Radial glial cells derived from the neonatal rat spinal cord: morphological and immunocytochemical characterization*

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Summary. Radial glial cells are transiently bipolar cells in the developing central nervous system, best known for their role in guiding migrating neurons. The aim of the present study was to investigate phenotypic characteristics of these bipolar precursor cells in a mixed glial cell culture system derived from the rat neonatal spinal cord. Morphological characterization was assessed by cell-specific immunocytochemical markers (nestin, vimentin, 3CB2) and transmission electron microscopy. Our study yielded substantial evidence showing that the bipolar cells exhibit immunocytochemical and ultrastructural features of radial glial cells. Immunohistochemistry of the neonatal rat spinal cord using the same cell-specific markers suggested these cells are likely derived from the subependymal zone, ventral commissure, and dorsomedial septum. We believe our data recommend this mixed glial culture system to be a valuable tool in studying radial glial cells *in vitro*.

Introduction

Radial glial cells, transient cells in the developing central nervous system (CNS), are known to be involved in guiding

migrating neurons (Rakic, 1971). Postnatally, after neuronal generation and migration are completed, they transform into astrocytes (Voight, 1989; Edwards *et al.*, 1990). Initially, radial glia were thought to act as glial progenitors. However, recent studies demonstrated that radial glia are also neural precursor cells (Malatesta *et al.*, 2000; Hartfuss *et al.*, 2001). In addition, evidence suggests that radial glial cells are involved in the onset of myelination and play important roles after CNS injury (Nakahara *et al.*, 2003; Shibuya *et al.*, 2003; Hasegawa *et al.*, 2005).

Radial glial cells are morphologically characterized by their bipolar morphology. They share molecular similarities both with neuro-epithelial cells and astrocytes. Primate radial glial cells express both the neural precursor cell marker nestin (Hockfield and McKay, 1985) and the astrocytic cell marker glial fibrillary acidic protein (GFAP) (Levitt and Rakic, 1980), whereas in rodents they express the neural precursor markers nestin and RC2 (Misson *et al.*, 1988), but lack GFAP expression (Pixley and de Vellis, 1984). However, these rodent radial glia express other astrocyte cell markers such as the glutamate aspartate transporter (GLAST) (Shibata *et al.*, 1997), vimentin (Dahl *et al.* 1981), and brain lipid binding protein (BLBP) (Feng *et al.*, 1994). Moreover, rodent radial glial cells can be identified by the use of more specific immunocytological markers such as RC1 (Edwards *et al.*, 1990) and 3BC2 (Prada *et al.*, 1995).

Functionally, radial glial cells are not a uniform cell type (Hartfuss *et al.*, 2001). Their origin and the mechanisms underlying their transformations to astrocytes or neurons remain elusive, especially in the spinal cord. Though they contribute to different cell lineages, they do so in apparently restricted lineages from distinct regions of the developing CNS (Li *et al.*, 2004).

The aim of this study was to morphologically characterize the bipolar precursor cells in primary mixed glial cell cultures. Cultures were obtained from neonatal

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rat spinal cords using a procedure originally described by Van der Pal *et al.* (1990) which was slightly modified. Also demonstrated was their *in situ* histological correlate, using cell-specific immunocytochemical markers and transmission electron microscopy (TEM).

Materials and Methods

Animals and materials

Wistar rats at 16–18 days of gestation were purchased from Harlan CPB (Zeist, The Netherlands). Dulbecco's Modified Eagle's Medium (DMEM), L-glutamine, penicillin/streptomycin, trypsin, DNase, and transferrin were obtained from Gibco BRL (Life Technologies Paisley, Scotland). Fetal bovine serum (FBS) was supplied by HyClone (Utah, USA). All other chemicals were purchased from Sigma (St. Louis, MO, USA).

Mixed glial cell cultures

Mixed glial cell cultures enriched for mature OLGs were prepared from 3 day old rat spinal cords as described by Van der Pal *et al.* (1990), with slight modifications. The spine was cut above the tail, a syringe with Hanks Balanced Salt Solution was inserted, and the spinal cord was pushed out by hydraulic pressure. After dissection, spinal cords were dissociated mechanically and enzymatically with

trypsin (0.4%) in the presence of DNase (100 $\mu\text{g}/\text{ml}$) for 30 min at 37°C in a shaking incubator. After trypsin inhibition and gentle trituration, the cell suspension was subsequently sieved through a 100 μm and a 30 μm mesh respectively. The single cell solution was suspended in DMEM containing 5% heat inactivated FBS and seeded at a density of 5×10^4 cells onto glass coverslips coated with 10 $\mu\text{g}/\text{ml}$ poly-L-lysine for immunocytochemistry and at a density of 1×10^5 cells on plastic coverslips (Thermanox[®], Electron Microscopy Sciences) coated with 5 $\mu\text{g}/\text{ml}$ poly-L-lysine for transmission electron microscopy. After 1 h, non-adhering cells were removed and the culture medium was replaced. After 24 h, the culture medium was switched to a serum-free, chemically defined medium (CDM, Van der Pal *et al.*, 1990) to induce the differentiation of progenitor cells into mature myelin-forming OLGs. Twenty-four h later, 5 μM cytosine-1-beta-D-arabinoside (Ara-C) was added to the medium to prevent any overgrowth of astrocytes. After 2 more days, the medium was replaced by CDM with 0.5% FBS and further changed every two days. Cells were fixed 6–7 days after plating.

Immunocytochemistry

Immunocytochemical stainings were performed using the peroxidase-based Envision System[®] (DakoCytomation, Glostrup, Denmark) as described by Vandenabeele *et al.* (2003) with some minor modifications.

Briefly, cells were fixed in 4% formaldehyde (Unifix,

Table 1. Markers used to identify cells.

Antibodies	Species	Dilution	Target neuronal type	Source
A2B5	Mouse IgM	1:200	OLG progenitors	Chemicon, USA
5A5*	Mouse IgM	1:100	Neuronal precursors	DSHB, USA
3CB2**	Mouse IgM	1:100	Radial glia	DSHB*, USA
CD11b	Mouse IgM	1:40	Microglia	Serotec, UK
GFAP	Mouse IgG1	1:200	Mature astrocyte	Novocastra, UK
MBP	Mouse IgG2a	1:200	Mature OLG	Serotec, UK
Nestin	Mouse IgG1	1:2000	Neural stem cells	Chemicon, USA
Neurofilament	Mouse IgG1	1:200	Neurons	Novocastra, UK
NG2	Rabbit polyclonal	1:200	OLG progenitors	Chemicon, USA
Vimentin	Mouse IgG1	1:500	Immature astrocyte	Dako, Denmark

DSHB: Developmental Studies Hybridoma Bank. * Developed by T. M. Jessell and J. Dodd and obtained from the DSHB developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, USA.

**Developed by E.J. De la Rosa and obtained from the DSHB.

Fig. 1. Oligodendrocyte enriched mixed glial culture system containing 4 different cell types. **a:** MBP-positive oligodendrocytes, **b:** Nestin-positive bipolar cells, **c:** GFAP-positive astrocytes, **d:** CD68+ microglia. Bars=50 μ m (a, b), 20 μ m (c, d)

Duiven, The Netherlands) for 20 min. After washing in 0.01 M phosphate buffered saline (PBS) (pH 7.2), they were permeabilized with 0.05% Triton X-100 (Boehringer, Mannheim, Germany) in PBS for 30 min for intracellular staining, and washed again. Non-specific binding sites were subsequently blocked with 3% normal goat serum (NGS) (DakoCytomation) in PBS for 20 min. After washing, the cells were incubated for 1 h with the primary antibodies (see Table 1; page 15), washed again, and incubated for 30 min with a peroxidase labelled polymer conjugated to a goat anti-mouse secondary antibody. A diaminobenzidine (DAB) chromogenic substrate system and Mayers' hematoxylin were respectively used to visualize the immunoperoxidase and for counterstaining. After mounting in an aqueous mounting medium (Aquatex, Merck, Darmstadt, Germany), cell-seeded coverslips were examined using a photomicroscope equipped with an automated camera (Nikon Eclipse 80i, Nikon Co., Japan).

Immunohistochemistry

Paraffin sections

Vimentin and nestin immunostaining were performed on paraffin sections. The spinal cords of 3-day-old rats were removed, preserved overnight in 4% formaldehyde, then put through a dehydrating series of graded concentrations of alcohol, and finally embedded in paraffin. Paraffin sections (5–8 μ m) were attached to poly-L-lysine coated glass slides. Sections were deparaffinized with xylol, followed by decreasing concentrations of alcohol, and then washed in PBS. Prior to immunohistochemical staining, antigen retrieval was performed (sections were microwaved in a 10 mM citrate buffer at pH 6.0), and endogenous peroxidase activity was quenched with 0.5% H_2O_2 . Non-specific binding sites were blocked with 3% NGS. Subsequently, the sections were stained with mouse monoclonal antibodies against vimentin or nestin (see

Table 1) for 1 h. The sections were next incubated with a peroxidase labeled polymer conjugated to a goat anti-mouse secondary antibody for 30 min (Envision System[®], DakoCytomation). Immunoreactivity was visualized with DAB. The sections were counterstained with hematoxylin, coverslipped with an aqueous mounting medium (Aquatex, Merck), and examined using a photomicroscope equipped with an automated camera (Nikon Eclipse 80i, Nikon Co.).

Frozen sections

3CB2 immunostaining was performed on frozen sections. Briefly, spinal cords of 3-day-old rats were removed and immersed in liquid nitrogen-chilled isopentane (Fluka Chemistry, Buchs, Switzerland) and frozen in liquid nitrogen. Afterwards they were cryoprotected in a 30% sucrose solution. Seven–ten μ m cryosections were cut and mounted onto poly-L-lysine coated glass slides. The sections were post-fixed in acetone for 10 min and dried for 30 min. The sections were subsequently blocked with 3% NGS and stained for 3CB2 (see Table 1) using the same protocol as described above.

Transmission electron microscopy (TEM)

For examination by TEM, cells were fixed overnight in a solution containing 2% glutaraldehyde in a 0.05 M cacodylate buffer (pH 7.3) at 4°C, postfixed in 2% osmium tetroxide for 1 h, stained with 2% uranyl acetate in 10% acetone for 20 min, put through a dehydrating series of graded concentrations of acetone, and embedded in epoxy resin (Araldite) according to the pop-off method (Bretsneider *et al.*, 1981). Ultra-thin sections (0.06 μ m) were mounted on 0.7% formvar coated grids, contrasted with uranyl acetate followed by lead citrate, and examined in a Philips EM 208 transmission electron microscope operated at 80 kV.

Fig. 2. Morphological and immunocytochemical characterization of bipolar cells in an oligodendrocyte enriched mixed glial culture system (7div). **a:** phasecontrast micrograph showing a cluster of bipolar cells within the culture system. Bipolar cells show immunoreactivity for nestin (**b**), vimentin (**c**), and 3CB2 (**d**). No immunoreactivity was observed for A2B5 (**e**), NG2 (**f**), neurofilament (**g**), or PSA-NCAM (**h**). Bipolar cells generally do not stain for GFAP. Note the presence of strong GFAP positive astrocytes within the culture system (arrows) (**i**). GFAP immunoreactivity is occasionally observed (**j**) within bipolar cells (arrows). Bars=50 μ m (**a**, **b**, **c**), 20 μ m (**d**, **e**, **f**, **g**, **h**, **i**, **j**)

Fig. 3. Transmission electron micrographs of bipolar cells. **a:** Bipolar cells have elongated and often indented euchromatic nuclei with a prominent nucleolus. **b** and **c:** High power electron micrographs showing details of the cytoplasm. Cells contain dilated RER (arrow) as shown in **b**. Sometimes a cilium is observed (arrow) as shown in **c**. **d:** Detail of a cell process containing microtubuli and intermediate filaments, ribosomes, and an elongated mitochondrion. Bars = 10 μ m (a), 2000 nm (b), 1000 nm (c, d)

Results

In vitro characterization of bipolar precursor cells

After 6 days *in vitro* (div), the mixed glial cell culture included 4 different cell types: 55% MBP+OLGs (Fig. 1a), 30% nestin+bipolar cells (Fig. 1b), 15% GFAP+astrocytes (Fig. 1c), and <1% CD68+microglia (Fig. 1d) (Moreels *et al.*, submitted). The percentage of nestin+bipolar cells at 6 div was comparable to that observed at 1 div. At 1 div, about 40% of cells showed nestin immunoreactivity. Also observed in addition to bipolar cells were nestin+astrocytes and oligodendroglial lineage cells.

Morphologically, nestin positive bipolar precursor cells were easily recognized by their long and fine, unbranched cell processes and a small, elongated cell body.

Additionally, some nestin positive cells showed a tripolar morphology. The cell processes extended up to 150 μ m and occasionally possessed broadened end-foot terminations. Bipolar cells were often clustered in cultures (Fig. 2a).

Cells were further characterized by the use of immunocytochemistry and TEM. In addition to nestin immunoreactivity (Fig. 2b), bipolar cells showed a specific immunoreactivity for vimentin and 3CB2 (Fig. 2c, d). They did not stain for A2B5, NG2 (Fig. 2e, f), neurofilament (Fig. 2g), or 5A5 (PSA-NCAM) (Fig. 2h). In general, bipolar cells were GFAP negative (Fig. 2i) although some cultures contained few GFAP positive bi/tripolar cells (Fig. 2j).

Figure 3 gives an overview of the most important ultrastructural characteristics of the cells. Bipolar cells had an ovoid euchromatic nucleus with a rim

Fig. 4. Legend on the opposite page.

of heterochromatin beneath the nuclear envelope in addition to prominent nucleoli. Their nuclei were often indented (Fig. 3a, b). Their cytoplasm contained a well-developed Golgi-apparatus, small elongated mitochondria, and numerous lysosomes. The rough endoplasmatic reticulum (RER) showed dilated cisternae containing material of moderate electron density (Fig. 3b). Sometimes a cilium arising from one member of a centriolar pair was observed (Fig. 3c). The cell processes were relatively electron lucent and contained a network of intermediate filaments, numerous microtubules directed parallel to the long axis of the cell process, strikingly long mitochondria, and ribosomes (Fig. 3d).

In situ characterization of bipolar precursor cells in the neonatal spinal cord

In order to immunolocalize radial glial cells *in vivo*, the distributions of the cytoskeletal proteins nestin, 3CB2, and vimentin were analyzed in the rat neonatal spinal cord (P3).

Strong nestin staining was observed in the dorsomedial septum and cells surrounding the ventral commissure. Moderate nestin immunoreactivity was confined to radial fibers both in the grey and white matter of the neonatal spinal cord (Fig. 4a, b). Nerve roots also showed strong nestin immunoreactivity (Fig. 4c).

3CB2 staining revealed radially oriented bipolar cells extending from the pial surface, especially in the ventrolateral white matter. Strong 3CB2 labeling could be observed in subependymal cells, the ventral median fissure, and the dorsomedial septum (Fig. 4d, e). No staining was observed in the nerve roots (Fig. 4f).

Vimentin immunoreactivity was most pronounced and could be detected in ependymal cells and cells surrounding the developing ventral commissure in the neonatal spinal cord. This immunoreactivity was most pronounced in the grey matter. Astrocyte cell processes and end-feet stained intensively. Nerve roots and meninges also showed vimentin staining (Fig. 4g, h, i).

Discussion

The aim of study was the morphological characterization of the nestin positive bipolar cells present in mixed glial cultures derived from the rat neonatal spinal cord by the use of immunocytochemistry and TEM. Additionally, we demonstrated their morphological correlate *in situ*.

Primary cultures were prepared according to the method described by Van der Pal *et al.* (1990), who used 7-day-old rats to obtain an oligodendrocyte-enriched (90%) culture system. In contrast, our cultures were derived from 3-day-old rats which resulted in a mixed glial cell culture system containing oligodendrocytes (55%), radial glial cells (30%), and astrocytes (15%). The age difference (7 days vs. 3 day old rats) might explain the presence of radial glial cells in our culture system as previous studies demonstrated that the numbers of these cells decrease significantly during the first week in the neonatal rat spinal cord (Barry and McDermott, 2005).

About 30% of cells in this culture system showed morphological features typical of radial glial cells (Hockfield and McKay, 1985; Culican *et al.*, 1990; Sancho-Tello *et al.*, 1995). Our immunocytochemical analysis was consistent with their radial glial cell phenotype: e.g. all bipolar cells showed immunoreactivity for nestin, vimentin, and the more specific radial glial cell marker 3CB2. No immunoreactivity for A2B5 and NG2—two markers typically expressed by O-2A progenitors—could be observed (Fredman *et al.*, 1984; Nishiyama, 2001). The absence of neurofilaments and PSA-NCAM excluded the possibility that the bipolar cells were either neurons or neuronal precursors, respectively. This is in agreement with the study by Barry and McDermott (2005) in which they suggested that radial glia in the rat spinal cord are most likely restricted to the astrocyte lineage.

The bipolar cells in our culture system resembled the ultrastructural features of radial glial cells described previously (Hinds and Ruffett, 1971; Tramontin *et al.*,

Fig. 4. *In situ* localization of radial glial cells in the neonatal spinal cord. **a:** Moderate nestin staining was observed in radial fibers in the grey matter and the dorsomedial septum. **b:** Detailed view of nestin positive bipolar cells in the dorsal and ventral subependymal zone. **c:** Nestin staining is detected within the nerve roots. **d:** 3CB2-immunoreactivity in the dorsomedial septum, ventral commissure, and radial fibers extending from the ventral pial surface. **e:** Detailed view of 3CB2-positive subependymal cells. **f:** No 3CB2 staining is detected in the nerve roots. **g:** Vimentin expression within the grey matter. **h:** Detailed view of vimentin expression in ependymal cells and the ventral and dorsal subependymal zone. **i:** Vimentin staining is observed in the nerve roots. Bars=200 μ m (a, d, g), 50 μ m (b, e, h), 20 μ m (c, f, i).

2003). Radial glial cells are ultrastructurally characterized by abundant RER and an extensive network of 24-nm microtubuli and 9-nm intermediate filaments, both in the cell body as well as in the cell processes. Moreover, radial glial cells possess one cilium, with a typical 9+0 organization.

Unlike primates, rodent radial glial cells do not contain GFAP (Pixley and de Vellis, 1984). Occasionally, we observed weak GFAP staining in some bi/tripolar cells. Recently, an *in vivo* study revealed that, after birth, radial glial cells are the major source of astrocytes in the rat spinal cord (Barry and McDermott, 2005). Furthermore, radial glial *in vitro* have the capacity to transform into astrocytes (Culican *et al.*, 1990; Sancho-Tello *et al.*, 1995). We propose that these GFAP expressing bipolar cells are a transitional cell type between radial glial cells and mature astrocytes. Interestingly, the majority of radial glial cells within our culture system maintained their bipolar morphology and did not transform into astrocytes (even after longer culture periods). This observation is consistent with the finding that radial glial cells transform into astrocytes *in vitro* in the presence of neurons (Culican *et al.*, 1990). Obviously, since there are no neurons present in our culture system, the transformation of radial glial cells towards astrocytes seems to be suppressed.

The expression pattern of nestin, vimentin, and 3CB2 in spinal cords from 3-day-old rats was similar to that observed previously by others (Yang *et al.*, 1993; Barry and McDermott, 2005). Nestin, vimentin, and 3CB2 were all predominantly present in the subependymal zone, the dorsomedial septum, and the ventral commissure, suggesting that the radial glial cells *in vitro* originate from cells located within these regions.

Our study yielded substantial evidence that the bipolar cells show immunocytochemical and ultrastructural features of radial glial cells. Immunohistochemistry of the neonatal rat spinal cord using the same cell-specific markers suggests these cells are likely derived from the subependymal zone, ventral commissure, and dorsomedial septum. Previous studies revealed that radial glial cells might play a role after CNS injury. Shibuya *et al.* (2003) demonstrated the emergence of radial glia after spinal cord injury and suggested their involvement in neural repair and regeneration. Acutely transplanted radial glia promote functional recovery following spinal cord contusion (Hasegawa *et al.*, 2005). *In vitro* studies have shown that the transformation of radial glial cells into astrocytes is bidirectional (Hunter and Hatten, 1995; Zhou *et al.*, 2001). It was recently demonstrated that a subpopulation of reactive astrocytes re-express the radial glial antigens RC-1 in the demyelinated adult rat spinal cord (Talbot *et al.*, 2005). Further characterization of radial glial cells

will be essential to elucidate their role during spinal cord development and injury. From a morphological point of view, our mixed glial cell culture system could provide a suitable model for studying radial glial cell biology and cellular interactions between different glial cells including oligodendrocytes, radial glial cells, and astrocytes.

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