fig. 4. A tumor can be observed growing on top of normal cerebral cortex. We have not been able to identify a predominant cell type. Therefore, cell cultures were prepared from the tumor to verify its identity. The cells from these cultures have round cell bodies and long extended processes and appear similar to both primary oligodendrocyte cultures and the transformed oligodendroglial-derived cell line (Fig. 5). The tumors were also biochemically characterized for CNPase activity, an oligodendrocyte marker. A significant amount of enzyme was observed in the homogenates, and these levels were in the same order of magnitude as those observed for the cells in culture (Table 2).

**Anchorage-independent Growth**. Only the transformed cell lines were capable of anchorage-independent growth in soft agar (Table 4). The growth capability was similar for each cell line and did not exceed 0.3%. This limited growth capability was not due to the culture conditions, since we have been able to obtain up to 20% growth for other types of transformed glial cell lines (data not shown).

**Fibrinolytic Activity**. Of the 4 transformed cell lines assayed, 3 exhibited significant amounts of extracellular plasminogen

---

### Table 1

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Prematernal treatment</th>
<th>Culture media</th>
<th>No. of days</th>
<th>No. of passages</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Buffer</td>
<td>DMEM:F12 + 7% FCS + 3% FHS</td>
<td>98</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>Buffer</td>
<td>DMEM:F12 + 7% FCS + 3% FHS</td>
<td>108</td>
<td>9</td>
</tr>
<tr>
<td>12</td>
<td>ENU</td>
<td>BME + 10% FCS</td>
<td>79</td>
<td>9</td>
</tr>
<tr>
<td>13</td>
<td>ENU</td>
<td>BME + 10% FCS</td>
<td>95</td>
<td>5</td>
</tr>
</tbody>
</table>

### Table 2

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell culture</th>
<th>Tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>388.7 ± 17.6</td>
<td>325.0 ± 23.1</td>
</tr>
<tr>
<td>4</td>
<td>445.0 ± 20.3</td>
<td>250.0 ± 13.3</td>
</tr>
<tr>
<td>12</td>
<td>265.0 ± 15.0</td>
<td>460.0 ± 16.5</td>
</tr>
<tr>
<td>13</td>
<td>526.6 ± 62.1</td>
<td>270.2 ± 21.2</td>
</tr>
</tbody>
</table>

---

*a* Cells (2 × 10⁵) were plated in 35-mm Petri dishes. After 10 days, the cells were assayed for CNPase activity. Units of enzyme activity were defined as nmol of substrates used per min at 30°C. 

**Notes:**
- **FEBRUARY 1983**
- **711**
The transformed cells displayed microtubules, a well-developed endoplasmic reticulum cisternae, and an eccentric nucleus. The cells also exhibited significant levels of CNPase activity in dendrocytes. The cell body appeared to be small and spherical, lines morphologically were similar to primary cultures of oligodendroglial-like cell can be specifically induced to transform either by a neurotropic carcinogen in an in vivo-in vitro system or by spontaneous release of radioactivity from the plate itself.

**Rate of Proliferation.** To quantitate the changes in proliferative capabilities, we determined the generation times for the transformed and nontransformed cell lines. As shown in Table 5, all 4 transformed cell lines exhibited much shorter generation times than the control cell line. The generation time of the transformed cell lines varied between 1.3 and 22 hr while that of the control line was 120 hr.

**Con A Agglutination.** Marked agglutination was observed by all of the transformed cell lines after being treated with Con A (200 µg/ml) (Table 5). The amount of agglutination varied according to the cell line. For example, with line 12, an 86% agglutination was observed, while line 13 displayed a 50% agglutination. Con A treatment of cells from the control cell line did not agglutinate.

**DISCUSSION**

The results presented in this study suggest that an oligodendroglial-like cell can be specifically induced to transform either by a neurotropic carcinogen in an in vivo-in vitro system or by altering the culture conditions of nontreated cells. Proof that the transformed cells were oligodendroglial-derived is based on a number of criteria. We found, using light, scanning, and transmission electron microscopy, that the transformed cell lines morphologically were similar to primary cultures of oligodendrocytes. The cell body appeared to be small and spherical, and processes were observed extending from the cell body. In addition, the cells displayed microtubules, a well-developed endoplasmic reticulum cisternae, and an eccentric nucleus. The cells also exhibited significant levels of CNPase activity in addition to Bt2cAMP-inducible LDH levels. On the other hand, GPDH induction could not be used as an oligodendroglial marker, since both the transformed and nontransformed oligodendroglial cell lines failed to express this property.

Systems designed to study the neoplastic transformation of nonfibroblast cells need to establish criteria that will verify the cell type before and after transformation. This proves to be difficult, since the processes of both malignant transformation and normal adaptation to cell culture conditions very often lead to a loss of differentiated properties. Glial cells present an even greater problem, since until recently little was known about their characteristics and differentiated properties. Two systems have been reported previously which address themselves to the study of chemical transformation of glial cells in vitro. In one report, investigators found that glial fibrillary acidic protein was retained in benzo-pyrene-induced transformed postnatal mouse glial cells (12). In the other, Laerum et al. (7) were not able to detect myelin basic protein or glial fibrillary acidic protein in prenatal rat glial cells transformed by ENU although S-100 protein was retained. Both reports demonstrated the presence of only one differentiation marker in transformed glial cells and did not determine whether the cells were astroglial or oligodendroglial derived. More markers are needed to identify the specific type of glial cell. The development of a library of markers is one important direction of future studies which will allow the investigator to trace the lineage of glial cells.

Besides differentiated properties, transformation-related properties were also investigated. Only those cell lines exhibiting a marked decrease in generation time were capable of producing tumors when injected intracranially into postweanling rats. The tumors produced obvious neurological stress and were lethal. Histologically, we were unable to determine the type of tumor, but biochemical analysis and cell cultures from the tumor confirmed their oligodendroglial origin. Other investigators have also had difficulty in histologically identifying tumors that are produced by injecting nonfibroblast cells transformed in an in vitro system (4, 15). This suggests that either the host failed to provide the necessary environment for the transformed cell to morphologically differentiate or the cell lost this ability. Studies are in progress to elucidate this problem.
Tumorigenicity is the best indicator for neoplastic transformation, but the appearance of a tumor requires a lengthy incubation period. This has prompted many investigators to find in vitro properties which correlate with tumorigenicity. In this study, we found 3 characteristics that correlated with oligodendroglial transformation. These were anchorage-independent growth, alterations in proliferative capacity, and agglutination by Con A.

Anchorage-independent growth has previously been demonstrated to correlate with tumorigenicity for a number of cell types, such as liver (19) and skin epithelia (4), fibroblast (21), and glial cells (6). Hence, we were not surprised that transformed oligodendroglial cell lines exhibited this property. Conversely, changes in proliferative capability are not considered to correlate with tumorigenicity for all cell types. For example, transformed fibroblasts (17) and mammary epithelia (23) have rates of proliferation similar to nontransformed cells, while other investigators have shown that transformation affects the proliferative capability of skin (4) and liver epithelia (20) and glial cells (6). Similarly, Con A agglutination has been demonstrated to be a marker for transformation in certain cell types. Transformed fibroblasts (22), unlike transformed liver (19) or mammary epithelia cells (1, 23), agglutinate after Con A treatment. In summary, changes in the rate of proliferation and sensitivity to Con A serve as markers for transformed oligodendroglial-derived cells but not as general tumor markers.

Increases in fibrinolytic activity have also been suggested to correlate with tumorigenic potential in specific cell types. For example, fibroblasts usually exhibit elevated levels of activity (9) while transformed liver epithelia (20) cells have similar levels to the nontransformed cells. Recently, Hince and Roscoe (5) observed that a transformed astrocyte cell line had higher levels of fibrinolytic activity than a nontransformed line. In this study, we observed that 3 transformed oligodendroglial-derived lines exhibited elevated levels of activity; however, one transformed line contained levels of activity similar to the control cell line. Therefore, elevated fibrinolytic activity does not appear to be an absolute requirement for tumorigenicity in oligodendroglial-transformed cells. We suggest that this property is also restricted to specific cell types despite the anatomical and embryological relatedness of the cells.

In summary, we have presented a model to study the transformation of an oligodendroglial-like cell in vitro. These cells retain various differentiated properties, and they can be characterized by an assortment of transformation-related characteristics. The importance of this model is that we can now study the immediate and long-term effects of biological modifiers on the oligodendrocyte transformation process. In addition, we can also conduct studies to determine the relationship between transformation and differentiation. By this methodology, we hope to better understand the mechanisms responsible for the occurrence of human gliomas.

REFERENCES

Fig. 1. Phase-contrast micrograph of cell line 12 after 7 days in culture (A) and a primary oligodendrocyte culture (B). About $5 \times 10^5$ oligodendrocytes and $1 \times 10^6$ cells of cell line 12 were plated. $\times$ 380.

Fig. 2. Scanning electron micrograph of cell line 12 after 7 days in culture (A) and a primary oligodendrocyte culture (B). $\times$ 440.

Fig. 3. Transmission electron microscopy of cell line 12 after 7 days in culture (A) and a primary oligodendrocyte culture (B). Please note: endoplasmic reticulum (large arrows); microtubules (small arrows); eccentric nucleus (n). $\times$ 7700.
Neoplastic Transformation of Rat Oligodendrocytes

Fig. 4. Tissue section from a 30-day-old tumor induced by injecting $1 \times 10^3$ cells of cell line 12 intracranially into 25-day-old rats. H & E, $\times$ 330.

Fig. 5. Phase-contrast micrograph of a cell culture derived from a trypsinized 30-day-old tumor induced with cell line 12. $\times$ 380.
Neoplastic Transformation of Newborn Rat Oligodendrocytes in Culture

Joseph P. Bressler, Ruth Cole and Jean de Vellis


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/43/2/709

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.