Differentiation of bipotential glial precursors into oligodendrocytes is promoted by interaction with type-1 astrocytes in cerebellar cultures

(cell cultures/cell lineage/cellular interactions/immunofluorescence/conditioned medium)

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ABSTRACT The differentiation of bipotential precursors of oligodendrocytes (OL) and type-2 astrocytes (AS) was followed in primary cultures from 8-day postnatal rat cerebellum by labeling the cells with the antibodies LB1 (which binds to the surface dialesialoganglioside GD3 present in glial precursors, type-2 AS, and immature OL), O4 (a marker of immature and mature OL binding to surface sulfatide), anti-galactocerebroside (GalCer, a marker of OL), and anti-gial fibrillary acidic protein (GFAP, a marker of AS). Two hours after plating, about 85% of the LB1+ cells were detectable. 40% of the O4+ cells were GalCer+, and none of the O4+ cells were GFAP+. Upon culturing cells plated at a density of 1 x 10^5 cells per cm^2 in the presence of fetal calf serum, most of the LB1+ precursors differentiated into type-2 AS, even if most of them had already expressed the O4 antigen. Thus, in culture, most type-2 AS seem to derive from progenitor cells that were differentiating in vivo into OL. In higher density cultures (2.5 x 10^5 cells per cm^2), however, many precursors differentiated into GalCer+ OL, rather than into AS. As a possible source of the signals responsible for the behavior of the glial precursors in high-density cultures, we focused our attention on type-1 AS, the most abundant cell type in the cultures. We found that, in low-density cultures maintained for 5-7 days in a medium conditioned by type-1 AS, the proliferation of the precursors was enhanced and their differentiation into OL or AS was prevented. In contrast, when cerebellar cells were co-cultured with type-1 AS dissociated from purified cultures, not only did the precursors proliferate more than in control cultures, but also a larger proportion of them differentiated into GalCer+ OL. In conclusion, type-1 AS appear to facilitate the differentiation of bipotential glial precursors into OL through direct cell-cell interactions. The influence of type-1 AS on the differentiation of the LB1+ and O4+ precursors is supported also by experiments with glial cortical cultures.

In serum-containing cultures from the rat central nervous system two populations of astrocytes (AS) have been distinguished on the basis of morphological, antigenic, and functional criteria (1-7): the epithelioid type-1 AS and the stellate type-2 AS. These two populations belong to different cell lineages. Although little is known about the progenitors of type-1 AS (8), type-2 AS derive from bipotential glial precursors that differentiate in optic nerve (1) and in cerebellar cultures (6) into (i) type-2 glial fibrillary acidic protein-carrying (GFAP+) AS in the presence of fetal calf serum (FCS) and (ii) galactocerebroside-carrying (GalCer+) oligodendrocytes (OL) in serum-free medium. As type-2 AS, the precursors express on their surface the gangliosides binding the monoclonal antibodies (mAbs) A2B5 (1, 6, 8), and LB1 (6, 9, 10), accumulate γ-amino-[3H]butyric acid ([3H]GABA) in a "neuron-like" fashion (6, 11), are sensitive to kainic acid (V. Gallo, R. Suergiu, C. Giovannini, and G.L., unpublished data), and express chondroitin sulfate (7). The bipotential cerebellar glial precursors pass through four distinct developmental stages during their differentiation into OL in serum-free cerebellar cultures (9). In the first stage, the cells are A2B5+ and LB1+. In the second stage, they can be stained also by the mAb O4, which binds to surface sulfatides and recognizes both immature and mature OL (12). In the third, short-lasting stage, the cells express on their surface also the well-established marker of OL, GalCer (13). In the fourth stage, however, the antigens recognized by A2B5 and LB1 are no longer detectable, and the cells remain positive for O4 and GalCer. Upon addition of FCS, the precursors maintain their ability to differentiate into type-2 AS until they are in the A2B5/LB1/O4-positive stage (9). The factors responsible for the choice of the differentiation route taken by the precursor cells have not been defined. It has been suggested that FCS may contain substances mimicking endogenous factors present in the developing brain and orienting the precursors toward an astroglial differentiation (14). On the other hand, it is known that OL can be grown and can even differentiate from precursor cells in serum-containing media (15-18). Recently, Goldman and collaborators have proposed that the interaction between glial precursors and AS may facilitate the choice of the oligodendrogial differentiation pathway (18).

The aim of the present study was to try to understand what promotes the differentiation of cerebellar bipotential glial precursors into OL under culture conditions (presence of FCS) that in principle should facilitate their astroglial differentiation. Our results indicate that type-1 AS stimulate the proliferation of the bipotential precursors through the secretion of soluble factors and facilitate their differentiation into OL through direct cell-cell interactions. Our observations also suggest that the type-2 AS differentiating in serum-containing cultures from neonatal rat central nervous system partly derive from bipotential progenitor cells that, before dissociation, were already in the process of differentiating into OL.

MATERIALS AND METHODS

Cells dissociated from 8-day-old Wistar rat cerebellums after mild trypsinization (4, 6) were resuspended in Eagle's basal medium (BME, Gibco) supplemented with 2 mM glutamine, gentamycin (Sigma) at 0.1 mg/ml, and 10% heat-inactivated FCS (different batches from Gibco); were seeded at 1 x 10^5 (low density) or 2.5 x 10^5 (high density) cells per cm^2 onto

Abbreviations: AS, astrocytes; OL, oligodendrocytes; GalCer, galactocerebroside; GFAP, glial fibrillary acidic protein; mAb, monoclonal antibody; DIV, days in vitro; GABA, γ-amino butyric acid; FCS, fetal calf serum.

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Poly(L-lysine) (10 μg/ml)-coated 12-mm round coverslips were placed in 35-mm dishes and were grown in the same medium at 37°C in humidified 95% air/5% CO₂ (4, 6).

Primary cultures from 1-day-old Wistar rat cerebral cortex were prepared as described by Labouret et al. (19) with minor modifications. In brief, brain hemispheres stripped of meninges were dissociated through a 82-μm nylon mesh; cells were seeded onto poly(L-lysine)-coated 60-mm plastic dishes (two hemispheres per dish) and grown in BME/10% vol/vol FCS, with medium changes every 3–4 days. After 20 days in vitro (20 DIV) the cells growing on top of these cultures were dislodged by gently syringing the culture medium on the cell layer, collected by centrifugation at 150 × g, seeded onto poly(L-lysine)-coated coverslips at a density of 3.5 × 10⁴ cells per cm², and grown in BME/10% FCS.

For the preparation of purified type-1 AS, freshly dissociated cerebellar cell suspensions were treated for 40 min at 37°C with A2B5 ascites fluid and detoxified rabbit complement [Cederlane; diluted 1:50 and 1:10, respectively, in RPMI 1640 medium (Sigma)] to kill glial precursors and neurons. The surviving cells were washed and cultured for 7 days in BME/10% FCS and then subcultured after dissociation with 0.05% trypsin in 0.02% EDTA (Sigma). After another week, these cultures did not contain neurons; A2B5+, LB1+ (first stage) or O4+ (second stage) glial precursors; or GalCer+ (third stage) cells.

Indirect immunofluorescence procedures for L1 and O4 antigens, GalCer, and GFA were carried out as described (6, 9). For combined [³H]GABA autoradiography (2) and immunofluorescence with anti-GalCer mAb, high density cerebellar cultures at 5 DIV were incubated with [³H]GABA at 2 μCi/ml (specific activity, 37 Ci/mmol; New England Nuclear; 1 Ci = 37 GBq) for 10 min, then stained with anti-GalCer mAb and a second fluorescein-conjugated goat anti-mouse antibody (9), fixed with 2.5% glutaraldehyde for 15 min, and rinsed three times for 4 min with NaBH₄ (1 mg/ml). The coverslips were then coated with Ilford K2 emulsion [1:2.5 (vol/vol) in H₂O] and developed after 1–2 days of exposure.

RESULTS

The literature reports that serum-containing cultures relatively enriched in OL can be obtained when cells are plated at fairly high density (15, 18, 20). As a first step for our investigation, we compared the differentiation of LB1+ and O4+ glial precursors in serum-containing low- and high-density cultures obtained from 8-day postnatal rat cerebella. The cells were double-stained with LB1 or O4 mAb and anti-GFAP or with O4 and anti-GalCer mAbs. In previous studies performed on serum-free cerebellar cultures, labeling of bipotential precursors obtained with the mAb LB1, which recognizes the ganglioside GD3 (10), was not distinguishable from that obtained with the mAb A2B5 (9). Two hours after plating at any cell density, hardly anystellate LB1+, GFAP+ type-2 AS and no O4+, GFAP+ cells were detectable, while about 40% of the O4+ cells were GalCer+ and <10% of the GalCer+ cells were LB1+. In low-density cultures (1 × 10⁵ cells per cm²), about 70% of the stellate LB1+ glial precursors became GFAP+ within 3–5 days (Fig. 1 A and D; Table 1), in agreement with previous results (4, 6). At 5 DIV, ≈80% of the LB1+ cells expressed the immature OL marker O4 as determined indirectly by a previously described procedure (9), and half of the O4+ cells also became GFAP+ and thus acquired the phenotype of type-2 AS (Fig. 1 B and E; Fig. 2). On the other hand, the percentage of O4+ cells expressing GalCer declined from ≈40% at 1 DIV to <10% at 5 DIV (Fig. 1 C and F; Fig. 2). This decline is largely explained by the decrease in the number of O4+, GalCer+ cells during the first 5 days of culturing while the number of O4+ cells almost doubled (Fig. 2).

When the initial cell plating density was increased to 2.5 × 10⁵ cells per cm², only ≈45% of the LB1+ cells (not shown) and 15% of the O4+ cells expressed GFAP within 5 days, while the proportion of O4+ cells expressing GalCer rose abruptly from ≈16% to 40–60% between 3 and 5 DIV, in spite of the 3-fold increase in the number of O4+ cells during the 5-DIV period (Fig. 2). Experiments with cells dissociated from the 1-day postnatal cerebella gave results qualitatively similar to those reported above (data not shown).

The progressive increase in O4+ cell number observed in both low- and high-density cultures (Fig. 2) could be due to cell proliferation and/or to the expression of the O4 antigen by preexisting LB1+ precursors. As in serum-free cultures (9), the contribution of cell proliferation was shown by incorporation of [³H]thymidine (6-hr pulse at 3 DIV) into the nuclei of 15% of the O4+ cells (average of two experiments). Combined autoradiography and immunofluorescence experiments showed that many of the GalCer+ OL growing in FCS accumulated [³H]GABA (Fig. 3). On the basis of previous work (4, 6), this functional property appears to be shared by

![Fig. 1.](image-url)
Table 1. Influence of type-1 AS on bipotential glial progenitor cell differentiation and proliferation in serum-containing cerebellar primary cultures

<table>
<thead>
<tr>
<th>Low-density culture conditions</th>
<th>LB1+ cells</th>
<th>O4+ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. per mm²</td>
<td>% expressing GFAP</td>
</tr>
<tr>
<td>Controls</td>
<td>33 ± 5 (7)</td>
<td>69 ± 4</td>
</tr>
<tr>
<td>Cells grown in type-1 AS-cond.</td>
<td>100 ± 13 (4)</td>
<td>24 ± 10</td>
</tr>
<tr>
<td>Cells plated with purified type-1 AS</td>
<td>60 ± 9 (4)</td>
<td>35 ± 5</td>
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Freshly dissociated cerebellar cells at low density (1 x 10^5 cells per cm²) were grown for 5-7 days under three conditions: (i) cells were plated on polystyrene-coated coverslips; (ii) cells were plated as in (i) and 2 hr later the coverslips were transferred into plastic dishes containing a monolayer of purified type-1 AS to be exposed to a medium conditioned by type-1 AS and to avoid direct contact with them; and (iii) cells were seeded together with purified type-1 AS (4 x 10^4 cells per cm²). The cultures were processed for double immunofluorescence staining with LB1 and anti-GFAP or with O4 and anti-GalCer mAbs (see the legends to Figs. 2 and 4), and the labeled cells were counted as in Fig. 2. Figures represent means ± SEM of four to eight experiments. In the experiments with the cocultures, counting the LB1+ /GFAP+ cells lying on top of the GFAP+ type-1 monolayer was difficult. In this case, the percentage of LB1+ cells expressing GFAP was determined after the cells were detached with trypsin/EDTA, replated at a lower density on new coverslips, and immunostained.

all of the cells of the type-2 AS-OL cell lineage, including bipotential glial precursors and type-2 AS. Evidence for the expression of a GABA transport system by cultured OL has been provided in mouse cortical cultures (21).

In the cultures used in the present study, neurons die off within the first 3–4 days, and type-1 AS represent the most abundant cell population (2,4). Thus, the preferential differentiation of the bipotential glial precursors into OL observed in high-density cultures could be related to soluble factors secreted by type-1 AS or to a direct cellular interaction with them.

To assess the effect of type-1 AS-conditioned medium, we compared the differentiation profile of the precursors when seeded on coverslips in control low-density cultures to that occurring when the coverslips were transferred (2 hr after the cells were plated) into culture dishes containing a monolayer of purified type-1 AS. After 5–7 DIV, the number of LB1+ and of O4+ precursors was much greater on the transferred than on control coverslips. However, the proportion of the LB1+ cells expressing GFAP (Fig. 4 A and D) and of the O4+ cells expressing GalCer was low (Table 1).

To determine the effect of a direct interaction between type-1 AS and glial precursors, cells freshly dissociated from the cerebellum were plated at low density (1 x 10^5 cells per cm²) together with type-1 AS (4 x 10^4 cells per cm²) dissociated from confluent cultures. At 5–7 DIV, the LB1+ and O4+ cells resided on top of a monolayer of type-1 AS and had fewer and shorter processes than in control cultures, in which the cells grew on a polystyrene substrate; moreover, they had a less sparse distribution, being arranged in defined cell groups (compare Fig. 4 B and C with Fig. 1 A–C). As in the case of AS-conditioned medium, the number of LB1+ and O4+ cells was greater than in control low-density cultures. However, the proportion of LB1+ cells expressing GFAP was lower than in controls, while the proportion of O4+ cells expressing GalCer was significantly higher (Table 1 and Fig. 4, compare B with E and C with F). Our comparative study was restricted to the first 5–7 DIV, since at later stages the control low-density cultures became more and more similar to high-density cultures because of the active proliferation of type-1 AS.

In a set of experiments, cells dissociated by a different method (mechanical rather than enzymatic) from a different brain area (cerebral cortex) were used. After 20 DIV, the cultures consisted of a confluent layer largely composed of GFAP+ /LB1− cells on top of which a large number of bipotential glial precursors at the LB1+ or LB1+ /O4− stage of differentiation was present. In these cultures ~10% of the LB1+ cells and <5% of the O4+ cells expressed GFAP, and 15–40% of the O4− cells (depending on the preparation) were GalCer−, as determined in cells stained after dissociation with trypsin/EDTA. When the cells of the top layer were mechanically detached and cultured in the virtual absence of type-1 AS, most of the precursors (73% of the LB1+ cells and 54% of the O4−, GalCer− cells, averages from three experiments run in duplicate) differentiated into type-2 GFAP+ AS within 3 days (Fig. 5), while the number of GalCer+ cells did not increase.

**Fig. 2.** Time course of the expression of GFAP and GalCer by O4+ bipotential glial precursors in cerebellar cultures at different cell densities. Cells were plated at low density (1 x 10^5 cells per cm²) (Left) or at high density (2.5 x 10^5 cells per cm²) (Right), cultured for up to 5 DIV, and double-stained with O4 mAb and either anti-GFAP or anti-GalCer mAb (9). The number of total O4+ cells and that of O4−, GalCer− and O4−, GFAP+ cells is reported in the ordinate. Each point represents an average ± SEM of cell counts obtained in four to six experiments (two coverslips per experiment, 20.0.44-mm² microscopic fields per coverslip). In one case, data from two experiments (without SEM) are presented.

**Fig. 3.** Double-labeling of cerebellar glial cultures with [3H]GABA autoradiography and anti-GalCer immunofluorescence. Cells plated at high density (2.5 x 10^5 cells per cm²) were cultured for 5 DIV before processing. (A) Autoradiography with bright field optics. (B) Immunofluorescence with fluorescein optics and the same microscopic field as in A. (Bar = 100 μm.)
FIG. 4. Effect of type-1 AS on the differentiation of cerebellar bipotential glial precursors: double immunofluorescence labelings (9). Cerebellar cells at low density \( (1 \times 10^3 \text{cells per cm}^2) \) either were grown in type-1 AS-conditioned medium (A and D) or were cocultured with purified type-1 AS \((B, C, E, \text{and } F)\) and were cultured for 5 DIV. At this time the cells were double-labeled with the following mAbs: LB1 \( (A\) and \( B)\) and anti-GFAP \( (D\) and \( E)\); O4 \( (C)\) and anti-GaCer \( (F)\). A and D, B and E, and C and F represent the same microscopic fields under fluorescein \( (A-C)\) and rhodamine \( (D-F)\) optics. Although only a few precursors differentiated into GFAP* cells both in AS-conditioned medium \((A \text{ and } D)\) and in the cocultures \((B \text{ and } E; \text{ see also Table 1})\), a substantial proportion of them expressed GaCer only in the cocultures \((C \text{ and } F)\). (Bar = 150 \( \mu m\).)

DISCUSSION

The literature on the destiny of bipotential precursors of OL and type-2 AS has indicated that, in monolayer primary cultures from rat central nervous system, the oligodendroglial differentiation route becomes compulsory in serum-free culture media \((1, 6, 22, 23)\) containing insulin or insulin-like growth factors \((24)\), whereas the astroglial differentiation route is by and large preferred in the presence of FCS \((1, 6, 8)\). However, other observations indicated that OL can be grown and can even differentiate from precursor cells in serum-containing media \((15-18)\). Thus, FCS may contain substances capable of facilitating the astroglial differentiation of the bipotential precursors but not of preventing their differentiation into OL in the presence of appropriate signals. To better understand the nature of these signals, we monitored the differentiation of cerebellar glial precursors grown in FCS-containing culture media, using three experimental protocols.

The first experimental protocol (cultures at different cell densities, Fig. 2) not only allowed us to confirm the previous reports \((18, 19)\) showing that OL grow better in high- than in low-density cultures, but also allowed us to add new insights to these observations. In fact, the experiments indicated that the larger number of OL present in high-density cultures is related to the proliferation of a larger proportion of LB1* bipotential precursors (many of which are also O4*) and to a preferential differentiation of the precursors into OL, rather than into type-2 AS. The additional observation that many of the type-2 AS developing in both low- and high-density cultures were positive for the marker O4 on immature OL \((12)\), suggests that, at the time of cell dissociation and plating, several progenitor cells had already received the in vivo signals capable of orienting them towards an oligodendroglial differentiation. In our experiments, cells were dissociated at a stage at which, at least in the rat optic nerve, OL are in the process of differentiation, while type-2 AS have not yet developed \((25)\). Probably the process of differentiation into OL would have continued if the cells had not been forced towards an astroglial differentiation by the presence of FCS in the culture medium. It is unlikely that type-2 AS must go through a stage of O4 positivity during their normal differentiation because, in our cultures, the number of O4*.-GFAP* cells (Fig. 2) was always lower than that of LB1* .GFAP* type-2 AS \((\text{Table 1})\), and O4* .GFAP* cells have not been described in vivo. However, the existence of glial cells with a mixed AS-OL phenotype has been reported during in vivo development \((26)\).

The fact that type-1 AS represented the most abundant cell population in our cultures \((2, 4)\) led us to investigate the possible influence of type-1 AS on the differentiation of bipotential glial precursors. Two experimental protocols were used to discriminate between effects mediated by

FIG. 5. Double immunolabeling of glial bipotential precursors purified from glial mixed cortical cultures. Cells were stained 3 days after isolation with LB1 (fluorescein optics in A) and anti-GFAP (rhodamine optics in B); phase contrast is shown in C. The same microscopic field is shown in A-C. Note that most of the stellate LB1* cells expressed GFAP. (Bar = 100 \( \mu m\).)
soluble factors and those mediated by cellular contacts. In both cases, freshly dissociated cerebellar cells were cocultured at low density with purified type-1 AS, but direct contact with type-1 AS was prevented in one case and permitted in the other. In the absence of direct contact, the LB1+ and O4+ precursors proliferated more than in control cultures, and their differentiation into either type-2 AS or OL was prevented or at least retarded. These data agree with the finding that, in serum-free media, type-1 AS produce factors that are mitogenic for bipotential glial precursors (27,28). In contrast to its mitogenic effect on glial precursors, the type-1 AS-conditioned medium has been shown (29) to depress the proliferation of type-1 AS themselves.

The mitogenic effect of type-1 AS-conditioned medium on glial precursors was present also when contact between freshly dissociated cerebellar cells and type-1 AS was permitted. However, in this condition the proportion of precursors differentiating into OL was 3- to 4-fold greater than in control low-density cultures (Table 1) and was comparable to that seen in high-density cultures (Fig. 2). A similar increase in GalCer expression was also observed in experiments (not shown) in which freshly isolated cerebellar cells were cultured on a monolayer of killed (air-dried) type-1 AS. Thus, the oligodendroglial differentiation route appears to be facilitated by a direct interaction between precursors and type-1 AS. Preliminary experiments in which membranes from purified type-1 AS cultures were added to low density cerebellar cell cultures showed only a modest increase in GalCer expression by O4+ OL precursors (not statistically significant). One of the possible reasons for this negative result may be that the interaction between glial precursors and type-1 AS is mediated by molecules of the extracellular matrix or by other molecules that are lost during the preparation of membranes. Indeed, it remains also to be determined whether the differentiation signals are directly provided by type-1 AS or whether the presence of a substrate of type-1 AS facilitates the establishment of homotypic cell-to-cell interactions among the progenitor cells themselves. In a recent study, O4+ OL precursors expressing vimentin were shown to differentiate into O4+ , vimentin- cells but not into mature O4+ OL when cocultured with AS (30).

The aim of the experiments with brain cortical cell cultures was to corroborate, in a different brain area and with a different experimental protocol, the conclusions drawn with cerebellar cells. In mixed, high-density cortical cultures at 20 DIV, most of the glial precursors were at the O4 differentiation stage; ∼5% of the O4+ cells were GFAP+, and at least 3 times as many were GalCer+ , a situation qualitatively similar to that present in high-density cerebellar cultures (see Fig. 2). On the other hand, similar to the finding of Goldman et al. (18), when the cells lying on top of the monolayer of type-1 AS were isolated and cultured in 10% FCS in the virtual absence of type-1 AS, most of the precursors differentiated into GFAP+ type-2 AS within 3 days (Fig. 5), as occurred in low-density cerebellar cultures (Fig. 1).

Our present findings lend themselves to the hypothesis that in vivo type-1 AS [which during development appear before OL (25)] can positively influence the proliferation and the oligodendroglial differentiation of bipotential glial progenitor cells. If this were the case, one should perhaps reconsider the widely accepted concept that, in demyelinating diseases, reactive AS represent only an obstacle to remyelination (31). In fact, reactive AS, which appear to belong to the type-1 AS cell lineage (32), might provide mitogenic factors capable of expanding the population of glial precursors present in the adult brain (33), and also facilitate their differentiation into mature OL.

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