Progenitor Cell Proliferation Outside the Ventricular and Subventricular Zones during Human Brain Development

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Abstract. The ventricular zone (VZ) and subventricular zone (SVZ) of the fetal brain are generally believed to give rise to all the neurons and glia that will populate the cerebral hemispheres. In rodents a mitotically active cell (progenitor cell) has been identified outside the VZ and SVZ, in the intermediate zone (IZ) of the cerebral hemisphere. The cell types that arise from these progenitor cells remain uncertain. We have set out to determine if a similar population of mitotically active cells is present in the IZ during human brain development, and to try and define the cell types that arise from this progenitor cell. Using a monoclonal antibody that recognizes proliferating cell nuclear antigen (PCNA), a replication-specific protein, the cerebral hemispheres from 9 human fetal and infant brains between ages of 15 and 38 weeks gestation were studied. PCNA-immunopositive cells were found in the internal capsule and cerebral white matter in approximately equal frequency and rarely in the cerebral cortex between 15 and 20 weeks gestation. In the internal capsule, the number of positive cells decreased by the end of the second trimester; however, a relatively constant number of PCNA-positive cells remained in the cerebral white matter. By the last trimester relatively little staining was found in any of the regions studied. Anti-GFAP immunostaining indicated that at least some of these progenitors were in the glial lineage. These data provide direct evidence that, in addition to ventricular zone proliferation, a population of progenitor cells continue to proliferate within nascent white matter tracts during development.

Key Words: Cell proliferation; Glia; PCNA; Ventricular zone.

INTRODUCTION

Identifying the location where neurons, astrocytes, and oligodendrocytes are generated in the cerebral hemispheres is important for understanding normal brain development and for the interpretation of pathological processes. It is believed that most, if not all, cells which give rise to the cerebral hemispheres are born (defined as undergoing their final mitosis) in the ventricular zone (VZ) and subventricular zone (SVZ) of the lateral ventricles (1, 2). Recently, proliferating cells located outside the VZ and SVZ have been identified in rodents. Whether a similar population of proliferating cells located beyond the VZ and SVZ exists in humans is unknown.

Several lines of evidence have come together indicating that a population of cells remain mitotically active after migrating from the germinal matrix in laboratory animals. For example, injection of 3H-thymidine into the ventricular zone of postnatal rats was found to label cells with the morphologic appearance of oligodendrocytes in nascent white matter tracts (3, 4). These authors proposed that glial progenitors colonize the white matter, where they continue to undergo replication and further differentiation; however, because the 3H-thymidine persists in nonmitotic daughter cells, their hypothesis could not be proven. Similar studies utilizing 3H-thymidine in conjunction with immunostaining methods have provided evidence that a “migrating progenitor” cell leaves the germinal matrix as a largely undifferentiated cell which expresses the oligodendrocyte marker galactosylceramide once positioned in the white matter (5). Most recently, replication-deficient retroviruses which require passage through the cell cycle for integration were injected into adult rat subcortical white matter (6); results showed clusters of labeled cells, indicating the persistence of a proliferating population in the adult white matter.

We have investigated the existence of a mitotically active cell population outside the ventricular and subventricular zones of human infants during development using an antibody to proliferating cell nuclear antigen (PCNA). Our data demonstrate the presence of a population of proliferating cells in the cerebral white matter. Double labeling with antibodies directed against PCNA and glial fibrillary acid protein (GFAP) indicate that at least some of these cells are in the glial lineage.

MATERIALS AND METHODS

Sections of fetal and newborn postmortem brains from the autopsy and OB-GYN surgical pathology departments at the Brigham and Women’s Hospital/Children’s Hospital Medical Center were fixed in buffered formalin, processed in a Technicon tissue processor, and embedded in paraffin. Deparaffinized tissue sections were placed in 10 mM sodium citrate and microwaved in short bursts until the temperature of the buffer reached 90 to 95°C. The slides, in hot buffer, were placed in a room temperature environment and allowed to cool. When the buffer reached 25 to 30°C, the procedure was repeated, for a total of 5 times. Slides were never allowed to dry during these treatments. Sections were incubated at 37°C with 50 to 100 μl of anti-PCNA antisera (Purified mouse monoclonal IgG, diluted 1:50 in phosphate-buffered saline [PBS], Biogenex, San Ramon, CA.), for 30 to 45 minutes (min). Sections were rinsed gently 3 times with PBS and incubated at 37°C with Multilink biotinylated anti-immunoglobulins (undiluted, Biogenex) for 30 min.

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The slides were washed with PBS and then incubated with Streptavidin-linked alkaline phosphatase (Biogenex, diluted 1:20) for 30 min at 37°C. After washing, slides were developed with the Fast Red (Biogenex) chromogen kit and counterstained with Meyer's hematoxylin. Separate slides labeled for GFAP were treated as above, except that the primary antibody used was rabbit anti-glial fibrillary acidic protein (anti-GFAP, polyclonal, Biogenex, diluted 1:3). It was found that slides subjected to antigen retrieval as described could be immunostained for GFAP with no adverse effect on the results. In experiments where double immunostaining with anti-PCA and anti-GFAP was done, the Multilink second antibody could not be used; rather slides treated with mouse anti-PCA were then incubated with anti-mouse immunoglobulins (DAKO, PAP Kit, Carpenteria, CA, undiluted, washed, incubated with Peroxidase anti-peroxidase soluble complex, undiluted), washed, and developed with hydrogen peroxide/chromogen substrate, either DAB or CAE. The slides were then washed, fixed for 1 min in 4% paraformaldehyde, washed with PBS, incubated for 30 min with rabbit anti-GFAP, washed, treated with anti-rabbit IgG, biotin-linked (Sigma Immunochemicals, St. Louis, MO, diluted 1:50) for 30 min at 37°C. The slides were then incubated with Streptavidin-linked alkaline phosphatase (Biogenex, diluted 1:20), washed, developed with Fast Red, and counterstained with Meyer's hematoxylin. Slides were covered with Immumount aqueous mounting media (Shandon, Pittsburgh, PA) and coverslipped.

RESULTS

Nine cases spanning the mid-second trimester to the end of the third trimester were selected and analyzed from the neuropathology files at the Brigham and Women's Hospital and Boston Children's Hospital (Table 1). Criteria for all cases was the presence of well-oriented blocks with high quality histology which lacked postmortem or processing artifacts. In addition, all slides from the cases were screened to insure that no specific neuropathological abnormalities were present. Identifying cases late in gestation without any evidence of gliosis proved most difficult, and slight white matter gliosis, based on GFAP immunoreactivity, was present in 2 cases (cases 7 and 9, Table 1). Coronal sections selected for immunostaining were at, or close to, the level of the mammillary body and contained representative areas of germinat matrix, basal ganglia (caudate, putamen, and globus pallidus), anterior thalamus, cerebral cortex, and the corresponding white matter. Fifty high power (40x objective) fields (hpf) were counted in the white matter of the internal capsule, the white matter of the centrum semiovale, subcortical white matter (defined as within three high power fields of the deepest layer of cortex), and the cerebral cortex. Equivocally-stained cells were not counted. In addition to determining the number of PCNA-positive cells per hpf, the number of PCNA-positive nuclei per 300 nuclei was counted in the white matter regions. Similar counts were not possible in the cerebral cortex because the extreme density of immature cells in the cortical plate resulted in unreliable cell counts. Each of the 2 counting methods controls for a different bias; by counting 50 hpf's one excludes a bias of counting the single highest field, and counting the number of positive cells per a given number of cells removes a cell density bias.

As expected, numerous cells in the germinat matrix (conventional VZ and SVZ elsewhere) were immunoreactive for PCNA (Fig. 1). PCNA immunostaining was always localized to the nucleus of the cell (Fig. 1 and Fig. 2A, inset). Consistent with the finding of PCNA-positive cells in the cerebral white matter, occasional mitotic figures were identified (Fig. 2B). The PCNA-positive cells in the VZ and SVZ were not quantitated but served as an internal control for the antibody staining. Controls without primary antibody and without secondary antibody were also performed and were always negative (data not shown). Trial experiments using the proliferation marker MIB-1 (Ki-67) were also attempted; however, this antibody did not prove reliable with our material.

Three brains were studied from fetuses 15 to 20 weeks gestation age. An average of 46 ± 4 PCNA-positive cells
per 50 hpf were seen in the internal capsule (Figs. 2 and 3) and 25 cells per 300 cells counted. These cells did not tend to cluster in groups and did not have significant amounts of cytoplasm. However, double staining for GFAP showed 10 to 20% of the PCNA-positive cells to have one to two delicate GFAP-positive processes (Fig. 4), features previously associated with “myelination glia” (7–10). Clusters of immature cells, resembling germinal zone cells, were often identified in finger-shaped clusters extending from the SVZ and crossing the internal capsule. These cells were excluded from analysis of cells in the internal capsule. The subcortical and deep white matter showed an average of 44 ± 10 PCNA-positive cells per 50 hpf (Fig. 3) and 15 PCNA-positive cells per 300 cells counted. PCNA-positive cells were also scattered in the white matter and did not show a tendency for clustering. The number of PCNA-positive cells in the hemispheric white matter was not statistically different from the number seen in the internal capsule at this age (p>0.1, two-tailed t test). In contrast, the cerebral cortex had only 4 ± 0.6 PCNA-positive cells per 50 hpf (Fig. 3). The endothelial and meningotheial cells of the leptomeninges showed a relatively high proliferation index at this stage of development (data not shown).

Three fetal brains at 23 to 25 weeks gestation were examined (Table 1). The internal capsule showed fewer PCNA-positive nuclei (19 ±16 per 50 hpf) compared to the 15 to 20 week group, but 40 PCNA-positive cells per 300 cells counted. The discrepancy between the two counts was believed to be a bias in areas selected to count when counting 300 cells. Therefore, all statistical data were based on the counts per 50 hpf since within each area cell density was fairly constant. The counts per 300 cells are given as a relative number of positive cells in any given number. The deep and subcortical white matter, however, maintained a similar proliferative activity to that of the 15 to 20 week fetuses (49 ± 14 per 50 hpf, vs 44 ± 10 per 50 hpf in the 15 to 20 week group; p>0.1). Twenty-four PCNA-positive cells were counted per 300 total cells in the subcortical white matter. Again, as in the 15 to 16 week group, many of the PCNA-positive nuclei in the white matter tracts showed one or two thin GFAP-positive processes, often giving the cell a bipolar appearance and supporting a glial lineage. At these time points in gestation there was an increased number of PCNA-positive nuclei in the cerebral cortex (Fig. 3). The intracortical PCNA-positive cells did not immunostain for GFAP.

Three infants in the last trimester of pregnancy, 30 through 37 and 5/7 week gestational age were studied (Table 1). GFAP immunostaining revealed a diffuse, mild astrocytosis in the subcortical and deep white matter in two of three brains. The astrocytes were interpreted as reactive because of the presence of GFAP-positive stellate cytoplasmic processes. Occasional binucleated astrocytes were also identified. The astrocytes with reactive changes were easily distinguished from the white matter cells described above as “myelination glia” that have a delicate bipolar morphology. PCNA staining showed very few cells in these cases: 9 ± 8 mitoses per 50 hpf in the internal capsule (5 PCNA-positive cells/300 total cells), 8 ± 7 per 50 hpf in the subcortical and deep white matter (8 PCNA-positive cells/300 total cells). The absence of significant cell proliferation in the presence of reactive astrocitosis was particularly striking. The cerebral cortex also showed a decreased number of PCNA-positive cells, compared to the previous time period studied (Fig. 3).

**DISCUSSION**

Classic and modern studies have identified the site of both neurogenesis and gliogenesis to be the VZ and SVZ. These studies have resulted in the prevailing model that progenitor cells reside in the VZ and SVZ (2). Once a cell exits the cell cycle, it is generally believed to migrate from the VZ or SVZ to the position where it will permanently reside. Although the VZ and SVZ are likely to provide the major contribution of neurons and possible glia to the nervous system, several studies have suggested that a progenitor cell may exist outside these regions. Rio-Hortega (11) and Penfield (12) hypothesized, based partially on the appearance of interfascicular oligodendroglia in late embryonic and early postnatal ages, that a precursor cell migrates into the white matter and then divides, forming differentiated oligodendrocytes or astrocytes. More recently, glia-enriched enzyme histochemistry was used to demonstrate that the number of oligodendrocytes increases markedly just prior to myelination (13). The identification of 3H-thymidine-labeled astrocytes and oligodendrocytes in myelinating cerebral white matter (corpus callosum) immediately after introduction of the label provided further evidence for the presence of a progenitor cell outside the VZ and SVZ (3). Similar results were obtained by LeVine and Goldman (14), who found that carbonic anhydrase immunopositive cells (a glial marker) in the subcortical white matter could incorporate 3H-thymidine. Although studies with 3H-thymidine support the contention that a cell progressing through the cell cycle exists in the white matter, false positive results with 3H thymidine have been obtained due to incorporation of label into mitochondrial DNA and amplification of selective DNA sequence without mitosis (15). Probably the most compelling evidence that a progenitor cell exists in the cerebral white matter of even adult rats comes from Gensert and Goldman (6), who have succeeded in infecting cells in the white matter of adult rats with a retrovirus. Since M-phase is required for retroviral
integration during the cell cycle (16), by definition these cells must be cycling.

The data from the present study indicate that many cells in the nascent white matter of the cerebral hemispheres, by virtue of their PCNA, are cycling. The co-labeling of cells for PCNA and GFAP indicates that at least some of the cycling cells are in the glial lineage. Whether the progeny of these cycling cells will become oligodendrogli or astrocytes remains unknown. Alternatively, the progeny of these cells could all die and not contribute to the development of the brain. Further studies are required to define the fate of these cells.

Assuming the progeny of the cycling cells in the white matter are contributing to CNS development, the pattern of proliferation in the different regions studied is intriguing. The highest numbers of proliferating cells were found in the cerebral white matter at 15 to 25 weeks gestation, the time when gliogenesis is believed to begin and peak. Friede and others have shown increased numbers of glial cells in tracts just preceding myelination, specifically an increase in the internal capsule from 24,000 to 136,000 cells per cubic mm. (17). The presence of progenitor cells in the appropriate location and at the appropriate time could provide a mechanism for the developing brain to rapidly increase the number of glia in the white matter. None of

Fig. 1. Anti-PCNA immunostaining of germinal matrix (20 weeks gestation). Numerous PCNA-positive cells are found throughout the germinal matrix. The PCNA positivity is localized to the nucleus. The ventricle is to the right in the photograph (×200).

Fig. 2. Anti-PCNA staining in the cerebral white matter of a 23-week fetus. A: Numerous PCNA-positive cells are found in a single low power field (arrows). Insert shows the PCNA immunostaining localized to the nucleus (×400). B: Mitotic figure found in the cerebral white matter of a 25-week fetus (×400).
These cells have been shown to accumulate in the white matter prior to myelination and are believed to represent either immature oligodendrocytes, astrocytes, or bipotential oligodendrocyte/astrocyte precursor cells, such as the "O2A" progenitor (3, 7, 8, and 18). More recently, increased GFAP messenger RNA and protein expression have been demonstrated, and were seen to parallel the onset of myelination (19, 20). Unfortunately, myelin/oligodendroglial protein marker have not been reliable in paraffin-embedded tissue; thus we were unable to subclassify cells as definitely oligodendroglia versus astrocytes.

Two of the cases (#7 and #9) had a diffuse mild reactive astrocytosis, yet there was no increase in the number of PCNA-positive cells. Although astrocyte proliferation has been found in response to injury in several experimental paradigms (21, 22), \(^3\)H-thymidine incorporation could reflect increased nuclear transcription rather than cell division (15). Other authors have suggested that astrocyte proliferation is minimal in pathologic situations, and that migration and hypertrophy of local astrocytes may be the primary response to injury (22, 23). Further evidence to support the hypothesis that proliferation is not the only mechanism for accumulating reactive astrocytes has recently come from Dyer et al (24). These authors have shown that a substantial number of GFAP-positive reactive "astrocytes" seen in the white matter in various pathologic situations also are immunopositive (and express the mRNA) for several myelin/oligodendrocyte markers. Thus, it is possible that a precursor cell or even a mature oligodendrocyte can be induced to transform into a nonmyelinating, GFAP-positive phenotype without mitosis.

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   :alt: Anti-PCNA and anti-GFAP double-labeling. PCNA-positive cells are identified by dark red nuclear staining (blue arrows). GFAP-positive cells show light red cytoplasmic staining (white arrows). A double-labeled cell (black arrow) shows both nuclear PCNA-positive and cytoplasmic GFAP-positivity. (23-week fetus; X400).

   Fig. 4. Anti-PCNA and anti-GFAP double-labeling. PCNA-positive cells are identified by dark red nuclear staining (blue arrows). GFAP-positive cells show light red cytoplasmic staining (white arrows). A double-labeled cell (black arrow) shows both nuclear PCNA-positive and cytoplasmic GFAP-positivity. (23-week fetus; X400).
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REFERENCES


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