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Establishment of Mouse Oligodendrocyte/Type-2 Astrocyte Lineage Cell Line by Transfection with Origin-Defective Simian Virus 40 DNA

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ABSTRACT. **A permanent glial cell line has been established from the neonatal mouse primary mixed glial cell cultures by transfection with replication origin-defective simian virus 40 DNA. This cell line, designated OS3, has morphological similarity to type-2 astrocyte and expresses an astrocyte-specific marker, glial fibrillary acidic protein (GFAP), when cultured in the presence of 10% calf serum (CS). OS3 cells do not express the O4 antigen, galactocerebroside (GalC) and A2B5 under this culture condition. When cultured in a medium containing 2% CS or a chemically defined medium, these cells undergo morphological transformation. Some of these cells express O4 antigen and/or GalC, and the percentage of GFAP positive cells decreases under these conditions. Thus depending on the culture conditions, the OS3 cells display either type-2 astrocyte properties or immature oligodendrocyte characteristics. Furthermore, the OS3 cells show similar responses to the various growth factors as do oligodendrocyte/type-2 astrocyte (O-2A) progenitors. Therefore, the OS3 cell line is an unique mouse bipotential permanent O-2A lineage cell line which may be useful to analyze the developmental properties of these glial cells.**

Recent studies have shown that oligodendrocytes and a subclass of astrocytes, the type-2 astrocytes, develop from common precursor cells, that is, the oligodendrocyte/type-2 astrocyte (O-2A) progenitor cells. O-2A progenitor cells were first identified in the cultures of postnatal rat optic nerve (33). These glial precursor cells differentiate into oligodendrocytes in serum-free chemically defined medium, and differentiate in vitro (44) into type-2 astrocytes in a medium containing 10% fetal calf serum (FCS). O-2A progenitor cells have bipolar morphology and are identified by the monoclonal antibody (mAb) A2B5 (13), which recognizes cell surface ganglioside GQlc (20) and other gangliosides (15). Recently A2B5-positive cells were also identified in cultures of developing rat cerebrum (5, 38) and cerebellum (21) and were studied for their differentiation and proliferation.

When O-2A progenitor cells differentiate into myelinating oligodendrocytes, they chronologically express stage-specific differential markers on their surface or in their cytoplasm. These include ganglioside GD_3 (8, 18), O4 antigen (40, 41, 46), galactocerebroside (32) (GalC), 2′,3′-cyclic nucleotide 3′-phosphohydrolase (25, 26), proteolipid protein (9, 23) and myelin basic protein (9, 23). When O-2A progenitor cells differentiate into type-2 astrocytes, they express astrocyte marker glial fibrillary acidic protein (GFAP) (6).

Several factors have been shown to influence the induction of differentiation and proliferation of O-2A progenitor cells and oligodendrocytes. Platelet-derived growth factor (PDGF), released by the type-1 astrocytes (37) of which precursor cells are distinct from O-2A progenitor cells (34), promotes division of O-2A progenitor cells (30) and in the absence of PDGF these cells cease proliferation and differentiate rapidly into oligodendrocytes (35). Other polypeptide growth factors have also been shown to influence the development of O-2A progenitor cells or oligodendrocytes. These include basic fibroblast growth factor (bFGF) (7, 11), insulin-like growth factor 1 (IGF-1) (27, 28) and epidermal growth factor (EGF) (1). These results suggest that several growth factors, either alone or synergistically, modulate the proliferation and differentiation of O-2A progenitor in vivo. Besides humoral factors, cell-cell interaction may also promote myelin formation (23). However, the precise mechanisms of proliferation and differentiation of these cells remain to be elucidated. To study further the effects of humoral factors or the role of cell-cell interaction, it is of great advantage to obtain a pure population of O-2A lineage cells free of macrophages, microglia, type-1 astrocytes and neurons. It

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would considerably facilitate such studies to establish a permanent cell line that retains the main differentiation and functional properties of the normal counterpart from which it was derived.

Transfection of cells with simian virus 40 (SV40) DNA is one of the most useful strategies to immortalize cultured cells to make cell lines (17). We have established permanent bipotential glial cell line of O-2A lineage cells by electric field-mediated DNA transfer method (29, 31, 45), termed electroporation, with replication origin-defective SV40 DNA. Since most of previous studies on O-2A progenitor cells used only primary cultures of optic nerve or cerebellum of rat, our mouse-derived cell line may be particularly useful in the future studies.

MATERIALS AND METHODS

Reagents. The monoclonal antibody O4, which recognizes sulfolipids (sulfated ester sulfatid) (3, 39) is a gift from S.E. Pfeiffer (University of Connecticut Health Center, Farmington, U.S.A.). Polyclonal antibodies against SV40 large T antigen is also a gift from T. Ide (Hiroshima University, Hiroshima, Japan). Rat mAb (IgG) against GFAP and mouse mAb (IgG) against GalC were kindly provided by S.U. Kim (University of British Columbia, Vancouver, Canada). The A2B5 mAb was purified on a protein A (GIBCO, Grand Island, U.S.A.) column from hybridoma culture medium (American Type Culture Collection no. CRL 1520, Rockville, U.S.A.).

Human PDGF and recombinant human IGF-1 were purchased from Collaborative Research, Inc. (Bedford, MA, U.S.A.). Recombinant human EGF and bFGF were purchased from Boehringer Mannheim (Germany).

Cell culture and transfection. Mixed glial cell cultures were prepared from whole brains of newborn C3H/HeN mice, from which meninges and optic nerves were removed as detailed previously (43), and cultured in Eagle's minimum essential medium supplemented with 10% calf serum (CS), 5 μg/ml insulin and 0.2% glucose.

At 3 or 4 days in culture, cells were digested with trypsin in the presence of DNase and dissociated mechanically by repeated pipetting. After washing 3 times with phosphate buffered saline (PBS), these dissociated cells were transfected with origin-defective SV40 DNA (obtained from the Japanese Cancer Research Resources Bank-Gene, Tokyo, Japan) and pSV2neo DNA (42) by electroporation (Shimadzu somatic hybridizer SSH-1) and then they were cultured in the medium containing 10%CS as described above.

To select transfected cells, the aminoglycoside antibiotic, G418 (42) (neomycin analogue), was added to the culture medium 48 hours later. The G418-containing medium was changed every three days and its G418 concentration was increased gradually up to 400 μg/ml. When growing foci were observed, each focus was transferred to a well of a 24-well culture plate (Falcon 3047, Becton Dickinson, Lincoln Park, NJ, U.S.A.) by applying a small piece of filter paper soaked with trypsin onto the focus. Subcloned cells were recloned by limiting dilution method. Each clone (cell line) was seeded on a poly-L-lysine coated 14 mm glass coverslip and cultured in the medium containing 10%CS, 2%CS, or in chemically defined medium (12). The medium containing 2%CS was also supplemented with 5 μg/ml insulin and 0.2% glucose.

Immunofluorescence. Cells were plated on 14-mm diameter glass coverslips coated with poly-L-lysine. Abs were diluted in EBSS-HEPES plus 5% normal goat serum.

Staining with Abs against A2B5, O4 (1 : 50) or GalC (1 : 5) was carried out at 4°C for 30 min on unfixed viable cells. Prior to immunostaining with GFAP or SV40 large T antigen, cells were fixed with acid alcohol (5% acetic acid, 95% ethanol) or ethanol and acetone (1 : 1) for 20 min at −20°C.

After incubation with the appropriate second fluorescent-labeled Abs, the coverslips were mounted in buffered glycerol and viewed under a Zeiss universal microscope equipped with phase contrast, fluorescein and rhodamine optics. The percentage of positive cells in each culture condition was enumerated.

In some experiments, double staining with anti-GFAP Ab and O4 or anti-GalC Ab was carried out. The cells were first exposed to O4 or anti-GalC mAbs followed by rhodamine-conjugated goat anti-mouse IgM or IgG, fixed with acid alcohol, and then exposed to rat anti-GFAP Ab followed by fluorescein isothiocyanate (FITC)-conjugated goat IgG anti-rat IgG.

For double staining with O4 and anti-GalC Abs, the cells were first exposed to O4 mAb followed by anti-GalC Ab, and thereafter, incubated with rhodamine-conjugated goat anti-mouse IgM and FITC-conjugated goat anti-mouse IgG.

Effects of growth factors on differentiation. To investigate the effect of growth factors on transfected cells, 1×10^4 cells were plated on each poly-L-lysine coated glass coverslip and cultured in 10%CS, 2%CS containing medium or in chemically defined medium. Under all three conditions, these cells were treated with PDGF (10 ng/ml), bFGF (10 ng/ml), IGF-1 (100 ng/ml), EGF (10 ng/ml) or PDGF (10 ng/ml)+ bFGF (10 ng/ml) for 4 days. The cells were immunolabeled with O4 and GalC to study the effect of these factors on the differentiation of the transfected cells. The percentage of cells positive for O4 and GalC was enumerated under a immunofluorescent microscope.

Effects of growth factors on proliferation. Incorporation of ^{3}H-thymidine into the line cells under different culture conditions in the presence of various growth factors were examined.

The cloned cells were plated in the wells of 24-well plates at a density of 2×10^4 cells/well with 500 μl of 10%CS containing medium. After 1 hour in culture, the culture medium was exchanged for 500 μl of fresh 10%CS, 2%CS containing medium or chemically defined medium (in triplicate). The cells were cultured for 48 hours and then the cultures were pulsed

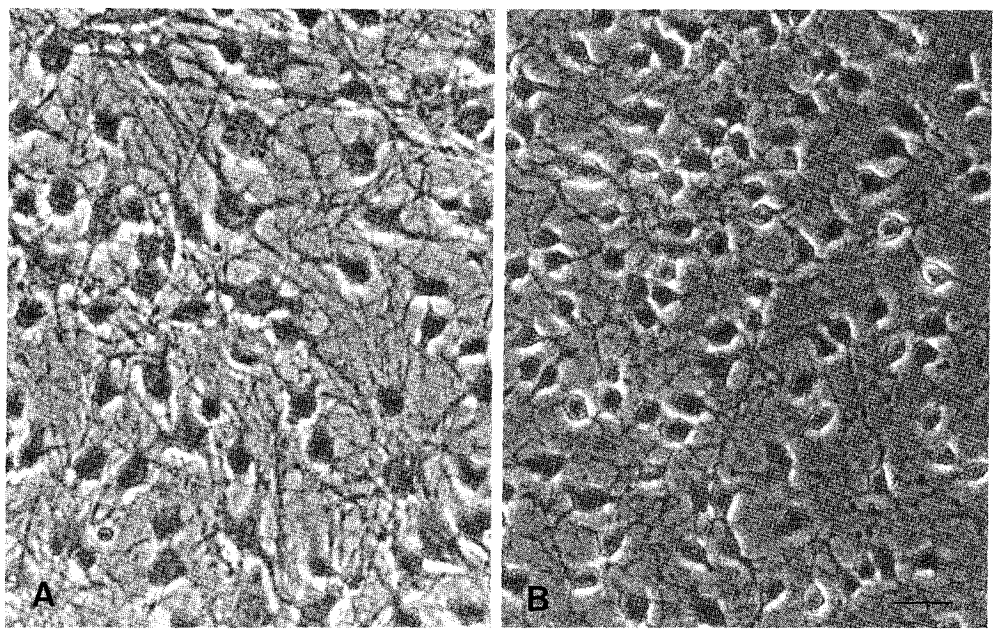

Fig. 1. Morphological transformation of the OS3 cells. When cultured in the medium containing 10%CS for a few weeks (A), the cells became morphologically similar to type-2 astrocytes with a rather flat cell body and filamentous processes. When cultured in the medium containing 2%CS or chemically defined medium (B), the cells had phase bright cell bodies with multiple processes similar to O-2A progenitors or immature oligodendrocyte. Bar = 30 μm.

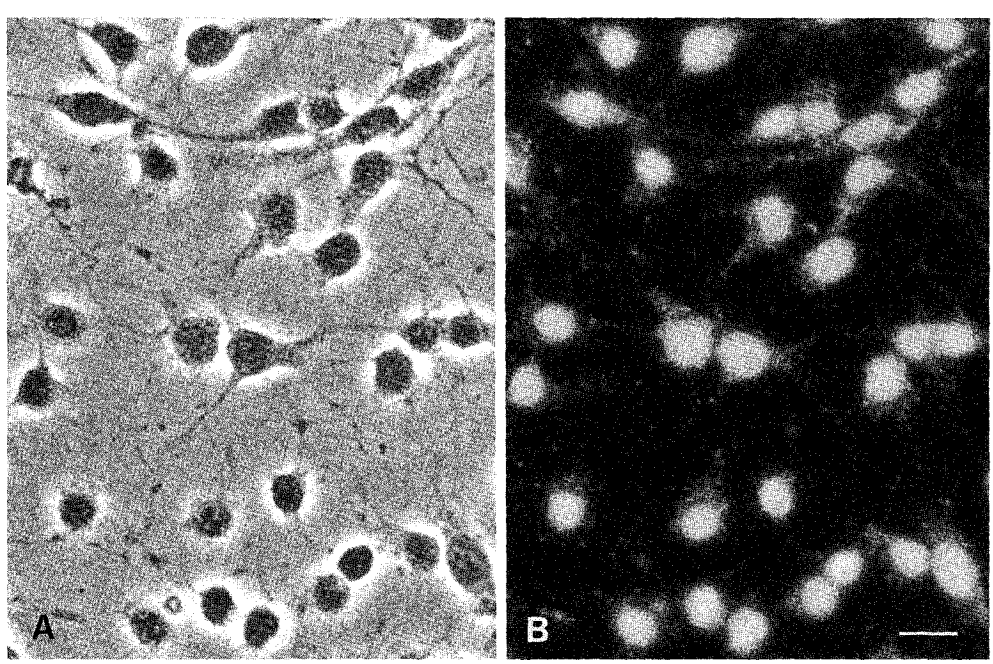

Fig. 2. Expression of the SV40 large T antigen. Phase contrast micrograph of the OS3 cells in the medium containing 10%CS (A). Staining with anti-SV40 large T Abs showed reactivity in the nuclei of all cells (B) (the same field as in A). Bar = 20 μm.

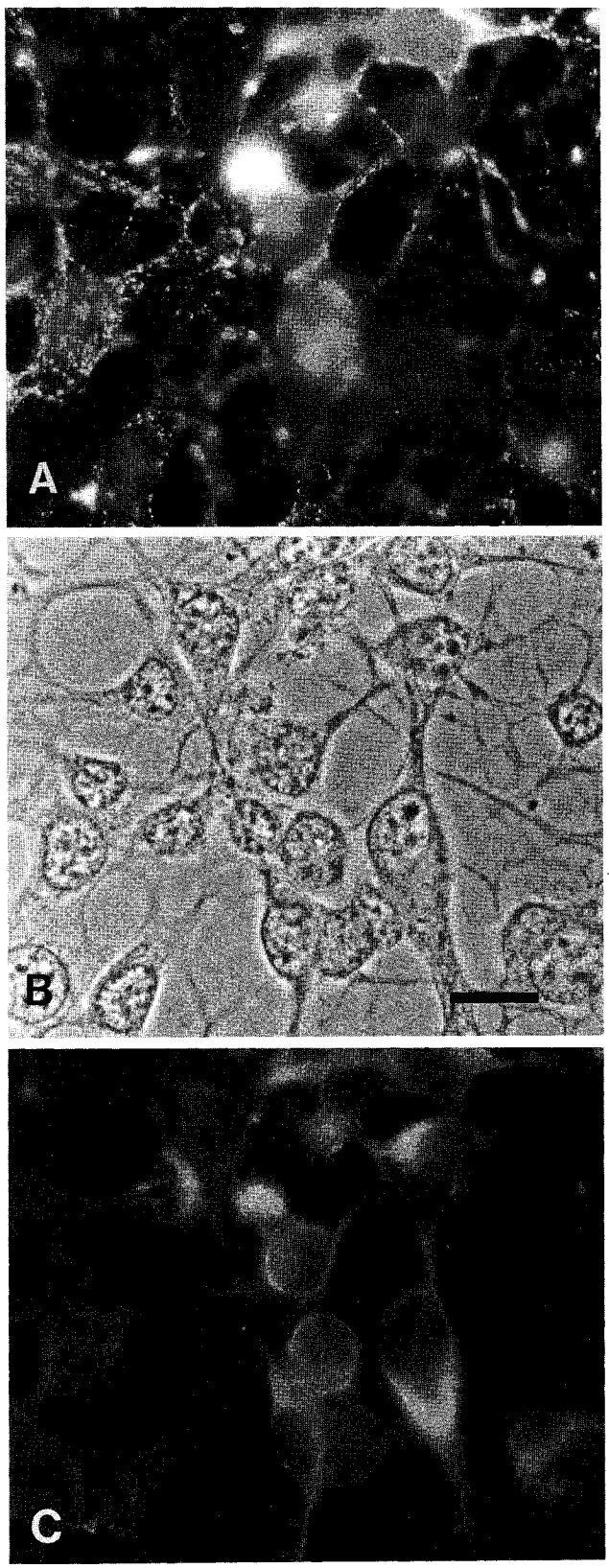

Fig. 3.

with 1 μCi/well ^{3}H-thymidine for another 16 hours. After washing twice with PBS, the cells were lysed and precipitated with 0.2 N NaOH, bovine serum albumin (1 mg/ml) followed by 1NHCl and ice-cold 50% (W/V) trichloroacetic acid (TCA) at 4°C. The insoluble contents were harvested onto glassfiber filters and the radioactivity of each sample was measured in a liquid scintillation counter.

RESULTS

Transfection. About 120 cell lines were established by transfection and most of them had the flat morphology as is characteristic of a type-1 astrocyte. Some clones bore processes which made them morphologically similar to type-2 astrocyte in the medium containing 10%CS. When they were cultured in the medium containing 2%CS, morphological transformation was observed in three cell lines. These three transfected cells had a phase bright cell body with multiple processes similar to immature oligodendrocyte under phase contrast microscopy. One of these cell lines, designated OS3 (Fig. 1), had multiple processes and an eccentric nucleus similar to that in type-2 astrocyte after a few weeks in culture with 10%CS and had some immunochemical markers in its cytoplasm and on its surface. OS3 cells were maintained in the medium containing 10%CS and were used for further analyses.

Characteristics of OS3 cells. When cultured in the medium containing 10%CS, almost all OS3 cells were GFAP-positive (GFAP$^+$), A2B5-negative (A2B5$^-$), O4-negative (O4$^-$) and GalC-negative (GalC$^-$). The SV 40 large T antigen was strongly positive in the nucleus of OS3 cells (Fig. 2). They grew rapidly with a doubling time of about 30 hours in this culture medium.

When the OS3 cells were cultured in the medium containing 2%CS, by 4 days in culture, about 50% of the cells became GFAP$^-$O4$^+$ (Fig. 3); less than 1% of the cells were doubly positive for GFAP and O4; and 2–3% of the cells expressed GalC. Most GalC$^+$ cells were also positive for O4 (Fig. 4) and none of the GalC$^+$ cells was positive for GFAP. These GalC$^+$ cells tended to appear in clusters. O4 and GalC were expressed on both the cell bodies and the processes. By 7 days in culture, the percentage of GFAP$^+$ cells decreased further and the percentage of O4$^+$ cells increased up to 60–70%. GalC$^+$ cells also increased but only slightly (Fig. 5A).

Cultured in the chemically defined medium, by 4 days in culture, about 50% of OS3 cells lost GFAP as seen in

Fig. 3. Double immunostaining of the OS3 cell cultured in the medium containing 2%CS for 7 days. Staining with mAb O4 (rhodamine optics) was shown in A. Staining with mAb against GFAP was shown in C (FITC). Most of the O4 positive cells were GFAP negative. A few of the cells, however, were doubly positive for O4 and GFAP. The phase contrast micrograph (B) shows the same field as in A and C. Bar = 20 μm.

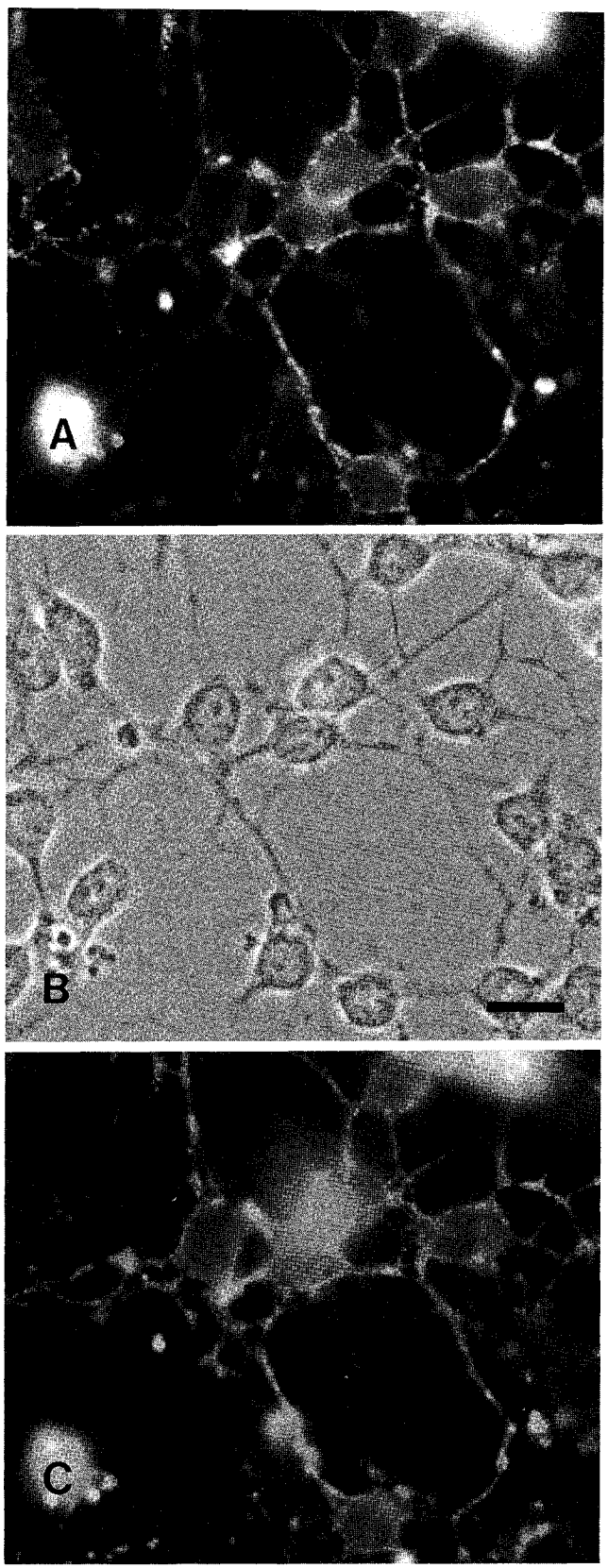

Fig. 4.

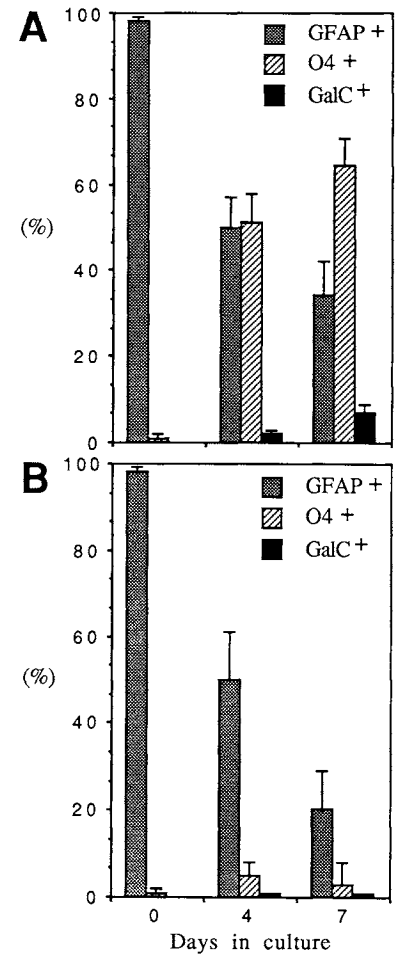

Fig. 5. Sequential changes in phenotypic markers under the different culture conditions, after being cultured in the medium containing 2%CS (A) or the chemically defined medium (B). The results are expressed as the mean ± standard deviation (SD) of cultures from three different platings.

the medium containing 2%CS but less than 10% of the cells expressed O4. The percentage of $GalC^+$ cells was less than 1%. About 40% of the cells lost all the immunochemical markers ($GFAP^-$, $O4^-$, $GalC^-$). By 7 days in culture, the percentage of $O4^+$ cells did not increase but the percentage of $GFAP^-O4^-GalC^-$ cells increased up to 70–80%. The percentage of $GalC^+$ cells remained less than 1% (Fig. 5B).

Effects of growth factors on OS3 differentiation. After treatment with PDGF, bFGF, IGF-1, EGF, or PDGF+bFGF there was no significant change in any immunochemical marker under any culture condition

Fig. 4. Double immunostaining of the OS3 cell cultured in the medium containing 2%CS for 7 days. Most of the cells labeled with mAb against GalC (A) (rhodamine optics) were also labeled with mAb O4 (C) (FITC). The phase contrast micrograph (B) shows the same field as in A and C. Bar = 20 μm.

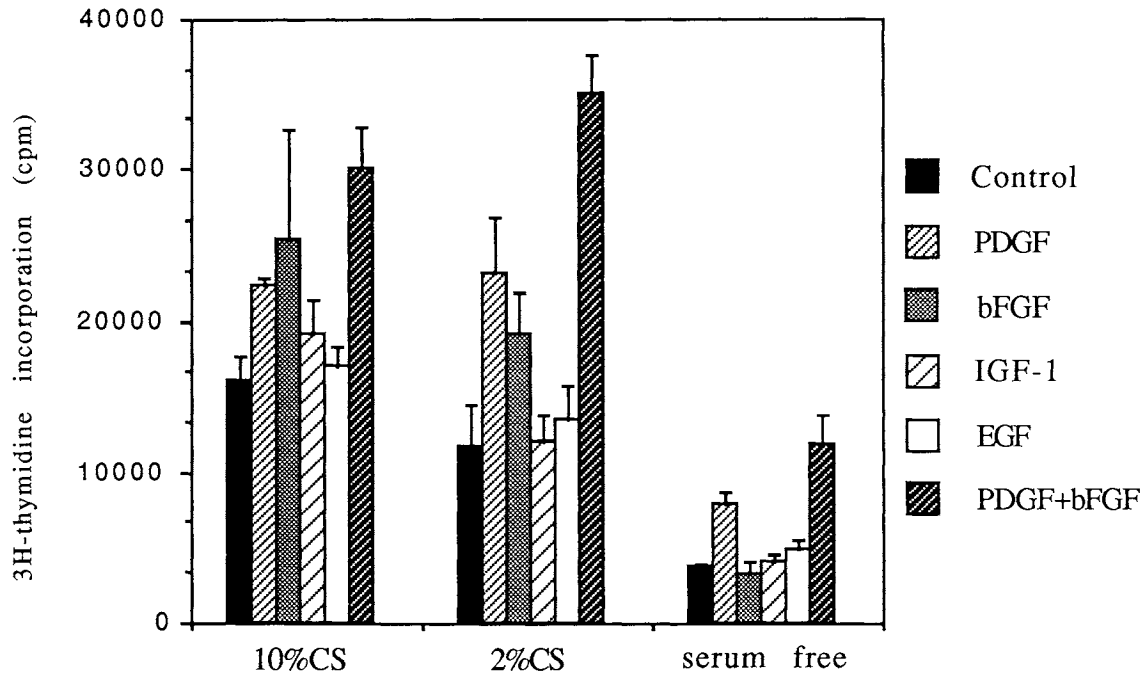

Fig. 6. ^{3}H-thymidine incorporation study under different culture conditions in the presence of various growth factors. The cells were cultured for 48 hours under each culture condition and then the cultures were pulsed with ^{3}H-thymidine and incubated for another 16 hours. Data from triplicate cultures are expressed as the mean ± SD. The concentration of all growth factors was 10 ng/ml except for IGF-1 (100 ng/ml).

(data not shown).

Effects of serum and growth factors on OS3 proliferation (Fig. 6). Without mitogen, DNA syntheses increased in proportion to the serum concentration in the culture medium. Under all three culture conditions, PDGF, bFGF, and PDGF + bFGF increased ^{3}H-thymidine incorporation in OS3 cells. Among these stimulants, the combination of PDGF and bFGF increased significantly DNA synthese (2 to 3-fold as compared to the untreated control). IGF-1 had some mitogenic effect in the medium containing 10%CS but not in the medium containing 2%CS or in the chemically defined medium. EGF had no mitogenic effect on the cells in any of the three culture conditions.

DISCUSSION

The sequential development of O-2A progenitor cells into oligodendrocytes and type-2 astrocytes in vitro depends on both intracellular and environmental controls (22). It is of use to establish a pure population of O-2A progenitor cells for studies on the mechanism of differentiation and proliferation of O-2A progenitor cells. In this respect, a transfection strategy with origin-defective SV40 DNA would be able to establish a stable mouse O-2A lineage cell line.

Throughout the long term culture period of over one and a half year, the OS3 cells expressed a stable morphological and immunocytochemical phenotype. When cultured in the medium containing 10%CS, OS3 cells had multiple processes and an eccentric nucleus similar to that of type-2 astrocytes after a few weeks in culture. When cultured in the medium containing 2%CS or a chemically defined medium, OS3 cells assumed oligodendrocyte-like morphology with phase bright cell body and multiple processes.

Immunocytochemically almost every OS3 cell was GFAP$^+$, O4$^-$, and GalC$^-$ in the presence of 10%CS. The cell surface antigens O4 and GalC were induced by reducing the serum level in the culture medium. These findings indicate that the OS3 cells line has some characteristics of O-2A progenitor cells although they are A2B5$^-$.

The ordered and partially overlapping expression of A2B5, O4, and GalC markers distinguishes three consecutive, phenotypically difined stages of oligodendrocyte lineage in vitro, represented by the bipolar A2B5$^+$O4$^-$GalC$^-$ glial precursor, multipolar O4$^+$GalC$^-$ progenitor, and the complex process-bearing O4$^+$GalC$^+$ oligodendrocyte (10, 16). The O4$^+$GalC$^-$ progenitor cells, termed proligodendrocytes by Gard and Pfeiffer (16), seems to be a major component of the OS3 cells in the medium containing 2%CS. Some of these cells differentiate into GalC$^+$ oligodendrocytes which tend to appear in cluster, therefore, cell-cell interaction may play an important role for differenti-

ation into GalC$^+$ oligodendrocytes in this cell line. Thus the OS3 cell line represents an early developmental stage of the oligodendrocyte lineage when cultured in low serum culture medium. Even in chemically defined medium, which is known to promote O-2A progenitors' differentiation into oligodendrocytes (33), however, GalC$^+$ cells did not increase significantly. Treatment of OS3 cells with cyclic AMP also failed to increase the percentage of GalC$^+$ cells (data not shown). Thus OS3 cells may have limited capacity to become GalC$^+$ mature oligodendrocytes, or further optimal condition may be required to induce differentiation into GalC$^+$ oligodendrocytes on OS3 cells. Compared to other reports of the immortalization of glio-neuronal precursor cells (4, 14, 36), the timing of transfection seems to be too late to obtain an A2B5$^+$O-2A progenitor cell line. Since A2B5 is one of the early immunochemical markers of the O-2A progenitor cell, OS3 cells may be derived from the cells which lose A2B5 immunoreactivity. Therefore we had tried to establish A2B5$^+$ precursor cell lines using the mixed glial cell culture of late gestational state embryonic mice, but we could not obtain any A2B5$^+$ cell lines.

Besides morphological and immunocytochemical similarity to O-2A progenitors, OS3 cells showed similar responses to several growth factors as do O-2A progenitors. It has been reported that purified human and porcine PDGFs, released by type-1 astrocyte (37), are strongly mitogenic for O-2A progenitors in vitro but after a set number of divisions these cells lose responsiveness to PDGF and differentiate (30). In the absence of PDGF, O-2A progenitors cease proliferation and differentiate rapidly into oligodendrocytes. Basic FGF, which is abundant in the central nervous system (19) has been shown to induce DNA synthesis in O-2A progenitors (30) as well as in oligodendrocytes (11). Basic FGF acts on O-2A progenitor cells to maintain high level expression of PDGF α-receptors and to increase the sensitivity of O-2A progenitor cells of PDGF (24). O-2A progenitors exposed simultaneously to PDGF and bFGF do not undergo any differentiation to oligodendrocytes but instead divide continuously as bipolar and motile progenitors (7). PDGF, bFGF and the combination of both acted as potent mitogens on OS3 cells as observed in O-2A progenitors. When cultured in the medium containing 10%CS, OS3 cells express the astrocyte marker GFAP but still have the potency to react to these growth factors as do O-2A progenitorss. IGF-1 is reported to promote proliferation of immature oligodendrocytes at the O4$^+$GalC-stage (28). However, only minimal mitogenic activity on OS3 cells was observed. Since we used a culture medium containing insulin, it is possible that insulin cross-reacted with IGF-1 receptors to block the effect of IGF-1 on OS3 cells. EGF induces proliferation and differentiation of oligodendrocytes, especially mature myelin-forming oligodendrocytes (1). The result that OS3 cells did not respond to EGF in any culture condition also supports the immature differentiation stage of OS3 cells, rather than myelin-forming oligodendrocyte.

There were a few reports about immortalization of glio-neuronal precursor cells (4, 14, 36). Two cell lines (4, 14) expressed either neuronal or glial markers depending on the culture conditions, but the differentiation capacity of these cell lines appeared to be limited and all of them differentiate only one way to an astrocyte. Others (36) also expressed either neuronal and glial markers, but they were not bipotential. There was only one report of a bipotential cell line that differentiates into both oligodendrocytes and type-2 astrocytes (2). This line was established by repetitive passage from rat neonatal forebrain culture for over a year, and did not consist of a single cell population. Therefore, the OS3 cell line is an unique mouse bipotential permanent O-2A lineage cell line and may be useful to analyze the developmental properties of these glial populations.

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