Myelin-forming Oligodendrocytes of Developing Mouse Spinal Cord: Immunocytochemical and Ultrastructural Studies


Abstract. The development and differentiation of oligodendrocytes (OC) in developing mouse spinal cord (MSC) were investigated by correlative analysis of light and electron microscopy (EM), and immunoperoxidase studies for glial fibrillary acidic protein (GFAP) and myelin basic protein (MBP). The sequential development of glial cells within the subpial region of the MSC bears a striking resemblance to the developing human fetal spinal cord. A rise in the level of mitotic activity among subpial astrocytes just prior to the onset of myelination was followed by the appearance of OC within the same region. The finding of "transitional" cells with the cytological and ultrastructural features of both astrocytes and OC and the finding of GFAP within the immature OC strongly support the hypothesis that OC in the developing central nervous system may arise from astroglial precursors. These observations also suggest that the MSC may be a suitable model for the study of OC differentiation and myelogenesis in man.

Key Words: Astrocyte; Electron microscopy; Glial fibrillary acidic protein; Gliogenesis; Immunoperoxidase stain; Mouse spinal cord; Oligodendrocyte.

INTRODUCTION

The study of gliogenesis in the developing central nervous system (CNS) was significantly advanced when electron microscopy (EM) and radioautography were first applied by Leblond and his co-workers (1–5). These investigators carefully correlated the images of glial cell nuclei in toluidine blue-stained semithin sections with those observed ultrastructurally, and identified cells undergoing DNA synthesis with the aid of tritiated thymidine radioautography. In a careful study of gliogenesis in the rat optic nerve, Skoff et al (6, 7) also applied EM and radioautographic techniques. They showed that immature cells that are sufficiently well-differentiated to permit their identification as astroglial or oligodendroglial cells, are still capable of incorporating tritiated thymidine. Their data suggested early differentiation of both glial cell types during development. However, neither the timing of this differentiation nor the nature of the cells from which astrocytes or oligodendrocytes (OC) originate has been clearly defined. More recently, the application of immunocytochemical techniques using conventional and monoclonal antibodies has greatly improved our ability to identify different cell types. These techniques have also contributed to our understanding of the glial cell lineage in the developing CNS (8). In spite of intensive efforts, however, the origin and mode of differentiation of OC remain major unresolved questions in neurobiology (9, 10).

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In previous studies, we presented evidence to suggest that in the developing human fetal spinal cord astrocyte-specific glial fibrillary acidic protin (GFAP) is present within radial glial cells by eight to ten weeks of ovulation age. We also suggested that these cells are the first ones of astroglial lineage that can be distinguished among the germinative cells in the ventricular zone and that some of them undergo transformation into astroglial cells (11). We have also shown that, just prior to the onset of myelination, "transitional" cells possessing the cytological and ultrastructural features of both astroglial and oligodendroglial cells appear. Light and EM immunoperoxidase studies also indicated that immature OC express GFAP (12, 13). Based on these findings, we proposed that the myelin-forming OC may be derived from precursor cells of astroglial lineage.

In order to gain further insight into the development and differentiation of OC and to determine whether or not our observations could be extended to other animal species, we have carried out correlative immunocytochemical and EM studies of the subpial region of the developing mouse spinal cord (MSC) during early myelination.

MATERIALS AND METHODS

C57BL/6J mice were mated at four-hour intervals to obtain precisely timed pregnant animals. The spinal cords were removed at varying ages ranging from embryonic day (E-) 9 to postnatal day (P-) 6. Perfusion with Karnovsky's solution was carried out in postnatal animals, but spinal cords from embryonic mice were immersion-fixed. Representative sections from the cervical, thoracic and lumbar regions were embedded in epon. Thin sections were cut with a diamond knife on a Sorvall MT 5000 Ultratome, stained with uranyl acetate and lead citrate and examined with a Philips EM 400 electron microscope.

Immuno peroxidase procedures as described by Sternberger (14) for myelin basic protein (MBP) and GFAP were carried out on de-eponized 1 μm sections and vibratome sections (10-30 μm). Thin sections prepared from epon-embedded vibratome sections to which GFAP immunoperoxidase staining had been applied were also examined. The dilution of primary antiserum was 1:500. The incubation period in primary antiserum varied from 18 to 48 hours, at 4°C. Reaction product was developed in a 0.025% solution of 3,3'-diaminobenzidine tetrahydrochloride in 0.05 M Tris buffer (pH 7.5) for five to ten minutes. All antibody steps included 5% fetal calf serum to block non-specific staining. The GFAP antiserum included our own, some provided by Dr. Larry Eng and some purchased from Dako, C. del Cerro of the Center for Brain Research, University of Rochester kindly supplied MBP antiserum.

RESULTS

The findings described in this report are derived from observations made within the ventral columns of the cervical region of the developing MSC. As shown in Figure 1, the subpial region of the developing MSC was initially relatively cell-free. By E-16, however, cells with relatively large pale nuclei began to accumulate beneath the pial surface (Fig. 2). These cells were identifiable as astrocytes both ultrastructurally and by light and EM immunoperoxidase staining for GFAP. The glia limitans (GL) at the pial surface, as well as the cytoplasm and processes of these cells, were strongly GFAP-positive. By P-1, scattered myelin sheaths could be seen (Fig. 3) near the pial surface. Myelin basic protein-positive myelin sheaths were closely associated with the subpial cells. By P-2, myelin sheaths were present in great abundance, and the majority of the subpial cells were identifiable by previously established criteria (1-7) as OC (Figs. 4, 5). Subpial OC were widely distributed but some extended long processes that were attached to the pia mater (Fig. 5A and B).

The pattern of GFAP immunoperoxidase staining in subpial OC at P-2 are shown in Figure 5C, D and E. Figure 5C shows a subpial OC with a GFAP-positive process

Fig. 1. Cell-free subpial region of the mouse spinal cord (MSC) at embryonic day (E-) 13. The region is occupied by cell processes (empty arrow), including those of radial glia. PM: pia mater. 1 μm section. Toluidine blue. ×280.

Fig. 2. Accumulation of cells with relatively large nuclei (thick arrows) in the subpial region of MSC at E-17. The astroglial nature of these cells was established by their strong immunoreactivity for GFAP both at the light and the EM levels (not shown). 1 μm section. Toluidine blue. ×560.

Fig. 3. Scattered MBP-positive myelin sheaths (empty arrows) in the subpial region of MSC at postnatal day (P-) 1. 1 μm section. MBP immunoperoxidase stain with toluidine blue counterstain. ×280.

Fig. 4. Abundant MBP-immunoreactive myelin sheaths (empty arrows) in the subpial region of MSC at P-2. The majority of the subpial cells at this age exhibit oligodendroglial morphology by conventional criteria. 1 μm section. MBP immunoperoxidase stain with toluidine blue counterstain. ×280.

extending to the GFAP-positive GL at one end. The perinuclear cytoplasm is also GFAP-positive. At the other end the GFAP-positive process branches and appears to be associated with myelin sheaths. Some OC showed GFAP immunoreactivity only in parts of the processes while the bulk of the cytoplasm remained GFAP-negative (Fig. 5D and E).
Fig. 5. A. Subpial cells of MSC at P-2. A myelin-forming OC (O) extends one of its processes (arrow) to the GL (thick arrow) of the PM while other processes appear to form myelin sheaths (arrowheads). The majority of the subpial glial cells at this age show oligodendroglial morphology. 1 µm section. Toluidine blue. ×2,800. B. Subpial OC (O) with extended processes (arrows) attached to the GL (thick arrows) of the (PM). On the right of the panel, the process of the cell with myelin enclosed within the cytoplasm appears to have broken away (arrowhead) from the PM. MSC at P-2. 1 µm section. Toluidine blue. ×2,800. C. A subpial OC (O) with GFAP-positive cytoplasm and processes (arrows) of MSC at P-2. The GFAP-positive process appears to extend into myelin sheaths (arrowheads) in the lower part of the panel. The GL (thick arrow) at the pia mater (PM) is also GFAP-positive. 1 µm section. GFAP immunoperoxidase stain with toluidine blue counterstain. ×2,900. D. Immunoperoxidase staining of
By EM, the cytoplasm or processes of astrocytes in the subpial region extended into and contributed to the formation of the GL (Figs. 6, 7). These cells contained bundles of filaments in addition to Golgi cisterns, mitochondria and ribosomes. Occasional mitotic figures were found among them (Fig. 7).

The cells that contributed to the formation of the GL had filament-containing processes that encircled axons in a spiral fashion (Figs. 8, 9). By P-2, many of the subpial glial cells were clearly identifiable as OC on the basis of standard light and EM criteria (1-7). Figure 10A illustrates a subpial OC at P-3 in the lower left part of the panel. It contains a relatively electron-dense nucleus and cytoplasm that is rich in ribosomes, rough endoplasmic reticulum and mitochondria. The extended processes of this cell form compact myelin sheaths. The cell in the lower right part of the panel of the same figure exhibits ultrastructural characteristics similar to those of the OC just described. The nucleus and the cytoplasm are electron-dense and the cytoplasm contains an abundance of ribosomes, rough endoplasmic reticulum, mitochondria and dense bodies. Many axons at the periphery of this cell are encircled by the cytoplasmic extensions, some show a spiral configuration that is strongly suggestive of early myelin sheath formation (Fig. 10C). The extended process of this cell, however, contains bundles of filaments within the matrix (Fig. 10B) and contributes to the formation of the GL at the pial surface. This cell, therefore, possesses the ultrastructural characteristics of both astrocytes and OC. The continuity of the process with the cell body is depicted at higher magnifications in Figures 11A and B.

Vibratome sections prepared for EM showed strong GFAP immunoreactivity of the filamentous profiles within the matrix of the GL (Fig. 12A). The cytoplasm of subpial astrocytes also contained GFAP immune reaction product (Fig. 12B).

DISCUSSION

The sequential development of glial cells within the subpial region of the MSC bears a striking resemblance to that observed within the developing human fetal spinal cord (12, 13). In the study of events taking place during and immediately before the onset of myelination in the developing human spinal cord, the MSC may be a suitable model. Since a similar sequence has also been described in studies of rat and human optic nerves by other investigators (6, 7, 15, 16), there appears to be a common pattern of oligodendroglialogenesis in the developing CNS. To recapitulate, a rise in the rate of mitotic activity among subpial astrocytes just prior to the onset of myelination is followed rather abruptly by the appearance of myelin-forming OC within the same region. These observations, coupled with the findings of "transitional" cells with cytological and ultrastructural features of both astrocytes and OC and with the finding of GFAP within the processes of immature OC, strongly suggest that OC are derived from astrogial precursors.

It has been postulated that the subpial glial cells or "glioblasts," which allegedly give rise to both astrocytes and OC, are derived from matrix cells that have migrated to the periphery of the spinal cord (17). Our immunocytochemical and EM studies,

GFAP in MSC at P-2. Scattered OC (O) in the subpial region bear the GL (thick arrow) and GFAP-positive processes (arrows). Immunoperoxidase stain. 1 μm section. ×2,900. E. Strong GFAP immunoreactivity within the bifurcated process (arrows) of an OC (O) while the bulk of the cytoplasm remains GFAP-negative. Note also the GFAP-positive GL (thick arrow). Immunoperoxidase stain, toluidine blue counterstain. MSC, P-2. 1 μm section. ×2,900.
Fig. 6. A subpial astrocyte of MSC at P-1. The cytoplasm of this cell closely abuts the basal lamina (thick arrows) of the PM. In addition there is an abundance of filaments (arrowheads), Golgi cisterns (G), rough endoplasmic reticulum and mitochondria. ×24,750.

Fig. 7. A subpial astrocyte in mitosis. Note the prominent centriole in the cytoplasm. This cell makes up part of the GL, closely abutting the basal lamina (thick arrows) of the PM. Bundles of glial filaments are also present within the cytoplasm (arrowheads). MSC, P-1. ×18,750.
Fig. 8. A subpial glial cell of MSC at P-3. The cytoplasm contains abundant filaments (arrows). The cytoplasmic extensions of the cell (empty arrows) enwrap an axon (A). The basal lamina (arrowheads) of the PM is closely apposed to the cytoplasm of the cell. × 34,650.

Fig. 9. A subpial glial cell of MSC at P-2. The matrix of the cell contains filaments (arrows) and the extended cytoplasmic processes (empty arrows) encircle an axon (A) in a spiral fashion. The arrowheads point to the basal lamina of the PM. × 43,750.

indicate that the cells that accumulate initially in the subpial region of the developing spinal cord already possess the characteristics of astroglial cells. Nagashima (18), in his study of neonatal rat spinal cord, observed the wrapping of axons by extended astroglial processes, and suggested that subpial glial cells might have the potential to differentiate into both astroglial and oligodendroglial cells. Although he dismissed the possibility of myelin formation by astrocytes on the basis of peculiarities in the configuration of the wrapping process, his illustrations show participation on the part of astroglial processes in what appear to be the early stages of myelin formation.

The possible role of cells with astroglial features in the formation of myelin sheaths within the developing CNS has been suggested by other investigators (19, 20). Immunocytochemical studies by Takashima and Becker (21) and Borit and McIntosh (22) indicated that “myelination glia” are frequently GFAP-positive. In a series of in vitro studies using cells derived from developing rat optic nerve, Raff and his colleagues reported the finding of a common progenitor cell for both astrocytes and OC and demonstrated the presence of cells showing immunoreactivity for GFAP and galactocerebroside (8, 23, 24). Recently, the presence of “bipotential” or “transitional” glial cells that express both OC and astrocytic phenotypes has been reported by Kim et al (25) in cultures of adult human OC. A monoclonal antibody that binds
Fig. 10. Composite EM. Subpial glial cells of MSC at P-3. The cell in the left lower part of the panel is relatively electron-dense and its cytoplasm contains rough endoplasmic reticulum, mitochondria and ribosomes. The extended processes of this cell encircle axons (A) and form compact myelin. A similar subpial cell in the right lower part of the panel also contains numerous mitochondria, ribosomes and rough endoplasmic reticulum. The extended process of this cell forms part of the GL closely abutting the basal lamina (large arrowheads) of the PM. The matrix of the process contains bundles of filaments (arrows) which are depicted at a higher magnification in Figure 10B. The same cell is shown to form what appears to be an early myelin sheath which is shown at a higher magnification in Figure 10C. Small arrows point to axons encircled by cytoplasmic extension of this cell. A: axon. 10A: ×4,970; 10B: ×36,500; 10C: ×38,700.
Fig. 11. A. Higher magnification of the subpial glial cell that is depicted in the lower right part of the panel in Figure 10A. The process forms part of the GL, and contains filaments (arrows) within its matrix, which abuts the basal lamina (arrowheads) of the PM. MSC, P-3. ×14,200. B. Enlargement of the enclosed area within Figure 11A, to show continuity of the process with the cell soma. The large arrowheads point to the cell membrane where the process originates from the cell body. The content and character of the matrix of the process are similar to those of the cell soma. The electron-dense matrix contains scattered ribosomes (empty arrows) and filamentous profiles (arrows). MSC, P-3. ×74,000.
Fig. 12. A. Strong GFAP immunoreactivity in the GL of the PM. Note filamentous content of the GFAP-positive GL (arrowheads). The basal lamina (empty arrows) closely abuts the GL. Mitochondria are numerous, large and elongated and are GFAP-negative. Immunoperoxidase EM. MSC at P-1. x17,000. B. A subpial astrocyte of MSC at P-1. Note the basal lamina (small arrow) hugging the cell. The cytoplasm is strongly immunoreactive for GFAP (large arrows). Numerous GFAP-negative mitochondria are present in the cytoplasm. Immunoperoxidase EM. x20,250.
to both astrocytes and myelin sheaths has also been reported by Dumas et al (26). Lanqui et al (27) reported the presence of both carbonic anhydrase isoenzyme II (specific for OC) and GFAP in immature rat glial cells in primary culture. All of these observations suggest that a close, dynamic relationship exists between astrocytes and OC during development as well as later in life. Myelin appears to be formed solely by OC and although there is no evidence to indicate that astrocytes form myelin, the expression of GFAP by immature OC may be taken as evidence to suggest that myelin-forming OC may originate from astroglial precursors.

Since the development and differentiation of OC and myelin sheaths progress rapidly in the developing MSC, the time period during which the observations are made is of critical importance in the study of the earliest stages of myelin formation. In the ventral columns of the cervical regions of the MSC, for example, the formation of myelin sheaths is already so extensive by P-5 that little can be learned of the cells that give rise to myelin-forming OC. The critical period for the demonstration of the "transitional" cells that we have described in the developing MSC within the same region appears to be between P-2 and P-3.

The identification of precursor cells for neurons and glial cells in the developing CNS has been extremely difficult because of morphological similarities among the cells within the proliferative zones of the embryonic neural tube. The introduction of immunocytochemical methods has been a significant advance, and has proven to be a valuable adjunct to the traditional morphological techniques used for the identification of cell types. These procedures require isolation and characterization of specific markers for any given cell type. Although a number of markers are purported to be specific for various CNS cell types, GFAP has proven to be the most consistent and reliable marker for cells of astroglial lineage. The immunocytochemical demonstration of GFAP within immature OC, and the demonstration of "transitional" cells with the morphological characteristics of both astrocytes and OC in the developing MSC, strongly suggest that OC within the developing CNS arise along the same pathway as that which leads to astroglial differentiation.

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