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The Properties of An Immature Glial Cell Line (HITS Glioma) Derived from Ethylnitrosourea (ENU)-Induced Rat Glioma

Yuji Ikeno

The First Department of Pathology, Nagasaki University School of Medicine, 1-12-4 Sakamoto, Nagasaki 852, Japan

In order to evaluate the proliferation and differentiation potentials of ethylnitrosourea (ENU)-induced glioma cells, the author attempted to obtain a cell line that maintains glial features in long term culture. One of five cell lines cultivated from ENU-induced rat gliomas deserved particular interest because of the differentiation of the neoplastic glia. This cell line, designated as HITS glioma, had a polygonal cell body and formed a monolayer with pile-up foci in vitro, in contrast to the other cell lines, which showed a mesenchymal drift through passages. GFAP-positive cells, found in the primary culture, disappeared in the late passages of HITS glioma as they did in the other cell lines. Galactocerebroside (GC) and GD3 ganglioside were not expressed in the cell lines during culture. Subcutaneous inoculation of HITS glioma into neonatal rats induced tumors with various histopathological components mimicking the histopathological appearance of ENU-induced gliomas. The components also had a fraction of GFAP-positive cells. These findings indicate that HITS glioma cells may be composed of immature glial cells and differentiate into astrocytic cells under certain conditions. Insulin-like growth factor I (IGF-I), which promoted the proliferation of GFAP-positive cells in neonatal glia, was used to evaluate the mechanism(s) of proliferation and/or differentiation of HITS glioma. IGF-I did not induce the expression of GFAP in HITS glioma, even though it promoted the proliferation of HITS glioma. Although the mechanism involving the astrocytic differentiation of HITS glioma is unknown, HITS glioma may serve as a research tool in evaluating the mechanisms of proliferation and differentiation of neoplastic glia.

Key Words: Ethylnitrosourea-Glioma-Cell line-GFAP

Introduction

Ethylnitrosourea(ENU)-induced gliomas in the rat are histopathologically similar to oligodendroglioma, mixed oligo-astrocytoma, astrocytoma, and anaplastic glioma in humans, and have been utilized extensively as an experimental model of human gliomas. Many investigators have been intrigued by the polymorphic phenotype of this tumor model, which may indicate the existence of pluripotent neoplastic glia and provide a way to investigate the differentiation of normal as well as neoplastic glia. However, there is still some controversy about this model. Reactive astrocytes, which appear within and around gliomas during the neoplastic process, complicated in vivo studies. Mauro et al. and Shimokawa indicated that most, if not all, GFAP-positive cells within tumors could be reactive cells, while other investigators considered them neoplastic cells. In our laboratory, Higami suggested the reactive nature of GFAP-positive cells in the glioma using primary culture cells. However, the possibility remained that GFAP-negative neoplastic glia differentiate into astrocytic cells under certain conditions. A cell line, derived from ENU-induced glioma, has expressed GFAP in a fraction of cells. The author have also cultured a cell line which expressed GFAP, not in vitro but only in transplanted sites of neonatal rats. In this report, the author take up the task of characterizing this cell line, designated as HITS glioma.

Recent advances in oncology have revealed the significant role of growth factors or oncogenes in the development of neoplasms. Insulin-like growth factor I (IGF-I), which is increased in the serum of animals at the onset of puberty, regulates the proliferation and differentiation of normal and neoplastic glia. The response of HITS glioma to IGF-I was examined to elucidate the mechanism(s) of cell proliferation and GFAP-expression in cells after transplantation.

Materials and Methods

Experimental Animals and Tumor Induction

Glioma induction was conducted according to the method of Koestner et al. Briefly, pregnant Wistar rats on the 15th day of gestation received an intravenous injection of a single 50mg/kg body weight dose of N-ethyl-N-nitrosourea (ENU, Nakarai Chemical, Ltd., Kyoto, Japan) dissolved in distilled water. After delivery, the offspring were fed ad libitum for 6-9 months and sacrificed under ether anes-
thesis when neurological symptoms appeared. Neonatal rats were used as controls and sacrificed on the day of birth.

Cell Culture
A half portion of macroscopic glioma or the cerebral hemisphere of neonatal rats was minced and digested with 0.25% trypsin (GIBCO, U.S.A.) at 37 °C for 15min. Isolated cells were suspended in Eagle’s Minimum Essential Medium (MEM, GIBCO) supplemented with 10% fetal bovine serum (FBS, GIBCO) and antibiotics (10 units/ml penicillin G sodium, 10 µg/ml streptomycin sulfate, 0.025 µg/ml amphotericin B, GIBCO), and incubated at 37 °C in 55cm² petri dishes in a 5% humidified atmosphere. In the glioma-derived cell cultures, passages were performed when the culture dishes became confluent. Cells were washed with Ca++ Mg++-free phosphate buffered saline (CMF-PBS), detached from the dishes with 0.05% trypsin/0.02% EDTA-PBS solution and inoculated into new culture dishes. The splitting ratio was 1:4. At the same time, a portion of the subcultivated cells was inoculated onto a cover slip sheeted into new dishes and grown for a day for immunocytochemical examination.

Subcutaneous Inoculation of Cultured Cells
HITS glioma and one other cell line (RT1005)-1 x 10⁶ cells in 0.5ml phosphate buffered saline (PBS)-were subcutaneously inoculated into 1-day-old neonatal rats. The tumors were harvested at a 3.0-4.0cm diameter. Each tumor was dissected free of connective tissue, fixed, and processed for hematoxylin-eosin and immunohistochemical examination.

Tissue Preparation for Histological and Immunohistochemical Examination
A portion of the original gliomas and transplanted tumors was fixed in 4% paraformaldehyde/0.1M PB at 4 °C overnight. After fixation, the specimens were dehydrated, embedded in paraffin and cut into 5 µm thick serial sections. Another portion of the specimens was cut in 2mm thick slices, placed in O. C. T. compound (Miles, U.S.A.) and quickly frozen in ethanol cooled by dry ice to -80 °C. The specimens were cut into 5 µm sections on a cryostat and fixed for 10min. with cold acetone. The serial sections were mounted on glass slides coated with poly-L-lysine (Sigma, Deisenhofen, FRG), and stained with hematoxylin-eosin or used for immunohistochemical examination.

Immunostaining
The primary antibodies used in this study and their optimal dilutions were as follows: Rabbit anti-cow GFAP (glial fibrillary acidic protein, 1:800, DAKO, Denmark), Rabbit anti-bovine galactocerebroside (1:200, Chemicon International, Inc., California, U.S.A.), Rabbit anti-human MBP (Myelin basic protein, 1:200, DAKO), Mouse anti-GD3 ganglioside (1:1600, Dr. A. Houghton, Sloan Kettering Memorial Institute, New York, U.S.A.). Galactocerebroside (GC) was used as an oligodendrocytic marker in vitro for frozen sections, since MBP displayed a frequent non-specific staining in culture cells. The avidin-biotin peroxidase complex (ABC) method was applied to demonstrate GFAP, MBP, GC and GD3 on tissue specimens and cultured cells. Commercial reagents were used (VECTASTAIN, VECTOR LABORATORIES, California, U.S.A.). Negative controls included replacement of the primary antibodies with normal rabbit serum (1:200-1600, DAKO). The immunoreactivity to rat positive control specimen of these primary antibodies was determined before use.

In the immuncytochemical analysis, GFAP-, GC-, and GD3- positive rates were calculated by counting more than 500 cells on a cover slip.

Bromodeoxyuridine (BrdU) Incorporation and Immunostaining
BrdU incorporation was performed to examine the proliferative activity of cultured cells, using an Amersham cell proliferation kit (Amersham, England). Culture cells grown on cover slips were incubated with the labelling medium containing BrdU (5-bromo-2'-deoxyuridine, BrdU;1:1000 dilution ratio) for 1 hour, and fixed in 4% paraformaldehyde for 20min. The indirect method was used for immunocytochemical visualization of BrdU incorporated cells. Cells were counterstained with hematoxylin. All steps were performed at room temperature. The BrdU incorporation rates were calculated by counting more than 500 cells on a cover slip.

Response of HITS Cells to Insulin-Like Growth Factor-I (IGF-I) in vitro
To elucidate mechanisms of cell proliferation and differentiation, the response to IGF-I was assessed by measuring the increased cell number, BrdU incorporation and GFAP-, GC-, GD3-expression in HITS glioma in vitro. To measure the increased cell number, 2 x 10⁴ cells were inoculated in 21cm² culture dishes with MEM + 10% FBS. The following day, the culture medium was replaced by the following culture mediums; (a) MEM + 10% FBS, (b) MEM + 0.1% FBS + Insulin-like growth factor-I (IGF-I, Collaborative Research, Inc., U. S. A.) in 10, 100, 200ng/ml amounts, (C) MEM + 0.1% FBS. Each culture medium was replaced by fresh medium on the 4th day, and the cells were harvested on the 7th day and counted using a hemocytometer.

To measure the effect of IGF-I on BrdU incorporation and GFAP-, GC-, GD3-expression, 5 x 10⁶ cells inoculated onto a cover slip were cultivated under the same condi-
tions. These cells were cultured for 12 hours in the experimental media. BrdU incorporation was performed for the last 1 hour of the culture, as previously described, and immunostaining was also performed. GFAP-, GC- and GD3-expression were observed on the 7th day of culture in the experimental media. The data were estimated by t-test (P < 0.05).

Transmission Electron Microscopic Examination

For transmission electron microscopy, the cells were fixed with 2.5% glutaraldehyde and postfixed with 1% osmium tetroxide in situ. Fixed cells were dehydrated, embedded in Epon-Araldite resin, sectioned with an ultramicrotome and observed with a JEM-1200EX (JEOL) transmission electron microscope.

Results

Original Gliomas

The five ENU-induced gliomas, from which cell lines in this study were derived, were basically oligodendrogliomas (Fig. 1). One contained a sarcomatous focus. Astrocytic (GFAP-positive) cells were scattered in and around the tumors (Fig. 2). Neither GFAP, GC nor MBP was detected in the oligodendroglioma cells.

Culture Cells

During passages, four of five cell lines showed mesenchymal drift developing spindle-shaped cells and losing glial features, morphologically. However, the HITS glioma possessed a polygonal cell body and a relatively small nucleus (Fig. 3), grew in a monolayer and formed pile-up foci after 30 days of culture (6th passage, Fig. 3). HITS glioma have been continuously cultivated for more than 300 days (over 50 passages) and still maintain these features.

Fig. 4 shows changes in the proportion of GFAP-positive, BrdU-incorporated cells in each cell line. Although approximately 10% of cultured cells expressed GFAP in the early passage of each cell line, the GFAP-positive cells decreased in proportion and finally disappeared. The proportion of BrdU-incorporated cells was within the range of approximately 10-15% in each cell line. There were no positive cells for GC and GD3 in any of the cell lines.

Subcutaneous Inoculation of HITS Glioma and RT1005 Cells

Subcutaneous inoculation of each cell line yielded tumors in all recipient rats. These tumors reached diameters of
Fig. 4. The proportion of GFAP-positive, BrdU-incorporated cells in each cell line. Although approximately 10% of cultured cells expressed GFAP in the early passage of each cell line, the GFAP-positive cells decreased in proportion and finally disappeared.

Fig. 5. Transplanted tumor of HITS glioma. The tumor was solid, white colored with gelatinous appearance. Cystic change was also noted in the tumor.

3-4cm, 2-8 weeks after inoculation. They were solid, white colored, soft tumors with gelatinous appearance and cystic change (Fig. 5). The tumors derived from HITS glioma consisted of oligodendroglioma (Fig. 6a) and anaplastic glioma (Fig. 6b). The tumors also contained a variety of minor components including sarcomatous (Fig. 6c), ependymomatous (Fig. 6d), astrocytoma-like foci (Fig. 6e), and perivascular proliferation (Fig. 6f). Immunostaining revealed GFAP-positive cells in all areas despite differing histopathology (Fig. 7 a-f), although no isomorphic proliferation of GFAP-positive cells was observed. MBP, GC and GD3 were not detected in any area of these tumors. Subcutaneous tumors derived from RT1005 cells showed only a sarcomatous appearance (Fig. 8). No GFAP- or MBP-positive cells were found.

Response of HITS Glioma to IGF-I in vitro

(1) Cell Proliferation: Cell Number
The effect of IGF-I (10, 100 and 200 ng/ml) on the proliferation of HITS glioma is shown in Tables 1 and 2. In the medium supplemented with 0.1% FBS, IGF-I induced a significant increase in the cell number of both neoplastic and neonatal glia, compared with controls (0.1% FBS alone).

(2) Cell Proliferation: BrdU Incorporation
The effect of IGF-I (10, 100 and 200ng/ml) on BrdU incorporation by the cultured tumor cells is shown in Table 2. In 0.1% FBS supplemented medium, IGF-I induced BrdU incorporation of both neoplastic and neonatal glia, compared with controls (0.1% FBS alone). IGF-I stimulated BrdU incorporation in a dose-dependent fashion in both neonatal and neoplastic glial cultures.

Effect of IGF-I on the Expression of GFAP, GC and GD3
IGF-I did not induce antigenic expression of GFAP in HITS glioma, although IGF-I increased the proportion and absolute cell number of GFAP-positive cells in neonatal glial cell culture, depending on the concentration (Table 3). IGF-I did not induce antigenic expression of GC or GD3 in neoplastic glia nor increase the population of GC or GD3 positive cells in neonatal glial cell culture.

Transmission Electron Microscope Examination (Fig. 9)
Each cell of HITS glioma contained a slightly irregular nucleus and a moderate amount of cytoplasm. The nucleus contained nucleoli and dense chromatin which were partially attached to the nuclear envelope. A moderate number of rER, mitochondria, abundant free ribosomes, and microtubules were observed in the cytoplasm (inset). There were thin cytoplasmic processes, but glial filaments were absent.
Fig. 6 (a-f). Light microscopy of transplanted tumors (HITS glioma). (H & E, x 400)
6a; Isomorphic proliferation of small round cells corresponding to oligodendroglialoma.
6b; Anaplastic glioma-like lesion composed of small polygonal cells.
6c; Sarcomatous focus composed of spindle cells.
6d; Ependymomatous focus composed of small polygonal cells and wide intercellular spaces.
6e; Astrocytoma-like lesion composed of multipolar cells.
6f; Perivascular proliferation composed of spindle cell proliferation in concentric circles.
Fig. 7 (a-f). GFAP immunostaining of transplanted tumors (HITS glioma). (GFAP)
7a; oligodendroglioma-like lesion. (x 400)
7b; anaplastic glioma-like lesion. (x 600)
7c; sarcomatous focus. (x 600)
7d; ependymomatous focus. (x 400)
7e; astrocytoma-like lesion. (x 400)
7f; perivascular proliferation. (x 600)
Fig. 8. Light microscopy of another transplanted tumor (KT1005) (H & E, x 200). The tumor composed of spindle cells lacks the morphological feature of primary glioma, histologically.

Fig. 9. Transmission electron micrographs of HITS glioma culture (x 4000). Each cell contained a slightly irregular nucleus and a moderate amount of cytoplasm. Microtubules were observed in the cytoplasm (inset).

Table 1. Effect of IGF-I on the Proliferation of HITS Glioma Cells and Neonatal Rat Glial Cell Culture (x 10^5): Cell Number After One Week

<table>
<thead>
<tr>
<th></th>
<th>IGF1 (-)</th>
<th>10ngIGF1</th>
<th>100ngIGF1</th>
<th>200ngIGF1</th>
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<td><strong>0.1%FBS</strong></td>
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<td></td>
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<tr>
<td>HITS glioma</td>
<td>8.40 ± 0.72</td>
<td>10.33 ± 1.04</td>
<td>16.77 ± 1.65</td>
<td>16.50 ± 0.73</td>
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<tr>
<td>control glia</td>
<td>2.30 ± 0.21</td>
<td>2.70 ± 0.21</td>
<td>5.07 ± 0.31</td>
<td>5.40 ± 0.38</td>
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<tr>
<td><strong>10%FBS</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HITS glioma</td>
<td>19.23 ± 0.45</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>control glia</td>
<td>8.07 ± 0.48</td>
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seeded cell number (approximately 2.0 x 10^5)
mean ± standard deviation (n = 3, P < 0.05)

Table 2. Effect of IGF-I on BrdU Incorporation of HITS Glioma Cells and Neonatal Rat Glial Cell Culture (%)

<table>
<thead>
<tr>
<th></th>
<th>IGF1 (-)</th>
<th>10ngIGF1</th>
<th>100ngIGF1</th>
<th>200ngIGF1</th>
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<tr>
<td><strong>0.1%FBS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HITS glioma</td>
<td>6.40 ± 0.50</td>
<td>9.00 ± 0.28</td>
<td>9.93 ± 0.21</td>
<td>10.30 ± 0.21</td>
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<tr>
<td>control glia</td>
<td>5.43 ± 0.12</td>
<td>6.33 ± 0.26</td>
<td>8.66 ± 0.35</td>
<td>9.60 ± 0.64</td>
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<tr>
<td><strong>10%FBS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HITS glioma</td>
<td>11.70 ± 0.29</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>control glia</td>
<td>11.00 ± 0.33</td>
<td>-</td>
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seeded cell number (approximately 5.0 x 10^5)
mean ± standard deviation (n = 3, P < 0.05)

Table 3. Proportion and Cell Number of GFAP Positive Cells of HITS Glioma Cells and Neonatal Rat Glial Cell Culture

<table>
<thead>
<tr>
<th></th>
<th>proportion of GFAP positive cells (%)</th>
<th>(cell number of GFAP positive cells x10^5)</th>
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</thead>
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<tr>
<td><strong>0.1%FBS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HITS glioma</td>
<td>0.0 ± 0.0 (0.4)</td>
<td>19.17 ± 0.76 (4.0)</td>
</tr>
<tr>
<td>control glia</td>
<td>0.0 ± 0.0 (0.4)</td>
<td>19.17 ± 0.76 (4.0)</td>
</tr>
<tr>
<td><strong>10%FBS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HITS glioma</td>
<td>51.60 ± 1.51 (4.1)</td>
<td>51.60 ± 1.51 (4.1)</td>
</tr>
<tr>
<td>control glia</td>
<td>51.60 ± 1.51 (4.1)</td>
<td>51.60 ± 1.51 (4.1)</td>
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</table>

seeded cell number (approximately 5.0 x 10^5)
mean ± standard deviation (n = 3, P < 0.05)
Discussion

Several types of neoplastic glia histologically similar to their normal counterparts (oligodendrocytes, astrocytes, and immature glia) have been recognized in ENU-induced gliomas. This fact may indicate the existence of pluripotent neoplastic glia. Identification of these pluripotent neoplastic glia and the elucidation of the mechanisms of their differentiation should therefore promote our understanding of their neurobiology. In vivo studies, however, have limitations in assigning pluripotent or immature glia to specific lineages, and are also complicated by reactive glia. In vitro studies circumvent these limitations, although antigenic instability or phenotypic change such as mesenchymal drift are often observed. In order to characterize the tumor cells in vitro, it is necessary to obtain cell lines which display morphological characteristics similar to the tumor cells in vivo.

In the present study, four out of five cell lines derived from ENU-induced gliomas showed mesenchymal drift, i.e. isomorphic proliferation of spindle-shaped cells in late passage, but one cell line, the HITS glioma, retained the morphological characteristics of cells in the primary culture for more than 300 days (over 50 passages) and showed the feature of neoplastic oligodendrocyte by electron microscopy. Furthermore, the HITS glioma have shown unique characteristics after subcutaneous inoculation into neonatal rats, yielding tumors similar to in vivo tumors of several histological types, including oligodendroglioma-like lesions, anaplastic glioma-like lesions, sarcomatous foci, ependymomatous foci, and astrocytoma-like lesions. There were apparent transitions among the various components. A fraction of the cells expressed GFAP, a specific astrocyte marker, and GFAP-positive cells in inoculated tumors varied in morphological features, consisting of oligodendrocytic, astrocytic and fibroblastic types. These GFAP-positive cells were negative for MBP, and so the author consider that the cells derived from inoculated culture cells rather than Schwann cells that migrated from the peripheral nerve of recipient rats. Since HITS glioma did not express GFAP in vitro, these GFAP-positive cells seemed to originate from GFAP-negative neoplastic glia as astrocytic differentiation of immature neoplastic glia. Using electron microscopy, Mandybur saw ENU-induced ependymomatous neoplasms not as true ependymal tumors but rather as primitive neuroepithelial neoplasms. In oligodendrogliomas, the existence of transitional cells between oligodendrocytes and gemistocytic astrocytes, and GFAP-positive neoplastic oligodendrocytes were reported. Some authors reported transient GFAP expression by immature oligodendrocytes. The morphological variety and GFAP expression in inoculated tumors suggest that HITS glioma consist of immature neoplastic glia.

Raff et al. found a type of progenitor cell (0-2A progenitor cells) in the rat optic nerve capable of differentiating to both oligodendrocytes and astrocytes depending on culture conditions. In humans, oligodendrogliomas may originate from 0-2A progenitor cells. In our laboratory, the antigenic expression of GD3, a marker for immature glia in 0-2A lineage, was found in ENU-induced gliomas in vivo. Although, HITS glioma cells in cell culture and inoculated sites showed loss of the GD3 ganglioside expression, HITS glioma cells had the ability to form the tumor histopathologically mimicking the primary glioma. Therefore, loss of the antigenic expression of GD3 ganglioside in cultured cells and inoculated tumor cells may be due to the change of environment or dedifferentiation of the cells. The subpopulation of HITS glioma that expressed GFAP may belong to cells of 0-2A lineage, although oligodendrocytic differentiation of HITS glioma has been proven neither in vivo nor in vitro in the present study despite the oligodendrocytic morphological characteristics in vitro. MBP and GC are expressed by mature oligodendrocytes, and the possibility of the mixture of various differential stages of glial cells in HITS glioma is not denied. In this respect, cloning of HITS glioma and other antibodies specific for immature oligodendrocytes is necessary for further investigation.

IGF-I, which is distributed throughout the central nervous system, regulates the proliferation and differentiation of glia. Specific receptors for IGF-I were also found in neoplastic glia. The present study suggested that IGF-I at physiological concentrations played a role in the proliferation of HITS glioma as well as neonatal glia in vitro. IGF-I induced the cell proliferation or differentiation of astrocytes in neonatal glial cultures, and not only the proportion but also the total number of GFAP positive cells was increased in the present study. However, GFAP was not detected in HITS glioma. Thus the expression of GFAP by HITS glioma in inoculated sites is probably mediated by other growth factors, for example, interleukins, fibroblast growth factor etc. The importance of the extracellular matrix in the development of gliomas was also noted. Further examination using other culture conditions that mimic in vivo environments is necessary to elucidate the glial cell differentiation in vitro.

The studies on the role of growth factors on ontogenesis in the fetal brain, and the mechanisms of proliferation of tumor cells by growth factors in vitro may clarify the differentiation of normal glial lineage and neoplastic glia. HITS glioma is a unique cell line that differentiates to astrocytic cells under certain conditions. This cell line might serve as a way to promote understanding of the proliferation and differentiation of neoplastic glia, although the mechanism of astrocytic differentiation remains to be elucidated.
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