Comparative Characterization of the Human and Mouse Third Ventricle Germinal Zones

Sonika Dahiya, MD, Da Yong Lee, PhD, and David H. Gutmann, MD, PhD

Abstract
Recent evidence indicates differences in neural stem cell biology in different brain regions. For example, we demonstrated that neurofibromatosis 1 (NF1) tumor suppressor gene inactivation leads to increased neural stem cell proliferation and gliogenesis in the optic chiasm and brainstem but not in the cerebral cortex. The differential effect of NF1 inactivation in the optic nerve and brainstem (in which gliomas commonly form in children with NF1) versus the cortex (in which gliomas rarely develop) suggests the existence of distinct ventricular zones for gliomagenesis in children and in adults. Here, we characterized the third ventricle subventricular zone (tv-SVZ) in young and adult mouse and human brains. In children, but not adult humans, the tv-SVZ contains nestin-positive, glial fibrillary acidic protein-positive, and sox2-positive cells with radial processes and prominent cilia. In contrast, the tv-SVZ in young mice contains sox2-positive progenitor cells and ciliated ependymal lining cells but lacks glial fibrillary acidic protein-positive, nestin-positive radial glia. As in the lateral ventricle SVZ, proliferation in the human and murine tv-SVZ decreases with age. The tv-SVZ in adult mice lacks the hypocellular subventricular zone observed in adult human specimens. Collectively, these data indicate the existence of a subventricular zone relevant to our understanding of glioma formation in children and will assist interpretation of genetically engineered mouse glioma models.

Key Words: Germinal zone, Third ventricle.

INTRODUCTION
With the emergence of the cancer stem cell hypothesis (1–4), considerable attention has focused on the lateral ventricle subventricular zone (lv-SVZ) as a potential germinal zone relevant to the genesis of high-grade gliomas in adults (5–7). Studies pioneered by Alvarez-Buylla (8) have shown that neural progenitor cells lining the lv-SVZ are authentic stem cells with the capability of producing all 3 major brain cell lineages (9–11). The existence of this specialized germinal zone in adults has prompted studies examining the consequence of introducing glioma-associated genetic mutations into stem-like cells within the lv-SVZ. The ability to generate high-grade gliomas supports the hypothesis that gliomas arise from this specialized stem cell niche in adults (12–15).

In contrast to their adult counterparts, gliomas in children arise less commonly in the cerebral hemispheres and more often develop in the optic nerve, brainstem, and cerebellum (16). This spatial pattern of gliomagenesis is illustrated by World Health Organization grade 1 gliomas (pilocytic astrocytoma), which arise in the cerebellum (33%), optic pathway (27%), brainstem (16%), and cerebrum (13%) (17). Moreover, pilocytic astrocytomas arising in the context of the neurofibromatosis 1 (NF1) cancer predisposition syndrome are located predominantly in the optic pathway (67%) and less commonly in other brain regions (18). The predilection for pediatric gliomas to form in these regions raises the possibility that additional germinal zones may contribute to the genesis of gliomas in children.

Because pediatric gliomas often arise in the optic pathway and hypothalamus, we sought to characterize an understudied potential germinal zone (i.e. the third ventricle) in human and mouse specimens. The location of the third ventricle (proximate to the hypothalamus and optic pathway) raises the possibility that optic nerve/hypothalamic gliomas arise from this unique ventricular zone. Support for regional heterogeneity in neuralglial cell function derives from our recent studies demonstrating that astrocytes and neural stem cells (NSCs) from different regions of the brain harbor region-specific transcriptional signatures (19) and exhibit differential cell-autonomous responses to 1 pediatric brain cancer–causing genetic mutation (Nf1 gene inactivation) (20).

In this report, we characterize the third ventricle subventricular zone (tv-SVZ) in both young and adult mouse and human specimens and demonstrate a time-dependent decrease in ventricular zone proliferation. We demonstrate that this zone shares some features with the better-characterized lv-SVZ, including the presence of radial glia, ciliated cells, and progenitor cells in children as well as the existence of a hypocellular zone in the immediate subependymal region of adults. However, in contrast to their human counterparts, GFAP+; nestin+ radial glia were not detected in the tv-SVZ of young mice, and no hypocellular zone was found in adult mice. Together, these data provide a more complete characterization of this potential germinal zone in young and adult mice.

From the Division of Neuropathology (SD) and Department of Neurology (DYL, DHG), Washington University School of Medicine, St Louis, Missouri.

Send correspondence and reprint requests to: David H. Gutmann, MD, PhD, Department of Neurology, Box 8111, 660 S Euclid Ave, Washington University School of Medicine, St Louis, MO 63110; E-mail: gutmannd@neuro.wustl.edu

This work was partially supported by a grant from the National Institutes of Health (1R01NS65547 to DHG).

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal’s Web site (www.jneuropath.com).
FIGURE 1. Age-dependent changes in the human third ventricle subventricular zone (tv-SVZ). (A, B) Section of the third ventricle from a child (A) reveals a single-layered, tall, columnar epithelium with prominent cilia at the ventricular surface (inset). In adults, the ependymal layer is flattened and contains a single layer of cuboidal cells and there is marked reduction of lining cilia (B). (C, D) Acetylated tubulin immunostaining reveals abundant cilia in the pediatric (C) and few in the adult (D) tv-SVZ. Scale bars = 20 μm. (E, F) Electron microscopy demonstrates a relative abundance of cilia in the pediatric tv-SVZ (E, arrow), and the presence of lipopigment in lining ependymal cells in the adult tv-SVZ (F, arrow). (A, B): Hematoxylin and eosin.
FIGURE 2. Radial glia in the third ventricle subventricular zone (tv-SVZ) of children. (A–D) Immunostaining for glial fibrillary acidic protein (GFAP) (A, B) and nestin (C, D) demonstrate moderate staining in the ependymal lining of tv-SVZ in a child (A, C) and an adult (B, D). Radial processes (inset, arrows) were only observed in the tv-SVZ of children; there is a cobweb-like pattern of expression in the adult third ventricle subependymal zone (SEZ) (inset). Arrowheads in C and D indicate blood vessels as internal positive immunostaining controls. (E) Radial glia in the pediatric tv-SVZ colabel (yellow) for GFAP (green) and nestin (red). Inset shows radial processes double positive for GFAP and nestin. (F) The radial glia also express brain fatty acid binding protein (BLBP, red; 4',6-diamidino-2-phenylindole, blue). Expression of BLBP is also seen in isolated cells within the SEZ. (G) A small number of cells within the lining ependyma and tv-SEZ are Sox2-immunoreactive (arrows). Scale bars = 20 μm.
vertebrates and form the basis for future studies aimed at understanding the role of this unique stem cell niche relevant to pediatric gliomagenesis.

MATERIALS AND METHODS

Human Brain Samples

Samples were obtained at autopsy from the brains of 10 children (7 days to 4 years; mean = 9.6 months) and 10 adults (47–87 years; mean = 64.3 years) (Table, Supplemental Digital Content 1, http://links.lww.com/NEN/A246). A midline section of the tv-SVZ was embedded in paraffin, and 5-μm-thick consecutive sections were cut. The lv-SVZ was represented in 10 cases (5 from each group). Mouse and human specimens are denoted by “m” and “h,” respectively. The SVZ was the region that encompassed both the ependymal lining and an adjacent 1- to 3-mm subependymal zone (SEZ) along the ventricular wall. Autopsy specimens were obtained in accordance with an approved human studies protocol at the Washington University School of Medicine.

Animals

Brain tissues were obtained from wild-type C57BL/6 mice at postnatal days 2 to 3 (young) and at 4 to 6 months of age (adult) after intracardiac perfusion with phosphate-buffered saline and 4% paraformaldehyde solution. After fixation, the specimens were paraffin-embedded. The mice were used in accordance with an approved animal studies protocol at the Washington University School of Medicine.

Routine Histology and Immunohistochemistry

Hematoxylin and eosin and immunohistochemical staining using lineage-specific antibodies were performed.

FIGURE 3. Age-dependent changes in neuronal architecture in the human third ventricle subventricular zone (tv-SVZ). (A–D) Immunostaining for (A, B) polysialated neural cell adhesion molecules (PSA-NCAM) (A, B) and Tuj1 (C, D) shows intense staining of the processes abutting the third ventricle SEZ in a child (A, C), which is absent in the adult SEZ (B, D). The hypocellular region in the adult tv-SVZ is denoted by bars. Scale bar = 20 μm.
after antigen retrieval (Table, Supplemental Digital Content 2, http://links.lww.com/NEN/A247). Immunohistochemistry was performed simultaneously on all sections with appropriate positive and negative controls. Double labeling was performed using separate antigen retrieval buffers when required. Three different peroxidase substrates were used: Vector-3′3′ diamobenzidine (SK-4100), Vector-NovaRED (SK-4800), and Vector-SG (SK-4700), all from Vector Laboratories, Burlingame, CA. The Ki-67 labeling index was defined as the number of Ki-67-positive cells divided by the total number of cells (including both Ki-67-positive and Ki-67-negative cells) counted. Only cells in the SVZ (both positive and negative for Ki-67) were counted. Appropriate Alexa Fluor-tagged secondary antibodies were used for fluorescence detection, and the cells were counterstained with 4′,6-diamidino-2-phenylindole.

For ultrastructural analysis, a portion of the SVZ was fixed in 2% glutaraldehyde, postfixed in phosphate-buffered 2% OsO4 for 2 hours, dehydrated in graded concentrations of ethanol and embedded in Embed-812 (Electron Microscopy Sciences, Hatfield, PA) with propylene oxide as an intermediary solvent. One-micrometer-thick plastic sections were stained with toluidine blue and examined under light microscopy to define the orientation of the ependymal layer and SVZ. Ultrathin sections were subsequently stained with uranyl acetate and lead citrate and examined with a JEOL 1200 electron microscope equipped with an ABT digital camera.

Statistical Analysis
Statistical significance (p < 0.05) was determined using the Student t-test and GraphPad Prism 4.0 software (GraphPad, Inc., La Jolla, CA).

RESULTS

Human tv-SVZ
We first compared the ventricular lining (ependyma) and SEZ extending 1 to 3 mm beneath the ependyma in human specimens. In children younger than 5 years (n = 10), the ependyma is a single-layered, tall, columnar epithelium with prominent cilia and a conspicuous terminal bar (Fig. 1A). In adults (~45 years, n = 10), the ependyma appears as a single layer of cuboidal cells with a marked reduction in cilia (Fig. 1B). The presence of cilia was confirmed using acetylated tubulin immunostaining (Figs. 1C, D) and electron microscopy (Figs. 1E, F; arrow). In addition, the SEZ is paucicellular in adults compared with children. On ultrastructural examination, the tv-SVZ in adults is also paucicellular and the ependymal cells contain lipofuscin.

Next, we performed immunohistochemistry to characterize the cellular composition of the human tv-SVZ. These studies employed a battery of antibodies that delineate neurons (Tuj1, PSA-NCAM, and NeuN), astrocytes (GFAP), and progenitor cells (nestin, vimentin, Sox2, and BLBP). Several differences were noted between the pediatric and adult tv-SVZ specimens. First, GFAP-immunoreactive cells in the third ventricle SEZ of children had a radial appearance with prominent radial glial processes (Fig. 2A); however, in adults, GFAP immunostaining revealed a cobweb appearance with haphazard arrangements of glial processes (Fig. 2B). Second, numerous long, perpendicularly oriented, nestin-immunoreactive processes arranged in parallel were found to extend from the ependymal layer into the SEZ in young children (Fig. 2C), whereas in adults, there was only coarse punctate nestin staining in the subependymal region with more labeling at the base of the ependymal lining (Fig. 2D). In children, numerous cells with radial processes in the ependymal lining were immunopositive for both nestin and GFAP (Fig. 2E) and for BLBP (Fig. 2F); these data support their designation as radial glia. A small number of cells in the ependymal layer and SEZ also expressed the Sox2 progenitor marker (Fig. 2G). No differences in vimentin immunoreactivity were noted between the 2 age groups (data not shown). Third, the Tuj1- and PSA-NCAM-immunoreactive cell processes abut the base of the ependymal layer in young children (Figs. 3A, C). In contrast, there are few neurons beneath the ependymal layer in adults, resulting in a hypocellular zone (Figs. 3B, D). NeuN immunohistochemistry demonstrated staining of neurons in the regions much deeper to the SEZ but was negative in the third ventricle SEZ of either age group (data not shown).

Ki-67 immunolabeling was used to determine the proliferative indices within the tv-SVZ (MIB-1). In general, proliferating cells were predominantly located in the immediate SEZ, with a very few Ki-67-positive ependymal cells. There was a 14-fold reduction in the Ki-67 labeling index in adults versus children (n = 10 specimens, p = 0.04) (Fig. 4). To determine which cells in the SEZ were proliferating, Ki-67 double labeling experiments were performed. Most of the

FIGURE 4. Proliferation in the human third ventricle subventricular zone (tv-SVZ) decreases with age. (A) Diagram of the region containing the human third ventricle (red box). (B, C) Ki-67 immunolabeling of the tv-SVZ in representative specimens from a child (B) and an adult (C). (D) Mean and standard deviations of Ki-67 labeling indices (p < 0.005). Scale bar = 20 μm (B, C).
FIGURE 5. Proliferating cells in the human third ventricle subventricular zone (tv-SVZ) are glial fibrillary acidic protein (GFAP)– and nestin-immunoreactive. (A) A small population of GFAP-positive cells in the third ventricle subependymal zone (SEZ) colabel with Ki-67. Inset demonstrates a representative Ki-67–positive, GFAP-positive cell. (B) Similar results are shown with GFAP/Ki-67 immunofluorescence double labeling. The arrow denotes a representative GFAP-positive, Ki-67–positive cell. (C) A small number of nestin-positive cells in the human third ventricle SEZ colabels with Ki-67. Inset demonstrates a representative nestin-positive, Ki-67–positive cell. Scale bar = 20 μm.
Ki-67-positive cells did not colabel with GFAP (Figs. 5A, B), PSA-NCAM (data not shown), nestin (Fig. 5C), or Olig2 (data not shown). Only 10% of the Ki-67-positive cells were GFAP-immunoreactive (glial progenitors or astrocytes), 5% to 7% were nestin-positive (glial progenitor or stem cells), 1% were Olig2-positive, and none were PSA-NCAM-immunoreactive.

FIGURE 6. Age-dependent changes in the cellular composition of the mouse third ventricle subventricular zone (tv-SVZ). (A, B) Representative hematoxylin and eosin–stained sections of the third ventricle in young and adult mice. (C, D) Glial fibrillary acidic protein (GFAP)–positive cells are barely detected in the third ventricle in young mice (C), whereas adult mice exhibit focal GFAP immunoreactivity in the SEZ (D). (E, F) Rare nestin-positive cells were found in young mice (E); whereas the adult mouse ependymal layer contains numerous nestin-immunopositive cells (F). Arrowheads indicate blood vessels, which serve as internal positive controls. (G, H) Acetylated tubulin immunostaining illustrates age-dependent reduction in the number of cilia. The cilia appear relatively shorter than those in the human tv-SVZ.
Several age-dependent features were noted in the mice. First, the third ventricle of young mice lacked cells with GFAP expression, whereas there was diffuse GFAP immunoreactivity in the ependymal lining in adult mice (Figs. 6C, D). Small numbers of GFAP-positive cells were also found in the third ventricle SEZ in the adult mice. Second, there was little nestin expression in the third ventricle of young mice, whereas widespread nestin immunoreactivity was found in the ependymal lining of adult mice (Figs. 6E, F). This absence of GFAP-positive, nestin-positive radial glia in the mouse TVZ sharply contrasts with the human specimens, both in the tv-SVZ (Fig. 2) and the lv-SVZ of children (Figure, Supplemental Digital Content 3, http://links.lww.com/NEN/A248). Third, as in human pediatric tv-SVZ specimens, sox2 immunostaining identified progenitor cells in the tv-SVZ of young mice (Figure, Supplemental Digital Content 4, http://links.lww.com/NEN/A249). There was also an age-dependent reduction in cilia, as evidenced by acetylated tubulin immunohistochemistry (Figs. 6G, H), but there were no differences in the pattern of neuronal (PSA-NCAM, Tuj1) staining between young and adult mice (Fig. 7). As in the human specimens (Fig. 4), Ki-67 labeling index in the tv-SVZ was much lower (45-fold) in adult versus early postnatal mice (n = 5 mice/group, p = 0.007) (Fig. 8). This age-dependent reduction in germinal zone proliferation within the human tv-SVZ was also observed in the human (Fig. 9A) and the mouse (Fig. 9C) lv-SVZ.

**DISCUSSION**

We used immunohistochemistry and electron microscopy to demonstrate the existence of an additional germinal zone within the third ventricle in humans. The designation of the tv-SVZ as a stem cell niche comparable to the better-studied lv-SVZ is supported by several observations. First, we identified cells with long radial processes that express both GFAP and nestin. Although nestin-immunoreactive cells have been previously reported in the rat tv-SVZ, it is not known...
whether these nestin-positive cells colabeled with GFAP (21), as seen in our study. Glial fibrillary acidic protein–positive/nestin-positive radial glia-like cells most likely represent type B cells, as originally proposed for the lv-SVZ (9–11). Second, there is robust expression of BLBP (FABP7), a marker of both radial glia and NSCs. In this regard, radial glia in the lv-SVZ have been shown to give rise to adult NSCs (22). In addition, we have previously used a BLBP-Cre transgenic mouse strain to demonstrate that BLBP-positive cells can self-renew in vitro and give rise to oligodendrocytes, astrocytes, and neurons (multilineage differentiation) in vitro and in vivo, consistent with their role as NSCs (23). Third, the pediatric tv-SVZ also contains Olig2-positive cells, another marker of progenitor cells in the brain (24). In this regard, Olig2 is universally expressed in gliomas (25), and its targeted inactivation abrogates gliomagenesis in genetically engineered mice (26). Fourth, we identified sox2-positive cells in the tv-SVZ of children. Sox2 is a transcription factor critical for the maintenance of neural progenitor identity (27), such that alterations in sox2 expression in genetically engineered mice lead to defects in astrocyte and neuronal differentiation (28, 29). Fifth, we found that the cells lining the tv-SVZ in children contained cilia. Previous studies in adult rats (30) and primates (31) identified cilia along the lateral walls of the tv-SVZ, but not in the ventral parts. In contrast, we did not identify significant numbers of cilia in the adult human tv-SVZ but could identify ciliated ependymal lining cells in the adult mouse tv-SVZ. The importance of cilia to NSC function is highlighted by several studies demonstrating that primary cilia are required for the formation of adult NSCs (32), regulation of hippocampal neurogenesis (33), and characterization of B cells within the adult lv-SVZ (34).

In addition, we found an age-dependent development of a hypocellular region within the third ventricle SEZ. Although the functional significance of this hypocellular region has not been fully elucidated, previous studies have demonstrated the existence of a similar paucicellular zone in the human lv-SVZ of adults (7, 10). Interestingly, this hypocellular region is very thin or not present in the lv-SVZ of other species (7, 35), similar to our findings in mice.

Next, we examined proliferation in the tv-SVZ. We describe an age-dependent decrease in the proliferative index in adults relative to their pediatric counterparts. This finding parallels a similar reduction in lv-SVZ proliferation in adults versus children and likely reflects an overall decrease in germinal zone capacity over time (36). To identify the proliferating cells within the pediatric tv-SVZ, we performed a series of double-labeling experiments. Although there were small numbers of proliferating nestin-positive, Olig2-positive, and GFAP-positive cells in the tv-SVZ, transient-amplifying type C cells could not be definitively identified. Similar to analogous studies in the human lv-SVZ, it is highly likely that some of the Ki-67–positive cells contained within the tv-SVZ are NSCs. Current studies are focused on defining the identity of the proliferating cells in the tv-SVZ and on determining whether distinct subpopulations of progenitor cells exist in this pediatric germinal zone.

To inform future studies using genetically engineered small animal glioma models, we compared the lv-SVZ and tv-SVZ in mice. As in humans, we found that proliferation decreased significantly with age in the murine tv-SVZ. In contrast to their human counterparts, we were unable to identify GFAP-positive, nestin-positive cells with clear radial processes in young mice. However, we could identify cells expressing the CD133 and BLBP progenitor markers in the young murine tv-SVZ (data not shown) as well as ciliated ependymal lining cells. The fact that BLBP-positive and CD133-positive cells can generate new neurons and astrocytes in the embryonic mammalian brain (37–40), as well as in the adult avian brain (41, 42), supports the presence of an analogous third ventricle germinal zone in mice. This contention is further strengthened by numerous studies examining a group of midline structures along the rodent third and fourth ventricles, termed circumventricular organs (CVOs). These CVO structures contain progenitor cells that express nestin, GFAP, sox2, and vimentin (43). Moreover, neurospheres generated from CVO tissues can proliferate and undergo multilineage differentiation in vitro and in vivo (44). Along these lines, preliminary results in our laboratory have established the existence of BLBP-positive NSCs in postnatal days 1 to 2 murine tv-SVZ with the ability to sustain long-term self-renewal and undergo multilineage differentiation into oligodendrocytes, astrocytes, and neurons in vitro (D.Y. Lee and D.H. Gutmann, unpublished observations). These preliminary findings parallel previous reports describing the presence of NSCs from the adult tv-SVZ with the capacity to self-renew and undergo multilineage differentiation (45, 46).
FIGURE 9. Age-dependent decreases in ventricular zone proliferation in the mouse and human third ventricle subventricular zone (tv-SVZ) and lateral ventricle SVZ (lv-SVZ). (A-D) The Ki-67 labeling index is reduced in the human lv-SVZ as a function of age (p = 0.01) (A). The Ki-67 labeling index is reduced in the murine lv-SVZ as a function of age (p = 0.0004) (C). Diagrams of the regions containing the human (B) and mouse (D) lv-SVZ (red boxes).
The observation that NSCs exist in the tv-SVZ is intriguing in light of mouse modeling experiments in which specific high-grade glioma-associated genetic changes introduced into lv-SVZ progenitor cells result in glioma formation. In these studies, coordinate loss of NF1, PTEN, and p53 function in mouse lv-SVZ cells is sufficient to generate high-grade gliomas in the subcortical and cortical regions (13). Similarly, the expression of mutant p53 proteins (15, 47), platelet-derived growth factor (14) or oncogenic KRas in lv-SVZ NSCs initiates gliogenesis (12). Although the relevance of the tv-SVZ to gliogenesis is currently not clear, studies in rodents have demonstrated that ciliated ependymal cells in the rat third ventricle can give rise to differentiated progeny (48, 49) and that GFAP-positive cells can derive from neuroepithelial cells lining the third ventricle to populate the optic tract along retinal ganglion cell projections (50) and hypothalamic parenchyma (51).

Current studies are focused on determining the consequence of introducing specific pediatric glioma-associated genetic changes (NF1 loss; BRAF activation) on third ventricle NSC function. Coupled with spatially distinct microenvironmental conditions, heterogeneity in NSC niches may contribute significantly to the pattern of gliogenesis and may uncover new targets for therapeutic drug design specific for glioma-maintaining stem cells in children and adults.

ACKNOWLEDGMENTS

The authors thank the excellent technical support from members of the Gutmann Laboratory and Lisa Snipes and Karen Green from the Division of Neuropathology. The authors also thank Robert E. Schmidt, MD, PhD, for his expert advice in interpreting the ultrastructural electron microscopy results.

REFERENCES


© 2011 American Association of Neuropathologists, Inc.