Cell proliferation in human ganglionic eminence and suppression after prematurity-associated haemorrhage

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In premature infants, germinal matrix haemorrhage in the brain is a common occurrence. However, cell proliferation and fate determination in the normal human germinal matrix is poorly understood. Human ganglionic eminence samples were collected prospectively from autopsies of premature and term infants with no evidence of pathological process (n = 78; dying at post-menstrual age 14–88 weeks). The ganglionic eminence was thickest at 20–26 weeks and involuted by 34–36 weeks. Proliferating cells, detected by Ki67 immunoreactivity, were abundant throughout the ganglionic eminence prior to 18 weeks, after which a sharp boundary between the dorsal and ventral ganglionic eminence appeared with reduced cell proliferation in the dorsal region. Ki67 immunoreactivity persisted in the majority of ventral cells until ~28 weeks, after which time the proportion of proliferating cells dropped quickly. The expression of cell lineage markers (such as Olig2, SOX2, platelet-derived growth factor receptor alpha) showed partitioning at the microscopic level. The hypothesis that germinal matrix haemorrhage suppresses cell proliferation was then addressed. In comparison to controls, germinal matrix haemorrhage (n = 47; born at post-menstrual age 18–34 weeks followed by survival of 0 h to 98 days) was associated with significantly decreased cell proliferation if survival was > 12 h. The cell cycle arrest transcription factor p53 was transiently increased and the oligodendroglial lineage markers Olig2 and platelet-derived growth factor receptor alpha were decreased. Cell death was negligible. A low level of microglial activation was detected. Haemorrhage-associated suppression of cell proliferation in premature human infants could partially explain the reduced brain size and clinical effects in children who suffer germinal matrix haemorrhage after premature birth.

Keywords: development of brain; cerebral palsy; prematurity; germinal matrix; intraventricular haemorrhage; transcription factors

Abbreviations: HLA-DR = human leucocyte antigen class DR; PDGFRα = platelet-derived growth factor receptor alpha; PSA-NCAM = polysialated neural cell adhesion molecule; TUNEL = terminal deoxynucleotidyl transferase dUTP nick end labelling
Introduction

Each year, more than 12 million children worldwide are prematurely born with considerable adverse consequences (Beck et al., 2010; McCormick et al., 2011). In infants born at <30 weeks post-menstrual (gestational) age, germinal matrix hemorrhage is a common occurrence (2.5/1000 live births) (Robertson et al., 1998). However, knowledge of the normal human germinal matrix, necessary to understand the consequences of haemorrhage, is incomplete. The developing neocortex is recognized to have several layers including the including the subcortical grey matter which is the predominant site of cell generation after 15 weeks when it begins to expand rapidly in some sites, forming prominent ganglionic eminences along the lateral walls of the frontal (and to a lesser extent the temporal) horns of the lateral ventricles. The ganglionic eminence is the source of neocortical neurons with long axons are generated from the ventricular zone as well as the preplate, a precursor to the cortical layer. The intermediate zone, a precursor of the white matter, appears by 8 weeks. Continuing to 14 weeks, the outer subplate and cortical plate are further distinguished. Cortical projection neurons with long axons are generated from the ventricular zone mainly during the embryonic and early foetal period (Bystron et al., 2008), although their synapse formation is not complete for many months or years (Lemire et al., 1975). The subventricular zone becomes the predominant site of cell generation after ~15 weeks when it begins to expand rapidly in some sites, forming prominent ganglionic eminences along the lateral walls of the frontal (and to a lesser extent the temporal) horns of the lateral ventricles. The ganglionic eminence is the source of neocortical GABAergic inhibitory interneurons, production of which continues until at least 20 weeks of gestation (Parnavelas et al., 2000; Zecevic et al., 2005, 2006, 2011), as well as neurons for the adjacent basal nuclei until ~25 weeks (Lemire et al., 1975; Marin et al., 2000; Ulfig, 2001a). Thalamus neuron generation continues until ~34 weeks, with cell migration occurring along the gangliothalamic body (Rakic and Sidman, 1969; Letinic and Kostovic, 1997; Letinic and Rakic, 2001). The subventricular zone also generates precursors of oligodendrocytes and astrocytes well into the third trimester (Gould and Howard, 1987, 1988; Zecevic et al., 2006; Jakovevski et al., 2009). The ganglionic matrix regresses gradually in a site-specific manner (Kahle, 1951), with final gross involuion of the ganglionic eminence by 34–36 weeks of gestation (Jammes and Gilles, 1983; Del Bigio, 2004). Small quantities of pluripotent subventricular zone cells persist into adulthood (Moe et al., 2005), a subpopulation of which, along the most rostral portion of the lateral ventricle, generates olfactory neurons (Curtis et al., 2007; Kam et al., 2009).

Despite improving care for premature infants, the incidence of germinal matrix haemorrhage is not declining because smaller infants are surviving (Wilson-Costello et al., 2005). Germinal matrix haemorrhage most often arises from the ganglionic eminence, which lies between the frontal horn of the lateral ventricle and the head of the caudate nucleus (Angevine et al., 1970; Leviton et al., 1983; Ulfig, 2002; Del Bigio, 2004). A potential consequence of germinal matrix haemorrhage is a permanent neurological deficit, such as cerebral palsy or post-haemorrhagic hydrocephalus (Luu et al., 2009). Many imaging studies in humans have shown that the volume of cerebrum, white matter and subcortical grey matter fails to increase normally following germinal matrix haemorrhage (Kuban et al., 1999; Ajayi-Obe et al., 2000; Kesler et al., 2004; Vasileiadis et al., 2004; Inder et al., 2005; Cheong et al., 2008). This was also found in our experiments, which showed that adult rats had diminished cerebral volume after autologous blood was injected into the periventricular region during the neonatal period (Balasubramaniam et al., 2006).

The mechanism of premature brain injury following haemorrhage remains somewhat conjectural with infarction and inflammation presumed to play a role (Kusters et al., 2009; Adler et al., 2010). In addition, autologous blood injection into neonatal mouse brains caused reduced expression of Ki67 protein, a marker of cellular proliferation, in the germinal matrix near the site of the haematoma (Xue et al., 2003). Cultured rat subventricular zone cells and oligodendrocyte precursors also exhibit reduced proliferation and migration following exposure to blood plasma, serum and thrombin (Juliet et al., 2009).

I therefore hypothesized that germinal matrix haemorrhage would be associated with suppressed proliferation in the human ganglionic eminence. However, first it was necessary to study cell proliferation in the undamaged foetal germinal matrix. Samples of the human cerebrum in autopsy specimens from foetuses, prematurely born infants and young children (14 weeks post-menstrual to 1 year post-natal) were studied. Ki67 nuclear antigen was used to identify proliferating cells in late GI through M phases of the cell cycle (Hall and Woods, 1990; Brown and Gatter, 2002) and H3 histone was used to identify S phase cells (Konishi et al., 1996). The nuclear transcription factor p53, which is associated with cell cycle arrest and differentiation (Billon et al., 2004; Tedeschi and Di Giovanni, 2009), was studied along with markers of cell death and reactive inflammatory changes.

Materials and methods

The study was conducted with approval of the Research Ethics Board of the University of Manitoba. For clarity, the term ‘post-menstrual age’ (gestational age + survival) will be used to describe the age of infants (Engle, 2004).

Autopsy specimens: control cases

Control brains with no malformation, haemorrhage or other pathological change were obtained from 78 human infants whose post-menstrual age ranged from 14 to 88 weeks (14–19 weeks, n=14; 20–21 weeks, n=13; 22–23 weeks, n=11; 24–25 weeks, n=5; 26–27 weeks, n=2; 28–29 weeks, n=4; 30–34 weeks, n=6; 35–40 weeks, n=11; 41–44 weeks, n=7; and 50–88 weeks, n=6). Necropsies were obtained following termination of the pregnancy because of foetal anomalies not involving the nervous system, or following spontaneous premature labour. In all cases, the control foetuses died intrapartum or immediately post-partum. The six previously healthy infants who had been born at full-term died suddenly from a variety of causes not affecting the nervous system. In no case was there gross or microscopic evidence of dying neurons (e.g. hypoxic-ischaemic damage) or autolysis with minor exception. Minimal acute damage (scattered slightly pyknotic neurons) was identified in a 30-week foetus (twin to twin transfusion), a 36-week foetus (presumed placental insufficiency), and a 1-year-old child (drowning with failed resuscitation).
Also examined were the brains of five premature infants born at 26–32 weeks who survived 2–9 days with clinical documentation of hypoxic episodes. These were found to have dying neurons in the basal nuclei, hippocampus and pons, suggestive of hypoxic-ischaemic damage, but had no germinal matrix haemorrhage. They represent disease controls whose purpose is to determine if hypoxia-ischaemia contributes to the changes observed following germinal matrix haemorrhage.

**Autopsy specimens: brain haemorrhage cases**

Forty-seven brains from premature infants with germinal matrix haemorrhage were examined. Twenty-one (post-menstrual age 18–28 weeks) who died <12 h after birth are designated as acute germinal matrix haemorrhage. In this group, 11 had small blood collections confined to the ganglionic eminence, nine had small amounts of blood in the lateral ventricles and one had a substantial intraventricular haemorrhage with enlargement of the ventricles. The diagnosis was exclusively based on the autopsy findings. Fifteen, who were born at 23–31 weeks post-menstrual age and who died ~1–16 days after birth, plus an additional infant with documented intraventricular haemorrhage in utero at 19 weeks who was born at 23 weeks and died 5 days later, are designated as subacute germinal matrix haemorrhage. In this group, two had small blood collections confined to the ganglionic eminence, two had small amounts of blood in the lateral ventricles, seven had substantial intraventricular haemorrhage with enlargement of the ventricles and four had intraventricular haemorrhage and extension of blood into the adjacent white matter and basal nuclei. Ten who were born at 25–34 weeks post-menstrual age and who died 24–98 days after birth are designated as chronic germinal matrix haemorrhage. Blood collections were no longer apparent and therefore the initial extent of haemorrhage was determined by the cranial ultrasound examination, acknowledging that it underestimates small blood collections (Mirmiran et al., 2004). All who survived >2 days had cranial ultrasound confirmation of blood, but imaging was not regularly scheduled and therefore the first documentation of intracerebral haemorrhage was as late as 7 days of age. For the purpose of the analysis, the haemorrhage is assumed to begin at or shortly after birth, recognizing that this might not in fact be accurate. In the chronic germinal matrix haemorrhage group, three had blood collections confined to the ganglionic eminence, one had a small amount of blood in the lateral ventricles, two had intraventricular haemorrhage with enlargement of the ventricles, three had intraventricular haemorrhage and extension of blood into the adjacent white matter and basal nuclei and one had intraventricular haemorrhage probably due to choroid plexus haemorrhage. There was no attempt to subdivide the analysis according to magnitude of haemorrhage because the resulting group sizes would be too small.

**Brain tissue sampling**

At the time of autopsy (8–36 h after death) brains were removed from the body and placed in 10% buffered formalin fixative. Post-mortem delays >36 h were associated with more pronounced autolytic changes and less reliable immunolabelling; such cases were excluded. After 7–14 days in fixative the cerebrum was sliced in the coronal plane. The standard coronal slice for microscopic examination of the ganglionic eminence was immediately anterior to the interventricular foramen (Bayer and Altman, 2004). This sample and others (typically 8–12 samples per brain including dorsal frontal, middle temporal and parietal cerebrum, thalamus, hippocampus and inferior temporal cerebrum, midbrain, pons, medulla, cerebellum and spinal cord) were embedded in paraffin. Sections (6 μm thick) were cut and stained with haematoxylin and eosin and solochrome cyanin for myelin. Maximum thickness of the ganglionic eminence was measured perpendicular to the ventricular surface using an ocular reticle. Distinction of the ventricular zone, subventricular zone and intermediate zone was done in accordance with current understanding of human neocortex development (Bystron et al., 2008).

**Cell proliferation markers**

Immunohistochemical labelling of proliferating cells was performed to demonstrate the Ki67 nuclear antigen by pressure cooking tissue sections for 10 min (120°C), reacting them with the primary antibody for 1 h and revealing the antigen with a peroxidase-conjugated secondary antibody followed by diaminobenzidine reaction and haematoxylin counterstaining (Dako Envision system). Negative controls had the primary antibody incubation omitted. The on-slide control was a section of lymph node. In a subset of cases, phosphorylated histone H3 in proliferating cells (Carney et al., 2007) was detected by immunohistochemistry and in situ hybridization was performed to demonstrate the H3 histone messenger RNA (Konishi et al., 1996; Hewitson et al., 2006). Paraffin sections were dewaxed, rehydrated, exposed to proteinase K (15 μg/ml) for 30 min, hybridized for 2.5 h at 37°C, reacted with alkaline phosphatase-conjugated anti-fluorescein isothiocyanate for 1 h, exposed to substrate overnight and counterstained with haematoxylin. The slides were processed in duplicate on separate occasions. Negative controls consisted of probe omission and a non-specific probe.

**Quantitation of cell proliferation**

Ki67 labelling indices were determined at ×400 ocular magnification using a calibrated ocular grid. Only strongly labelled nuclei were counted. Unlabelled nuclei counterstained with haematoxylin were counted in the same region so that the proportion of positive cells could be calculated. In the subventricular zone of the ganglionic eminence, areas 250 × 250 μm were assessed taking care not to include large blood vessels or haematomata within the counting region. For reasons explained in the ‘Results’ section, reported values are from the ventral region of the ganglionic eminence. For the ventricular zone, bands 50 × 200 μm overlying the ventral ganglionic eminence were assessed. In the intermediate zone dorsal to the ganglionic eminence, areas 250 × 250 μm were assessed. Counts were done with only partial blinding; the precise age and survival period of the individual were masked but the overall morphology and the presence of blood could not be obscured.

**Characterization of cell lineage markers, cell death, reactive changes and inflammation**

At all ages described, the lineage status of cells in the ganglionic eminence was assessed with antibodies to glial fibrillary acidic protein, NeuN (Sarnat et al., 1998), NG2 (Staugaitis and Trapp, 2009), Olig2 (Jakovcevski and Zecevic, 2005a), platelet-derived growth factor receptor alpha (PDGFRα) (Rakic and Zecevic, 2003a; Wilson et al., 2006), polysialated neural cell adhesion molecule (PSA-NCAM) (Ulfög and Chan, 2004), S100 (Wilkinson et al., 1990), SOX2 (Pevny and Nicolis, 2010) and vimentin (Sasaki et al., 1988; Howard et al., 2006).
Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) was used to detect dying cells with fragmented DNA. Apoptotic cells were detected using antibody to activated caspase 3. Tissue was stained with Perls Prussian blue to detect haemosiderin. Reactive astrocytes were detected with antibody to glial fibrillary acidic protein. Inflammation was demonstrated in several ways. The chloroacetate esterase method was used to stain neutrophils (Leder, 1964). Antibody to human leucocyte antigen (HLA)-DR was used to detect activated microglia. Antibodies to CD3, CD4 and CD8 were used to assess lymphocyte infiltrates. For all antibodies positive control tissue was run in parallel, and negative controls were processed with omission of the primary antibody. Localization of antibodies was demonstrated with the Dako Envision system and diaminobenzidine precipitation. Table 1 summarizes the antibodies and staining methods used.

### Statistical analysis

The proportion of Ki-67-positive cells was calculated in age clusters of 22–23, 24–25, 26–27, 28–29 and 30–33 weeks. The mean values of the groups were compared using analysis of variance and Fisher’s least significant difference test.

### Table 1 Antibodies and reagents used for cell identification

<table>
<thead>
<tr>
<th>Detection target</th>
<th>Cell characteristic</th>
<th>Antibody (or other reagent)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki67</td>
<td>Proliferating cells (late G1–M phase)</td>
<td>Mouse monoclonal anti-Ki67, MIB1 clone; 1/200 dilution; heat retrieval</td>
<td>Immunotech S.A</td>
</tr>
<tr>
<td>H3 histone protein</td>
<td>Proliferating cells (S phase)</td>
<td>Rabbit polyclonal anti-phospho-histone H3 Ser10</td>
<td>Cell Signalling Technology</td>
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<tr>
<td>H3 histone messenger RNA</td>
<td>Proliferating cells (pre-S phase)</td>
<td>Fluorescein-conjugated NCL histone-513 probe</td>
<td>Novocastra Laboratories Ltd</td>
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<tr>
<td>Caspase-3 (activated)</td>
<td>Apoptotic cells</td>
<td>Rabbit polyclonal anti-activated caspase 3; 1/20 dilution</td>
<td>Genetex Inc.</td>
</tr>
<tr>
<td>TUNEL</td>
<td>DNA fragments in dying cells</td>
<td>TUNEL; Apoptag HRP kit</td>
<td>Oncor Inc.</td>
</tr>
<tr>
<td>Glial fibrillary acidic protein</td>
<td>Astrocytes, maturing ependyma (Sarnat, 1992)</td>
<td>Rabbit polyclonal anti-gliarial fibrillary acidic protein; 1/3000 dilution</td>
<td>DakoCytomation</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Glial processes including radial glia (Honig et al., 1996; Howard et al., 2006)</td>
<td>Mouse monoclonal anti-vimentin, clone V9; 1/100 dilution; heat retrieval</td>
<td>DakoCytomation</td>
</tr>
<tr>
<td>S100</td>
<td>Maturing astroglia (Wilkinson et al., 1990)</td>
<td>Mouse monoclonal anti-S100 clone 15E2E2; 1/200 dilution</td>
<td>Biogenix</td>
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<tr>
<td>Olig2</td>
<td>Oligodendroglial precursors (Takebayashi et al., 2000; Jakovec and Zecevic, 2005a)</td>
<td>Rabbit polyclonal anti-Olig2; 1/100 dilution</td>
<td>Chemicon</td>
</tr>
<tr>
<td>PDGFRα</td>
<td>Oligodendroglial precursors (Rakic and Zecevic, 2005a)</td>
<td>Rabbit polyclonal anti-human PDGFRα, SC 338; 1/400 dilution Mouse monoclonal clone 2-2B; 1/2000 dilution</td>
<td>Santa Cruz Biotechnology, Inc.</td>
</tr>
<tr>
<td>PSA-NCAM</td>
<td>Migrating cells (Gago et al., 2003; Ulfig and Chan, 2004)</td>
<td>Mouse monoclonal clone 2-2B; 1/2000 dilution</td>
<td>Chemicon/Millipore</td>
</tr>
<tr>
<td>NG2</td>
<td>Gliol precursors and microglia (Nishiyama et al., 1997; Staugsaitis and Trapp, 2009; Geha et al., 2010)</td>
<td>Rabbit polyclonal anti-rat chondroitin sulphate proteoglycan; 1/125 dilution</td>
<td>Chemicon/Millipore</td>
</tr>
<tr>
<td>SOX2</td>
<td>Neural stem cells and neuronal progenitors (Pevny and Nicolis, 2010)</td>
<td>Goat polyclonal anti-human SOX2; 1/500 dilution; heat retrieval</td>
<td>Neuromics</td>
</tr>
<tr>
<td>NeuN</td>
<td>Neuronal lineage (Sarnat et al., 1998)</td>
<td>Mouse monoclonal anti-NeuN clone MAB377; 1/500 dilution; heat retrieval</td>
<td>Chemicon/Millipore</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>Activated microglia (Esiri et al., 1991)</td>
<td>Mouse monoclonal anti HLA-DR, clone TAL.185; 1/500 dilution; heat retrieval</td>
<td>DakoCytomation</td>
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<tr>
<td>CD3</td>
<td>T lymphocytes</td>
<td>Mouse monoclonal anti CD3, clone F7.2.38; dilution; 1/200 dilution; heat retrieval at pH 9</td>
<td>DakoCytomation</td>
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<tr>
<td>CD4</td>
<td>‘Helper’ T lymphocytes</td>
<td>Mouse monoclonal anti CD4, clone 4B12; 1/25 dilution; heat retrieval</td>
<td>Novocastra</td>
</tr>
<tr>
<td>CD8</td>
<td>‘Killer’ T lymphocytes</td>
<td>Mouse monoclonal anti CD8, clone 4B11; 1/40 dilution; heat retrieval at pH 9</td>
<td>Novocastra</td>
</tr>
<tr>
<td>Chloroacetate esterase</td>
<td>Neutrophils</td>
<td>Mouse monoclonal anti-p53, clone DO-7; 1/100 dilution; heat retrieval</td>
<td>DakoCytomation</td>
</tr>
<tr>
<td>p53</td>
<td>Cell cycle arrest protein</td>
<td>Mouse monoclonal anti-p53, clone DO-7; 1/100 dilution; heat retrieval</td>
<td>DakoCytomation</td>
</tr>
</tbody>
</table>
square difference test for post hoc intergroup comparisons (StatView 5.1 software, SAS Institute Inc).

Results

Germinal layers in control brains

The wall of the lateral ventricle frontal horn in mid-gestation foetuses is lined by the ventricular zone, a pseudo-stratified epithelium (~50–70 μm thick) of tightly packed elongated nuclei oriented perpendicular to the ventricle wall. Mitotic figures are often identified immediately adjacent to the ventricle. The underlying subventricular zone varies considerably in thickness and makes up the bulk of the ganglionic eminence. Here the germinal cells are smaller, rounder and less densely packed with abundant capillaries and veins. Mitotic figures are identified in random locations. The ganglionic eminence is thickest at 20–25 weeks post-menstrual age, after which time it quickly involutes and is reduced to a discontinuous narrow band (<250 μm) of cell clusters by 34–36 weeks (Fig. 1). Thereafter, the residual subventricular zone becomes progressively more dispersed with small subependymal and perivenous clusters persisting for up to eight post-natal weeks. Where the ventricular zone and subventricular zone begin to involute, ciliated ependymal cells appear on the ventricle surface.

Cell proliferation in control brains

Successful Ki67 labelling was achieved in all of the brain specimens. In control brains, Ki67 immunoreactivity is strong and abundant (>50% of cells) in the germinal matrix including the ganglionic eminence (Figs 1 and 2). Positive nuclei tend to be larger than unlabelled nuclei. Within the narrow ventricular zone at all ages, nuclear labelling is scattered throughout. In foetuses <18 weeks, throughout the subventricular zone the pattern of labelling is fairly homogenous with occasional patches exhibiting fewer labelled cells. However, in foetuses ≥18 weeks an abrupt transition appears with diminished Ki67 labelling in the dorsal ganglionic eminence and the extra-ganglionic germinal matrix. The posterior ganglionic eminence at the level of the interventricular foramen is smaller than over the caudate nucleus but exhibits

![Figure 1](http://brain.oxfordjournals.org/fig) Whole mount photomicrographs showing coronal sections of human foetal brain at the level of the ganglionic eminence (GE) along the wall of the frontal horn of the lateral ventricle at select ages. The upper row shows haematoxylin and eosin (H&E) stained slices and the lower row shows the corresponding Ki67 immunostained slices (brown diaminobenzidine reaction product with haematoxylin counterstain). Note that the photos are shown at the same size to illustrate proportionate changes; the magnification differs by a factor of 10 across the range. At 15 weeks post-menstrual age there is a prominent groove (arrow) dividing the dorsal and ventral regions of the ganglionic eminence (which correspond the lateral and medial ganglionic eminence referred to in the animal literature). The ventral ganglionic eminence is densely positive for Ki67, the dorsal portion slightly less so. The ventricular zone (VZ) lining the roof of the lateral ventricle is also strongly positive. A moderate proportion of cells in the intermediate zone (IZ) are positive. At the base of the brain, Ki67-positive cells are also evident around the olfactory ventricle. At 20 weeks the ganglionic eminence remains thick, but only the ventral portion is highly proliferative (arrow). From 26 weeks to 36 weeks, the densely cellular ganglionic eminence progressively involutes and the proportion of Ki67-positive cells is progressively reduced.
similar dorsoventral segmentation. Germinal tissue in the temporal lobe behaves in a manner similar to the dorsal ganglionic eminence (data not shown), with reduced proportion of Ki67-positive cells after 24–28 weeks. In foetuses 20–29 weeks, the subventricular zone of the ventral ganglionic eminence remains heavily labelled. However, within the ventral ganglionic eminence there is separation of proliferative and non-proliferative zones. Near mid-gestation (17–22 weeks) there is a clear laminar pattern, but after 24 weeks the pattern is less regular with sinuous bands of Ki67-positive cells surrounding clusters of small Ki67 negative cells (Fig. 2). By 30 weeks only the most ventral portion of the ganglionic eminence contains abundant Ki67 immunoreactive cells. Few labelled cells remain in the very thin subventricular zone of near term foetuses. Scattered Ki67 immunoreactive cells are evenly distributed in the intermediate zone, diminishing rapidly after 27 weeks but persisting in small quantities (~0.1% of cells).
Expressions of differentiation markers in the ganglionic eminence

In all of the specimens, several glial and neuronal lineage markers were evaluated by immunohistochemistry (Table 1 and Fig. 4). Olig2 is abundantly expressed in nuclei of subventricular zone cells in the ganglionic eminence from 15–29 weeks post-menstrual age. Initially the distribution is uniform but it transforms into a distinct regional pattern that reflects the pattern of Ki67-positive cells (Fig. 4D–F). In the intermediate zone an estimated 10% of cells are Olig2 positive from 15 to 22 weeks, and the proportion decreases gradually over time (data not shown). SOX2 is only expressed in the nuclei of subventricular zone cells. By 34 weeks there are only scattered cells in the subventricular zone adjacent to the developing caudate nucleus.

At 26 weeks, nests of small round PSA-NCAM cells are surrounded by bands of larger Ki67-positive cells. PSA-NCAM expression by small cells persists at term gestation in the subventricular zone and in perivascular germinal cells clusters, a time when there are few proliferating cells (Fig. 4M–O). NG2 immunostaining was present, but because of the range of cell types labelled it was not pursued. NeuN is not expressed in the ventricular zone. Within the subventricular zone, NeuN is expressed only in the hypocellular islands (data not shown), likely the site of future neurons of the basal nuclei (Sarnat et al., 1998). Glial fibrillary acidic protein is only expressed by ependymal cells that have acquired a mature morphology.
Figure 4  Immunohistochemical detection of factors related to proliferation and differentiation in the ventral ganglionic eminence of 18, 26 and 34 week post-menstrual foetuses. Ki67 (A–C) is fairly uniformly distributed in the ventricular zone (VZ) and subventricular zone (SVZ) of 18 week brain, and exhibits regions of exclusion at 26 weeks. By 34 weeks the ventricular zone has involuted and has been replaced by the ependymal cell layer with Ki67 expressed only in small clusters of the subventricular zone. Olig2 transcription factor (D–F), a nuclear marker of oligodendroglial lineage differentiation, is abundant in a deep band of the 18-week subventricular zone. Expression at 26 weeks overlaps with that of Ki67, with distinct islands of exclusion. In the 34 week subventricular zone Olig2 is present in scattered nuclei generally in the regions that do not express Ki67. SOX2 (G–I), a nuclear marker of neural stem cells and neuronal precursors, is
**Cell death in control brains**

In control brains at 15–22 weeks post-menstrual age, rare TUNEL-labelled cells (estimated <0.01%) are identified in the subventricular zone of the ganglionic eminence, usually near the boundary of the caudate nucleus. Labelled cells are not seen in other locations. In near term brains, very rare cells labelled by TUNEL and activated caspase-3 antibody are present in the involuting subventricular zone and rarely in the neocortex (data not shown).

**Reduced cell proliferation following ganglionic eminence haemorrhage**

Haematomas are irregular in shape and can involve both the dorsal and ventral regions of the ganglionic eminence. There was no attempt to separate small haematomas, which typically are present near the boundary of the developing caudate nucleus, from large haematomas, which can obliterate the ganglionic eminence. In infants with survival of <12 h, the erythrocytes stained strongly with eosin. Thereafter, erythrocytes show progressive degenerative changes with reduced eosinophilia. Regardless of haematoma size, in brains of most infants who survived <12 h, the Ki67 immunolabelling is not obviously different from controls of the same age (Fig. 5A, D and G). In infants who survived 1–16 days, Ki67 labelling in the ganglionic eminence is greatly reduced (Fig. 5B, E and H). Often a gradient is apparent, with the most pronounced effect nearest the blood and near normal Ki67 ~2.5 mm from the haematoma edge. Qualitatively, the same effect is seen in the dorsal ganglionic eminence, however because the baseline level of Ki67 is much lower, the haemorrhage-associated decrements are less obvious. Infants who survived 24–98 days have generally small amounts of residual subventricular zone and 4/10 had cystic degenerative changes in the subventricular zone. Ki67 expression remains low, but seems to be greater than in the 1–16 day survival group (Fig. 5C, F and I).

Quantitative analysis show that Ki67 is significantly reduced in the ventral subventricular zone when survival is >12 h. Ki67 expression is also substantially reduced in the ventricular zone as well as in the nearby intermediate zone (Fig. 6); statistical analysis was not possible in the latter two anatomical regions because of smaller sample sizes. In control brains from premature infants with clinical history of hypoxic periods but no haemorrhage, subventricular zone expression of Ki67 is not different from controls (Fig. 6).

**Differentiation markers following ganglionic eminence haemorrhage**

Alteration of oligodendrocyte or neuronal generation following premature birth might have adverse consequences on subsequent brain development. As with the Ki67 labelling, acute germinal matrix haemorrhage (<12 h survival) is associated with no change in Olig2, PDGFRα or PSA-NCAM. Olig2 and PDGFRα expression is reduced and restricted to scattered cells when survival is >24 h, while PSA-NCAM is not obviously changed (Fig. 7A–C). In control brains, scattered p53-positive nuclei are found in the subventricular zone at 15–20 weeks with very rare cells identified as late as 22 weeks, and none at >23 weeks (data not shown). In comparison to controls, haemorrhage was associated with p53 expression in 4/22 infants surviving <12 h, 5/15 infants surviving 1–16 days and 1/10 infants surviving 24–98 days. The earliest p53 expression was seen in an infant who survived 8 h and abundant expression was seen in 1–3 day survivors (Fig. 7D–F). The p53 seems to be upregulated shortly before Ki67 is reduced.

**Cell death following ganglionic eminence haemorrhage**

Because the germinal cells exhibit a profound reduction in proliferation when blood is nearby, it is critical to know if these cells are dying. The vast majority of germinal cells admixed with blood have normal nuclear morphology, although rare karyorrhectic nuclei can be found in the ganglionic eminence of premature infants with germinal matrix haemorrhage who survived 1–2 days. Rare activated caspase-3 positive cells were present in the subventricular zone of a single case that survived <12 h. More abundant cells were identified in the subventricular zone of three cases that survived 1–3 days (Fig. 7G–I), but the quantity is less than the p53-positive cells.

**Inflammation following ganglionic eminence haemorrhage**

To assess reactive inflammatory changes, immunostaining was performed to detect microglia and invading leucocytes. HLA-DR is not detected in control brains, but is detected in the subventricular zone and nearby structures 2–63 days after haemorrhage (Fig. 7J). The same pattern of microglial detection is seen using

![Figure 4 Continued](http://brain.oxfordjournals.org/)

expressed in a pattern that overlaps with but is not identical to that of Olig2. SOX2 is expressed in mature ependymal cells and in a larger proportion of SEZ cells at 34 weeks (I). Note that the clusters of small cells negative for Ki67, Olig2, and SOX2 are surrounded by vimentin immunoreactive processes (H*). (J–L) PDGFRα, also a marker of oligodendroglial lineage differentiation, is present throughout the ventricular zone at 18 weeks. At 26 weeks it remains in the ependymal layer at the ventricle surface, and is expressed in the small cell clusters that do not express Ki67. A 34 weeks, PDGFRα is expressed only by scattered cells deep to the Ki67 expressing clusters (arrows). (M–Q) PSA-NCAM, a surface molecule involved in glial differentiation and cell migration, is highly expressed in the ventricular zone at 18 weeks, as well as in the subventricular zone, often in a radial column pattern (arrow). This parallels vimentin immunoreactive cell processes (data not shown). At 26 weeks, strong expression persists; clusters of cells parallel to the ventricle surface show the highest expression (arrow). At 34 weeks, expression persists in the ependymal layer and around scattered cell clusters in the subventricular zone (arrows). Scale bar = 100 μm.
antibodies to CD45 and CD68 (data not shown). Some macrophages stain for haemosiderin with the Perls Prussian blue method. The earliest haemosiderin was detected in a few cells of one case at 3 days. Only half the cases with survival ≤10 days had haemosiderin, while all that survived 16–98 days had scattered haemosiderin-containing macrophages in the ganglionic eminence (Fig. 7L). Infiltrating neutrophils were detected in the vicinity of subventricular zone haematomas in infants who survived 1–2 days (Fig. 7K). Extremely rare CD3 and CD8 (but not CD4) immunoreactive T-lymphocytes were detected in 2–63 day survivors (data not shown). In hypoxic-ischaemic disease control brains with dying neurons but no haemorrhage, microglial reactions are more diffuse while infiltration by neutrophils and lymphocytes is not seen.

**Figure 5** Photomicrographs showing ganglionic eminence haemorrhages and effects on Ki67 expression. In an infant born at 25 weeks post-menstrual age with ~3 h survival (acute germinal matrix haemorrhage), blood is present in the deep ganglionic eminence and in the ventricle (A). Ki67 partitioning in the ventral ganglionic eminence is as in controls (D) and the proportion of Ki67-positive cells is high even immediately adjacent to the blood (G, arrow). In an infant born at 24 weeks post-menstrual age with 31 h survival (subacute germinal matrix haemorrhage), blood is present in the ventral ganglionic eminence (B). Ki67 expression is greatly reduced (H) except in the most ventral portion of the ganglionic eminence (E, arrow). In an infant born at 25 weeks post-menstrual age with 8 weeks survival (chronic germinal matrix haemorrhage), no blood is evident in the ganglionic eminence or ventricle (C). The ependymal layer over the ganglionic eminence is irregular and cystic spaces are present in the residual subventricular zone (F, arrow). Ki67 expression is evident immediately deep to the ependymal layer (I). The proportion of Ki67-positive cells is roughly the same as in control cases of the same post-menstrual age. Haemosiderin-containing macrophages are present on the ependymal surface (arrow). Scale bar = 2 mm for A, B, D, E; 1 mm for C; 250 μm for F; 50 μm for G, H, I.
Discussion

Development and involution of the ganglionic eminence

The general morphology of the developing human brain is well documented in the embryonic period (up to 8 weeks of gestation) (Hochstetter, 1919; Bartelmez and Dekaben, 1962; Yokoh, 1968; O’Rahilly and Müller, 2006) and in subsequent foetal development.

Figure 6 Continued

(i.e. brains with no pathological process evident; blue circles) the values climb rapidly after 14 weeks and are highest between 20 and 27 weeks, although variability is quite evident. Thereinafter the proportion of proliferating cells drops rapidly reaching values slightly > 0 by 35 weeks. Rare scattered positive cells can be detected as late as 84 weeks (full range not shown). In brains with acute germinal matrix haemorrhage (GMH; red circles) the Ki67 proportions are not obviously different than the control range. In those with subacute germinal matrix haemorrhage (i.e. survival of 1–16 days after birth; orange triangles) the values are lower than in controls. In those with chronic germinal matrix haemorrhage (i.e. survival of 24–98 days after birth; green squares) the values are also low, but because these infants are older the values are similar to the control values. In brains with probable hypoxic damage but no haemorrhage (i.e. clinical history of hypoxia, encephalopathy and histological evidence of dying neurons; cyan inverted triangles) there is no evident suppression of Ki67 immunoreactivity in the subventricular zone.

(B) Bar graph showing comparisons of the proportion of Ki67-positive cells in the ventral ganglionic eminence in control foetal brains and in brains with germinal matrix haemorrhage (GMH). In comparison to controls and brains with acute germinal matrix haemorrhage at 24–25 weeks post-menstrual age, subacute germinal matrix haemorrhage is associated with a significant reduction in proliferating cells (*P < 0.015). At 26–27 weeks, Ki67 counts in both acute and subacute germinal matrix haemorrhage are reduced in comparison to controls (*P < 0.045). At 28–29 weeks, the subacute germinal matrix haemorrhage remained significantly below control values (*P < 0.009). In the 22–23 week groups, there was no difference between control and acute germinal matrix haemorrhage groups. In the 30–33 week group, there were too few cases to make a meaningful analysis (data not shown). The data are expressed as means ± one standard error. (C) Scatter plot showing the proportion of cells immunoreactive for Ki67 in ventricular zone (VZ) overlying the ventral ganglionic eminence (GE) as a function of the corrected post-menstrual age. The proportion of positive cells is high and relatively stable from 15 to 23 weeks and drops thereafter as the ventricular zone matures. Subacute germinal matrix haemorrhage is associated with decreased expression of Ki67. Because there is often physical destruction of this layer by the haemorrhagic process there were too few cases to analyse statistically. (D) Scatter plot showing the proportion of cells immunoreactive for Ki67 in intermediate zone (IZ) dorsal to the ganglionic eminence and corpus callosum as a function of the corrected post-menstrual age. A moderate proportion of cells are in the proliferative phase from 15 to 23 weeks. Subacute germinal matrix haemorrhage is associated with decreased expression of Ki67.
Figure 7 Photomicrographs showing changes following germinal matrix haemorrhage. (A) Olig2 expression in ventral ganglionic eminence of a 24-week infant who survived 31 h. Expression is reduced in comparison to controls (Fig. 4E). (B) PDGFRα expression in ventral ganglionic eminence of a 28-week infant who survived 2 days is reduced to rare cells (arrow). (C) PSA-NCAM expression in the ventral ganglionic eminence of a 23-week infant who survived 10 days is not appreciably different than in controls of same age (Fig. 4M). (D) p53 expression in the ventral ganglionic eminence of a 28-week infant who survived 9 h. There are many positive cells in the vicinity of the blood (arrow). (E) p53 expression in the ventral ganglionic eminence of a 24-week infant who survived 31 h. There are many positive cells in the ventricular zone and subventricular zone. (F) p53 expression in the ventral ganglionic eminence of a 26-week infant who survived 33 days. This is the only case with p53 expression long after the haemorrhagic event; most positive cells are within the buried ependymal lining or among macrophages (arrow). (G) TUNEL labelling of rare dying cells (arrows) in subventricular zone of a 21-week control brain. (H) Activated caspase-3-immunoreactivity (brown) showing dying cells in the ventral ganglionic eminence of a 29-week infant with
The ganglionic eminence is not a uniform structure

A laminar expression pattern of the cell proliferation markers Ki67 and histone H3 was shown in the ventricular zone and subventricular zone. Histone H3 messenger RNA expression (S phase of the cell cycle) is present mainly in the ventricular zone furthest from the ventricle, while Ki67 (G1 to M phase of cell cycle) is present throughout the ventricular zone, and ventricular zone mitotic figures are present only at the ventricle surface. Their exclusive localization supports the existence in humans of ‘interkinetic nuclear migration’, which is the movement of neuroepithelial cell nuclei along the apical-basal axis of the cell in synchrony with the cell cycle (Sauer, 1935; Taverna and Huttner, 2010). This phenomenon, which generates the pseudostratified organization of the ventricular zone, is well established in other primates and lower mammals (Takahashi et al., 1995; Kornack and Rakic, 1998). Proliferating cell nuclear antigen labelling, of 6–20 week human foetal brains, showed a similar laminar pattern within the ventricular zone (Mollgard and Schumacher, 1993). In the subventricular zone, mitosis initially occurs at random locations (Fujita, 1960; Duckett, 1968; Zecevic, 1993). Vimentin immunoreactive glial processes are associated with the subsequently appearing clusters of proliferating cells, although it is not clear if the radial glia processes actively segregate the clusters. These proliferating cell clusters exhibit Olig2 and SOX2 suggesting that they might be a mix of stem cells and oligodendroglial precursors. Musashi1 and nestin were not reported to show clustering in the human ganglionic eminence (Chan et al., 2006). However, the germinal regions of mouse brain have recently been shown to have a mosaic pattern with distinct proliferative potentials and cell fates (Platel et al., 2009).
The intermediate zone decreased dramatically by 28 weeks but the subventricular zone, the number of proliferating cells in established existence of secondary proliferative populations. As in NCAM, suggesting a different cell lineage that could be migratory. Ki67 expression in the intermediate zone also supports the well-established existence of secondary proliferative populations. As in the subventricular zone, the number of proliferating cells in the intermediate zone decreased dramatically by 28 weeks but persisted at low levels into early infancy. This confirms a previous study that used proliferating cell nuclear antigen detection (Kendler and Golden, 1996).

Suppression of cell proliferation following haemorrhage

The basic histological features of human ganglionic eminence haemorrhages are the same as previously described (Leech and Kohnen, 1974; Takashima et al., 1979; Rorke, 1982). The novel data presented here indicate that proliferation of germinal cell populations is suppressed within 24 h (and more likely within 12 h) after haemorrhage. Our earlier mouse experiment, in which blood was injected into the periventricular region (Xue et al., 2003), and a similar rat experiment (J. Balasubramaniam and M.R. Del Bigio; unpublished results) show the same effect occurring within 6–8 h. The inherent uncertainty of haemorrhage timing in humans may explain the discrepancy (see below). The data suggest that suppression persists for up to 4 weeks and there is no evidence of rebound above normal levels in germinal matrix haemorrhage brains, suggesting that the adverse effect is lasting and that the germinal matrix continues to involute on schedule rather than compensating following germinal matrix haemorrhage. This is unlike the transient suppression with delayed compensatory increase documented in 3-day old mice subjected to 1 or 7 days hypoxia (Fagel et al., 2006). Despite the profound decrease in proliferation, there appears to be minimal cell death. In this study, only few apoptotic cells were observed in the subventricular zone after haemorrhage, although in another small study of human brains higher levels (~3%) of TUNEL-positive cells were reported after germinal matrix haemorrhage (Chamnanvanakij et al., 2002). In the mouse model, we showed that Ki67 suppression was not directly related to apoptosis (Xue et al., 2003).

The mechanism of suppressed cell proliferation in the germinal populations of human brain is not clear. The rapid effect suggests a direct cell-signalling pathway, perhaps mediated by p53 (Kurkaya-Yapicier et al., 2002; Billon et al., 2004; Tedeschi and Di Giovanni, 2009), which in this study was transiently upregulated before Ki67 was suppressed. Suppression of brain cell proliferation follows experimental hypoxia-ischaemia (Levision et al., 2001); however, our data indicate that human brains damaged by hypoxia do not suffer reduced subventricular zone proliferation even when there is evidence of dying neurons elsewhere in the brain. The current data suggest a gradient effect, suggesting that an inhibitory factor diffuses from the haematoma. In the mouse model, we showed that the plasma proteins thrombin and plasmin play an important role in the damage (Xue et al., 2005; Xue and Del Bigio, 2005b). We also demonstrated in cell culture systems a similar effect of thrombin and plasmin, as well as complement and kallikrein, on oligodendroglial precursor proliferation and migration (Juli et al., 2009). Thrombin receptors might act through the p53 pathway in brain cells (Choi et al., 2003; Guo et al., 2004), although p53-independent pathways must also be considered (Huang et al., 2000). Toxic neurotransmitters such as glutamate and γ-amino butyric acid, which could be released from damaged brain cells or platelets (Rainesalo et al., 2005), can suppress proliferation through membrane depolarization (Cameron et al., 1998; Luk and Sadikot, 2004). Haemoglobin and breakdown products might also be toxic through free radical mechanisms (Wang et al., 2002). While a direct signalling pathway could explain the initial suppression of proliferation, sustained suppression might occur through a different mechanism. In this study and others (Sherwood et al., 1978; Ulfig, 2001b), inflammatory cells infiltrated and were activated in the vicinity of the germinal matrix haemorrhage. Cytokines such as tumour necrosis factor alpha and interferon gamma (Wu et al., 2000), as well as the chemokines CXCL1 and CXCR2 (Tsai et al., 2002), might be involved in regulating cell proliferation. In neonatal mouse studies, we showed that prior systemic activation of the immune system aggravates brain damage after intracerebral blood injection (Xue and Del Bigio, 2005a).

Potential delayed effect of haemorrhage

Although not analysed in this experiment, we must consider the potential consequences of suppressed brain cell proliferation following premature birth and germinal matrix haemorrhage. Reduced oligodendrocyte development, suggested by decreased Olig2 and PDFGRα immunoreactivity, would have an adverse effect on cerebral myelination (Jakovcevski and Zecevic, 2005a, b), although it will be difficult to separate this effect from the hypoxic-ischaemic damage that also occurs in premature infants (Folkert, 2005). Germinal matrix haemorrhage in extremely premature infants has the potential for disrupting the production of GABAergic interneurons for the neocortex (Petanjek et al., 2009). Furthermore, the ventral ganglionic eminence supply of neuron precursors to the thalamus could be interrupted (Rakic and Sidman, 1969; Letinic and Rakic, 2001). Not directly related to cell proliferation, developing cortical association pathways located adjacent to the ganglionic eminence might also be damaged by haemorrhagic events (Vasung et al., 2011). Golgi impregnation studies have shown that germinal matrix haemorrhage can disrupt radial glia and can be associated with subtle changes in the outer neocortex (Marin-Padilla, 1996).

Limitations

This study is limited by factors related to use of human autopsy material. Because fixation conditions vary, immunohistochemical reactions might not be optimal for some of the cell lineage markers. Further work will be needed to assess co-expression of Ki67 with glial and neuronal differentiation factors. The actual timing of...
the haemorrhagic event is seldom known with certainty. We can be fairly confident that those defined as acute haemorrhage are accurate to within a few hours. For those who lived longer (the 1–16 days subacute germinal matrix haemorrhage group and the 24–98 days chronic germinal matrix haemorrhage group), the potential for misclassification is greater. It has been shown on serial ultrasound studies that up to half of haemorrhages are detected within 8 h of premature birth, but haematomas can appear or enlarge days after premature birth (Dolfin et al., 1983; Beverley et al., 1984; Rumack et al., 1985; Paneth et al., 1993). The weakness of these studies is the sensitivity of ultrasound imaging. Until MRI is routinely feasible in the immediate post-partum period, we will probably not know when these haemorrhages truly begin. We are thus forced to make assumptions about timing, which could be wrong in up to half of the cases. The decline of Ki67 expression appears to occur at the 12–24 h interval, during which we have reasonable timing certainty. Note that haemosiderin was detected in only a few cases with haemorrhage and survival of <10 days. This suggests that the processing of blood in the immature brain might be slower than in the adult brain, in which haemosiderin typically appears after 3–4 days (Oehmichen et al., 2009). Alternately, the assumed survival after haemorrhage is incorrect. However, reanalysis of the data assuming that the absence of haemosiderin indicated a survival of <3–4 days did not change the Ki67 result. Note also that the irregular shapes of the haematomas and the frequency of blood extension into the ventricles prevents absolute analysis for a gradient effect.

Conclusion

The highly proliferative human ganglionic eminence exhibits partitioning at macroscopic and microscopic levels during mid-foetal development. At the macroscopic level this might reflect production in the ventral ganglionic eminence of neuronal precursors destined for the thalamus. Microscopic organization might be a way of creating local environmental conditions necessary for the maintenance of germinal cell populations. Given that these features persist into the late foetal period, the consequences of premature birth might adversely affect these proliferating populations, which in turn could be associated with abnormal brain development. This finding may help to explain the reduced cerebral volume and the impaired developmental outcomes in survivors of such insults. The molecular mechanism for the suppression is not established by examination of autopsy brains but p53 might play a role. Future detailed analysis of transcription factors in human brains and in animal models, as well as exploration of mediators in animal and cell culture models, will be needed to elucidate the precise cellular mechanism of this injury.

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