Oxidative Injury in the Cerebral Cortex and Subplate Neurons in Periventricular Leukomalacia

Rebecca D. Folkerth, MD, Felicia L. Trachtenberg, PhD, and Robin L. Haynes, PhD

Abstract

We previously identified immunocytochemical evidence of nitrative and oxidative injury in premyelinating oligodendrocytes in periventricular leukomalacia (PVL). Here, we tested the hypothesis that free radical injury occurs in the overlying cerebral cortex and subplate neurons in PVL. We immunostained for nitrotyrosine, malondialdehyde, and hydroxynonenal adducts and scored neuron staining density in PVL (n = 11) and non-PVL (n = 15) cases (postconceptional ages from 34 to 109 weeks). Analysis of covariance controlled for age. Mean malondialdehyde scores in PVL cases were increased over controls (p = 0.005). Hydroxynonenal scores increased with age only in PVL cases (diagnosis vs age interaction; p = 0.024). Nitrotyrosine scores were not significantly increased. In 11 PVL and 23 control cases between 20 and 183 postconceptional weeks, cells morphologically consistent with subplate and Cajal-Retzius neurons showed qualitatively increased free radical modification in PVL over control cases with statistically significant odds ratios for hydroxynonenal and nitrotyrosine in both subplate neurons and Cajal-Retzius cells. Glial fibrillary acidic protein and CD68 scores for reactive astrocytes and microglia, respectively, were not significantly increased, suggesting a minimal inflammatory response. Thus, oxidative/nitrative damage to cortical and “pioneer” neurons, although mild overall, may contribute to cortical volume loss and cognitive/behavioral impairment in survivors of prematurity.

Key Words: Cerebral palsy, Dendritogenesis, Free radical injury, Prematurity, Synaptogenesis.

INTRODUCTION

Periventricular leukomalacia (PVL), the major neuropathologic substrate of the motor deficits of cerebral palsy, is characterized by focal periventricular necrosis and diffuse gliosis in the surrounding immature white matter. Preterm infants with and without PVL also develop significant cognitive and behavioral abnormalities (1). In premature infants weighing less than 1,500 g at birth, for example, 25% to 50% will develop cognitive impairments ranging in severity from subtle learning disabilities to mental retardation (1). Term-born infants with congenital heart or respiratory disease also are at risk for PVL and its complications (2). Recently, we noted the previously underemphasized occurrence of cerebral gray matter gliosis and neuronal loss in autopsy cases of PVL in the modern era of neonatal intensive care (3). Neuroimaging studies likewise indicate reduced cerebral cortical gray matter volume in premature infants studied at term equivalent, with or without PVL, compared with term control infants (4, 5). Furthermore, several studies report reduced cortical volumes that correlate with gestational age at birth and neurodevelopmental disability (4–10).

Although the pathogenesis of PVL is multifactorial, a major cause seems to be cerebral ischemia/reperfusion in the immature white matter that preferentially targets vulnerable premyelinating oligodendroglial cells (11–13). These cells are characterized by expression of O4 and O1, but not myelin basic protein, and are susceptible to free radical injury in PVL (14, 15). Here, we hypothesized that subtle oxidative and nitrative injury to the developing cerebral cortical gray matter, subplate-like neurons, and/or Layer I neurons (including Cajal-Retzius-like cells) is also present in the brains of infants dying with PVL compared with controls of similar ages who died without PVL. We evaluated the degree of oxidative and nitrative injury in neurons in the cerebral cortex (Layers I, III, and V) and subplate in postmortem brain specimens in cases with and without PVL using immunocytochemical methods on the same data set (i.e. same tissue sections) in which we had found significant levels of such injury in the white matter (15). We applied antibodies against malondialdehyde-protein adduct (MDA) and 4-hydroxy-2-nonenal (HNE; markers of lipid peroxidation), nitrotyrosine (NT; a marker of protein nitration), and CD68 (a macrophage/microglia marker), and glial fibrillary acidic protein (GFAP; a marker for reactive astrocytes), as previously published (15). The latter 2 antibodies are used commonly in diagnostic neuropathology practice and were included as indicators of cellular response to neuronal injury. Because microglia are sources of nitric oxide (NO) and peroxynitrite, and contribute to developing oligodendrocyte injury and death (16, 17), we also sought to determine whether microglial...
activation was associated with the markers of nitrative and/or oxidative stress.

MATERIALS AND METHODS

Clinicopathologic Database Information

Cases were collected from the autopsy services of the Departments of Pathology, Children’s Hospital, and Brigham and Women’s Hospital, Boston, with permission according to hospital protocols. All cases were autopsied between 1993 and 2001 and were classified by the systematic examination of standardized microscopic sections (maximum of 19 sections per case), stained with hematoxylin and eosin/Luxol fast blue, from each brain and spinal cord (the latter available on 8 cases). We defined PVL based upon the combination of ‘focal’ and ‘diffuse’ components as determined by gross and microscopic examination (18). The focal component consists of periventricular necrosis in which all tissue elements are destroyed analogous to the “core” of an infarct. The diffuse component is composed of reactive gliosis and microglial activation in the deep white matter surrounding the necrotic foci. The areas of predilection for PVL are the frontal, parietal, and occipital white matter regions. Acute PVL is identified histopathologically by coagulative necrosis, characterized by nuclear pyknosis (shrinking) of all cell types and axonal swellings (spheroids), and corresponds to injury within the preceding 8 to 24 hours (18). Subacute or organizing PVL develops between 3 and 5 days after the insult and is identified by infiltration of activated microglia, macrophages, and hypertrophic astrocytes at the onset of tissue loss and cavitation (18). Chronic PVL is recognized as cystic cavitation, glial scar formation, and/or mineralization associated with diffuse astrogliosis and microgliosis within weeks to months after the insult (18). Control cases were defined by the absence of PVL upon gross and microscopic neuropathologic examination. Among the PVL cases, we selected blocks for study from areas of the brain affected only by PVL because some cases had gray matter injury elsewhere in the brain (Table 1). Because our hypothesis addressed the presence or absence of neuropathology in PVL and control cases, respectively. Five PVL and 5 control cases had also been subjected to the intensive survey of gray matter injury reported previously (3). “Cortex overlying PVL” was defined as the segment of cortex nearest the foci of periventricular necrosis associated with diffuse white matter gliosis. When the section orientation allowed, the segment of cortex counted was located radially along a perpendicular line from the ventricular to the pial surface. Postmortem interval for the entire cohort ranged from 1.5 to 132 hours, with a mean of 18.3 and a median of 14 hours. There was no obvious effect of postmortem interval upon the degree of immunostaining. Statistical analyses were also performed to determine the effect of postmortem interval on the scored staining results.

Immunocytochemistry in Formalin-Fixed, Paraffin-Embedded Tissue

Standard methods on deparaffinized tissue sections (4 µm) were applied as previously described (15). Antibodies specific for the following markers were used at the indicated dilutions: HNE (1:100; from the laboratory of Dr. Luke I. Szewda, or purchased from Calbiochem, San Diego, CA); MDA (1:100; Abcam, Cambridge, UK); NT (1:100; Upstate, Lake Placid, NY); GFAP (1:9000, Sternberger Monoclonals Incorporated, Lutherville, MD), and CD68 (1:50, Cell Marque, Austin, TX). These were characterized for their specificity as previously reported (15). Optimal dilutions were determined using Alzheimer and amyotrophic lateral sclerosis brain and spinal cord tissue, in which free radical injury is known to occur, as positive controls. “Positive” control staining consisted of the glial immunoreactivity in the white matter in the same blocks as previously reported (15). In addition, a case with a segmental cortical infarct was stained as a positive neuronal control. Tonsil was used as the positive control for CD68 immunostaining; negative controls were determined using Alzheimer and amyotrophic lateral sclerosis brain and spinal cord tissue, in which free radical injury is known to occur, as positive controls. “Positive” control staining consisted of the glial immunoreactivity in the white matter in the same blocks as previously reported (15). In addition, a case with a segmental cortical infarct was stained as a positive neuronal control. Tonsil was used as the positive control for CD68 immunostaining; negative controls were the omission of the primary antibodies. To check for nonspecific staining, the NT antibody was preabsorbed and incubated overnight at 4°C; 3,3’-diaminobenzidine HCl detection was then performed as described.

Grading Method and Statistical Analysis

Grading of the immunostained tissue sections was performed by counting positive cells per high-power field (hpf) at 400× magnification (0.173 mm² on an Olympus BH-2 microscope), as previously described in the white matter in the same immunostained tissue sections (15). The hpfs were selected on the basis of the greatest density of immunostaining after visual survey of all fields at low magnification. Immunostaining was cytoplasmic for all the antibodies analyzed. Positively stained neurons were counted if they
were located within Layers II to VI of the cerebral cortex. The grading system was as follows: 0, no cell staining; ½, 1 immunopositive cell/hpf; 1, 2 to 10 cells/hpf; 2, 11 to 20 immunopositive cells/hpf; and 3, more than 20 cells/hpf, according to our published protocol (15, 19–21). For assessment of the immunostaining of the subplate and Cajal-Retzius neurons, a binary scheme was used as follows: 0, no staining; and 1, staining of the specific cell type present. Subplate neurons and Cajal-Retzius cells were separately scored according to this grading system because the densities of these cell types are relatively low, and they are not amenable to the cell counting procedure used in the cerebral cortex in which neurons are more densely packed. Cajal-Retzius cells were identified by their characteristic large polygonal morphologies as previously described (22). Separate counting of the different cell types was not done. In view of the lack of specific markers for these 2 cell types in human archival

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material, we relied on histomorphology, recognizing that the terms subplate-like and Cajal-Retzius-like may be more appropriate. To simplify discussion, however, we use the shorter designations in this report. Two observers scored each case without knowledge of clinical variables, including age. Because the presence of focal necrosis and diffuse gliosis are the defining features of PVL, however, blinding to this diagnosis was not possible.

Analysis of covariance was performed to test for significant differences in marker scores between PVL and control cases, controlling for postconceptional age. Cases (n = 11) and controls (n = 15) over similar developmental intervals (34–109 postconceptional weeks) were used for the cortical neuron density values, and cases over the entire age range (n = 11 PVL and 23 controls; 20–183 postconceptional weeks) were used for the binary subplate and Cajal-Retzius values. If there was a significant interaction between diagnosis and age, it was included in the model. Correlations between markers in the cortex and white matter were calculated. The latter values were previously published as a separate report (15). Additionally, regressions of postmortem interval, as well as postnatal age, on markers were performed. Finally, the Spearman correlation of subplate and Cajal-Retzius immunostaining to cortical immunostaining as computed. In all analyses, p < 0.05 was considered significant; values between 0.05 and 0.10 were considered to indicate a trend. Because of the exploratory nature of this work, we report p values between 0.05 and 0.10.

RESULTS
Clinical and Neuropathologic Findings

Findings are summarized in Tables 1A and B. The PVL cases ranged in postconceptional age from 34 to 95 weeks (median, 40 weeks), and the controls ranged from 20 to 183 postconceptional weeks (median, 40 weeks). Of the 11 PVL cases, 9 (81.8%) were born prematurely (<37 weeks); 3 of these 9 infants survived beyond the neonatal period (>44 postconceptional weeks). In the control group, 9 of 23 (39.1%) were born prematurely, but none survived beyond the perinatal period. The premature born PVL cases had Potter syndrome/renal dysplasia (n = 3), skeletal dysplasia (n = 2), complications of prematurity (n = 2), and congenital heart disease and pulmonary hypertension (n = 1 each) (15). The clinical courses of the full-term PVL infants were complicated by an inborn error of the urea cycle in 1 case and congenital heart malformation requiring surgery and extracorporeal membrane oxygenation in another (15). In 6 PVL cases, focal cystic cavities measuring up to 3 mm were visible macroscopically in the periventricular white matter. Microscopically, 4 cases had acute coagulative necrosis characterized by fragmentation of all tissue elements and pyknotic or absent nuclei, indicative of an acute insult of 24 to 48 hours’ duration (18); 7 cases had necrotic foci with subacute changes (i.e., with infiltration of macrophages and beginning cavitation) (18), and 7 cases had chronic changes (i.e., cyst, glial scarring, or mineralization), indicating the site of prior focal necrosis (18). Five cases had focal lesions of more than 1 histopathologic age. Six PVL cases had expanded ventricles and/or decreased white matter volume, and 3 had hypomyelination; these cases all had either organizing or chronic PVL lesions (15). Affected lobes from which blocks were used for this study included parietal (n = 4), parietooccipital (n = 1), occipital (n = 1), and frontal (n = 2); 3 blocks were from cerebral cortex, not otherwise specified. With respect to gray matter injury among the PVL cases, 1 had focal neuronal loss associated with focal cortical hemorrhage, and 1 had a focal cortical infarct; for both cases, the lesions were in a different section than the 1 graded semiquantitatively in the study. In the blocks used for immunohistochemistry, the overlying cortex was free of hypoxic-ischemic injury by light microscopy. In the cases without PVL, 4 were extremely premature and had respiratory insufficiency and/or necrotizing enterocolitis; 1 was born at 35 weeks and died of germinal matrix hemorrhage. The other 4 premature controls had a congenital cardiac anomaly (n = 2, one as a component of the VACTERL association), 1 had parvovirus-associated anemia and hydrops, and 1 was an unexplained stillbirth at 34 weeks. Of the controls born at term, 2 had congenital heart disease, 2 had congenital renal dysplasia, 3 had a chromosomal abnormality (trisomy 21, 12–14 translocation, Noonan syndrome; n = 1 each), and 1 each had spinal muscular atrophy, pulmonary hypoplasia, pulmonary hypertension, primary biliary atresia (status post liver transplant), viral gastroenteritis and dehydration, sudden unexpected death in childhood, and bronchopneumonia. As in the PVL cases, cerebral cortical blocks were free of infarcts, inflammation, or significant hemorrhage. Control cortex and white matter blocks were chosen on the basis of availability of periventricular white matter and well-oriented overlying cortex; they were comparable in size to those obtained from PVL cases. The locations included parietal lobe (n = 4), occipital lobe (n = 2), frontal lobe (n = 7), and cerebral cortex, not otherwise specified (n = 10).

In the case with the cortical infarct used as a positive control, the gestational age was 40 weeks, postnatal age was 4 weeks, and postconceptional age was 44 weeks. The infant had an unattended delivery with asphyxia and was found to have a congenital diaphragmatic hernia requiring extracorporeal membrane oxygenation. Although the child also had PVL, the block chosen for comparative staining contained an infarct of several days’ duration; this case was not scored.

Markers of Free Radical Injury in PVL and Non-PVL Cortex

Overall, there was mild oxidative and nitrative injury as determined by immunostaining of Layer V and, to a lesser extent, Layer III pyramidal neurons (Figs. 1A–C). This injury was detectable as individual immunopositive neurons within these layers in the cerebral cortical ribbon present on the section and by variable immunostaining of the background neuropil. By semiquantitative scoring, PVL cases showed age-adjusted mean scores for MDA immunostaining in cortical neurons of 0.65 (range, 0–2; median, 1), whereas controls showed age-adjusted means of 0.06 (range, 0–1; median, 0; p = 0.005; Fig. 2; Table 1). In the age range studied, PVL HNE scores were significantly higher than control HNE scores (p = 0.024; Fig. 3; Table 1), with an increase in HNE score with
Therefore, conclusions past approximately 60 postconceptional weeks are tentative. Nitrotyrosine immunostaining was present in cerebral cortical neurons in only 2 PVL cases (mean score, 0.22; range, 0–1; median, 0; Fig. 1C), whereas none of the controls were labeled, a marginal difference (p = 0.091; Fig. 2; Table 1). Staining in the cortex overlying PVL was qualitatively less than that seen in a representative section of a cortical infarct (Fig. 1E). Negative controls (omission of primary antibody and excess protein preabsorption) demonstrated no staining (data not shown).

We next addressed the question of whether free radical injury in the cortex overlying PVL correlates with the degree of free radical injury in the white matter (Fig. 1F) that we previously documented (15), but no significant relationship was identified between the white matter and cortex scores for any of the markers used (data not shown). To determine whether infants that survived longer had greater degrees of cortical injury, the effect of postnatal age on the study population was explored, but we found no influence of postnatal age (either as a continuous variable or in analysis restricted to cases with postnatal age greater than 5 days) on marker scores in the cortex (data not shown). Likewise, no effect of gestational age (i.e. degree of maturity at birth) or of postmortem intervals on marker scores was identified (data not shown).

The subplate was stained by at least 1 free radical adduct marker in 10 of 11 PVL cases, with 5 cases immunostained for all 3 markers (Fig 4A; Table 2A). Cajal-Retzius neurons were also immunopositive for at least 1 of these markers in 9 of 11 PVL cases and in 3 cases for all 3 markers (Fig. 4B; Table 2B). For both cell populations, approximately 2- to 3-fold increases in number of positive cells were detected for each marker in PVL versus control cases (Table 2). In control cases, subplate and Cajal-Retzius cells were immunopositive for HNE and MDA even if there was no staining of cortical Layers II to VI (Table 2). Subplate and Cajal-Retzius neurons showed qualitatively increased...
evidence of free radical modification in PVL cases over controls, with statistically significant odds ratios of 20.9 (confidence interval [CI], 2.2–199) for HNE and 9.1 (CI, 1.3–63.1) for NT in subplate neurons, and 12.6 (CI, 1.2–135) for HNE and 7.3 (CI, 1.2–45.1) for NT in Cajal-Retzius cells (Tables 3, 4).

Markers of Inflammatory Reaction in the Cerebral Cortex Overlying PVL

Fibrillary gliosis assessed by GFAP staining was mild in the cerebral cortex overlying PVL (Figs. 1G, 2). The age-adjusted mean score of GFAP-positive astrocytes in the cortex in the PVL cases was 0.97 (range, 0–3; median, 0.5) compared with a mean score of 0.39 (range, 0–1; median, 0) in the controls (p = 0.100; Table 1). Anti-CD68 staining showed variable densities of immunopositive reactive microglia in the PVL cortex, with an age-adjusted mean score of 0.73 (range, 0–1; median, 1), compared with 0.33 for controls (range, 0–2; median, 0; p = 0.060; Figs. 1H, 2). There was no significant correlation between any of the oxidative and nitrative marker scores and the scores for the inflammatory cell markers (data not shown).

DISCUSSION

The major finding of this study is the presence of oxidative and nitrative injury in the cerebral cortex overlying PVL and in subplate neurons and Cajal-Retzius cells. This finding, albeit subtle, supports the occurrence of mild gray matter and white matter injury in PVL. The concept of gray matter injury in PVL is not entirely new, but it is gaining in importance as prematurely born infants survive longer and reach school age, and cognitive and behavioral deficits emerge that are not readily explained by cerebral white matter damage alone (1, 23). Thus, interest has arisen concerning the extent, associations, and causes of such gray matter injury. Quantitative magnetic resonance imaging data suggest a significant reduction in cerebral cortical volume in premature infants studied at term equivalent compared with term infants, a finding that is especially pronounced in infants with PVL (4, 5). In our

![FIGURE 3](https://example.com/figure3)

**FIGURE 3.** Relationship between age and 4-hydroxy-2-nonenal (HNE) marker scores in periventricular leukomalacia (PVL; gray dots) versus controls (black dots) across 34 to 109 postconceptional weeks. With increasing age, HNE adduct formation increases significantly in the cerebral cortex in PVL but not in controls (see text for discussion).

![FIGURE 4](https://example.com/figure4)

**FIGURE 4.** Immunostaining of subplate neurons for 4-hydroxy-2-nonenal (A) and Cajal-Retzius cells for malondialdehyde-protein adduct (B) 40th postconceptional weeks, periventricular leukomalacia case. Original magnification: 400× for each.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Control</th>
<th>PVL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subplate neurons</td>
<td>HNE</td>
<td>7/22 (32%)</td>
</tr>
<tr>
<td></td>
<td>MDA</td>
<td>11/22 (50%)</td>
</tr>
<tr>
<td></td>
<td>NT</td>
<td>2/21 (10%)</td>
</tr>
<tr>
<td>Cajal-Retzius cells</td>
<td>HNE</td>
<td>8/19 (42%)</td>
</tr>
<tr>
<td></td>
<td>MDA</td>
<td>8/21 (38%)</td>
</tr>
<tr>
<td></td>
<td>NT</td>
<td>0/21 (0%)</td>
</tr>
</tbody>
</table>

Numbers in each cell indicate the fraction of cases with positive immunostaining for the marker over the total number of cases with available staining (percentage). HNE, 4-hydroxy-2-nonenal; MDA, malondialdehyde-protein adduct; NT, nitrotyrosine; PVL, periventricular leukomalacia.

### TABLE 2. Age-Adjusted Means of Marker Scores in Cerebral Cortical Neurons in PVL and Control Cases

<table>
<thead>
<tr>
<th>Marker</th>
<th>PVL</th>
<th>Controls</th>
<th>p</th>
<th>DX × PCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA</td>
<td>0.65 (0.14)</td>
<td>0.06 (0.12)</td>
<td>0.005</td>
<td>NS NS NS</td>
</tr>
<tr>
<td>HNE</td>
<td>1.23 (0.15)</td>
<td>0.00 (0.13)</td>
<td>NS NS</td>
<td>0.024</td>
</tr>
<tr>
<td>NT</td>
<td>0.20 (0.09)</td>
<td>0.00 (0.07)</td>
<td>0.091</td>
<td>NS NS</td>
</tr>
<tr>
<td>GFAP</td>
<td>0.97 (0.26)</td>
<td>0.39 (0.22)</td>
<td>0.100</td>
<td>0.099 NS</td>
</tr>
<tr>
<td>CD68</td>
<td>0.73 (0.15)</td>
<td>0.33 (0.13)</td>
<td>0.060</td>
<td>0.082 NS</td>
</tr>
</tbody>
</table>

NS and p > 0.100, ≥0.05, and <0.100 are included to indicate possible trends for future study; p values <0.050 are captured in bold. Range of marker scores is 0 (no staining) to 3 (>20 positive cells per high-power field; see text for details). Ages of analyzed cases are 34 to 109 postconceptional weeks (see text for details).

DX, diagnosis (PVL vs control); GFAP, glial fibrillary acidic protein; HNE, 4-hydroxy-2-nonenal; MDA, malondialdehyde-protein adduct; NS, not significant; NT, nitrotyrosine; PCA, postconceptional age; PVL, periventricular leukomalacia; SE, standard error of the mean.
TABLE 4. Logistic Regression Odds Ratios of Markers in PVLs Versus Controls

<table>
<thead>
<tr>
<th>Marker</th>
<th>Odds Ratio (95% CI)</th>
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<tr>
<td>Subplate neurons</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HNE</td>
<td>20.92 (2.20–198.62)</td>
<td>0.008</td>
</tr>
<tr>
<td>MDA</td>
<td>4.66 (0.80–27.19)</td>
<td>0.087</td>
</tr>
<tr>
<td>NT</td>
<td>9.15 (1.33–63.11)</td>
<td>0.025</td>
</tr>
<tr>
<td>CJal-Retzius cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HNE</td>
<td>12.57 (1.17–135.2)</td>
<td>0.037</td>
</tr>
<tr>
<td>MDA</td>
<td>7.27 (1.17–45.11)</td>
<td>0.033</td>
</tr>
<tr>
<td>NT</td>
<td>Infinite*</td>
<td>NS</td>
</tr>
</tbody>
</table>

* All controls have a score of 0; 3 of 9 PVL cases have a score of 1; NS and >0.10; p ≤ 0.05 and <0.100 are included to indicate possible trends for future study; p values <0.050 are captured in bold. Ages of analyzed cases are 20 to 183 postconceptional weeks (see text for details).

CI, confidence interval; HNE, 4-hydroxy-2-nonenal; MDA, malondialdehyde; NT, nitrotyrosine; PVL, periventricular leukomalacia.

Our investigation of the neuropathology of prematurity in the modern era of intensive care, we have detected widespread and variable gliosis and neuronal loss in centers involved in cognitive processing (i.e. cerebral cortex, thalamus, and cerebellar relay nuclei) in infants with PVL (3). Although the gliosis and neuronal loss in that cohort varied from mild to severe, they suggest a heretofore understated vulnerability of the immature gray matter in addition to the severe, they suggest a heretofore underemphasized vulnerability of the immature gray matter in addition to the well-recognized vulnerability of the developing white matter (12, 24). This has prompted us to coin the term “perinatal panencephalopathy.”

In the current study, we hypothesized that the cerebral cortex overlying PVL undergoes cellular modification by free radicals, given the findings by us (25) and others (26, 27) for a role for free radicals in the pathogenesis of the white matter injury. We therefore assessed, in a semiquantitative fashion, the density of cells showing immunopositivity for markers of oxidative and nitritative injury (HNE, MDA, and NT), as well as standard neurohistologic markers of cell reactions (i.e. GFAP for astrocytes and CD68 for microglia/macrophages) in a data set that we had already analyzed similarly for evidence of white matter injury (25). We found that MDA-positive cell density in neurons in Layers II to VI was mildly but significantly elevated compared with controls adjusted for postconceptional age. We also found a significant increase in HNE-immunopositive cells with increasing postconceptional age in PVL, in contrast to controls, in whom the density remained constant with increasing age (i.e. age vs diagnosis interaction). In contrast, neither of the standard neuropathologic markers of gliosis (GFAP) or microglial activation (CD68) consistently identified differences between PVLs and controls, although there were trends toward significance. This result supports the contention that the cerebral cortex is relatively spared in reference to the white matter in PVL (25).

The degree of free radical injury to other gray matter sites (e.g. thalamus) is the focus of ongoing work in the laboratory.

The reasons for the disparity between the markers of oxidative stress and the inflammatory markers of injury are uncertain, but could include a time window necessary for the free radicals to stimulate microglial and astrocytic response or some inherent limitation in the capability of the cortex to respond that contrasts with the marked GFAP and CD68 response in the injured white matter in PVL at this age (25). We were not able to identify a correlation between postnatal age (survival time) and cerebral cortical marker scores, indicating that infants who survive longer did not necessarily have more injury. In addition, if the injury to the cortical neurons is sublethal, astrocytic and microglial reactions may be more modest than in the areas of frank neuronal necrosis.

Might the microglial response to injury in the cerebral cortex of the premature infant be developmentally regulated? Across normal development, the density of activated microglia is transiently higher in the cerebral white matter of the fetus or premature infant (<37 postconceptional weeks) relative to the term infant and to the overlying cortex in either age group (19). This developmental pattern suggests a basis, at least in part, for the increased free radical injury in white matter relative to cortex (i.e. the normal transient increase in microglia may “prime” the white matter for injury in the occurrence of hypoxia-ischemia) and give rise to proliferation of activated CD68-positive microglia seen in the diffuse component of PVL (25). Microglia generate peroxynitrite via inducible NO synthase and superoxide via nicotinamide adenine dinucleotide phosphate (reduced form) oxidase, leading to oligodendrocyte free radical injury (15, 26) and death (17). Although less dense in the cerebral cortex than in the white matter, it is possible that when they are exposed to hypoxia-ischemia, cytokines, and excitotoxicity, cortical microglia could also generate sufficient free radicals to cause adduct formation in scattered neurons overlying PVL.

Vulnerability to Free Radical Injury

The localization of free radical marker immunostaining to Layer V and, to a lesser degree, Layer III neurons in this study was striking. It is possible that the visibility of staining could have been enhanced in these neurons because of their more abundant cytoplasm versus the smaller, granular, or compact cells of the other layers. This localization may, however, reflect a differential susceptibility of these neurons compared with those in Layers II, IV, and VI within the cerebral cortex. Between 25 and 37 postconceptional weeks, in deep layers of the cortical plate, glutamate receptor subunit 1 expression is high, whereas glutamate receptor subunit 2 expression is low, a receptor configuration that confers increased vulnerability to calcium-permeable excitotoxicity to neurons in that location (28). Nearer term and in the postnatal period (i.e. 38–46 postconceptional weeks), cortical pyramidal and nonpyramidal neurons in all layers express low levels of glutamate receptor subunit 2 relative to subunit 1 (28). We speculate that the developmental association for HNE (age × diagnosis interaction; [i.e. the older the infant with PVL, the greater the cerebral cortical labeling with HNE]) may reflect a component of development-related vulnerability to excitotoxicity and the subsequent generation of oxygen free radicals and membrane adduct formation. The findings could also indicate a greater capability to produce free radicals with age, for example, increased metabolic capacity. The HNE staining in controls was always 0, regardless of age, indicating the need potentially for a pathologic inciting event such as hypoxia-ischemia, excitotoxicity, and/or cytokine toxicity.
The qualitative patterns of subplate and Cajal-Retzius cell staining may reflect a particular susceptibility of these early-born “pioneer” neurons to free radical injury. Overall, the markers HNE and NT were increased 2- to 3-fold in PVL over controls in subplate neurons, and HNE and MDA were similarly increased in Cajal-Retzius neurons. During the developmental period studied, subplate neurons and Cajal-Retzius cells are relatively deficient in glutamate receptor subunit 2, rendering them susceptible to calcium-permeable excitotoxicity (28) (D.M. Talos, personal communication). In addition, these cells express NO synthase (29, 30), and this could explain, at least in the subplate, the basis for NT modification in our cohort.

The potential consequences of injury to the subplate and Cajal-Retzius (Layer I) neurons in PVL are of great interest with respect to the cognitive and behavioral deficits in survivors of prematurity and the volumetric gray matter losses identified on quantitative neuroimaging. Subplate and Cajal-Retzius cells are required for normal organization and function of the developing cortex (31, 32) and may retain a modulatory role in the adult rat cortex (33, 34). As discussed later on, free radicals could induce loss or dysfunction of these neurons at critical periods in thalamocortical or corticothalamic axonal outgrowth and synaptogenesis. In the human telencephalon, the subplate population is thought to have its peak density during the PVL window of vulnerability (24–32 postconceptional weeks) (35), functioning as a “waiting zone” for ingrowing afferents to the cortical plate (36). These afferents penetrate the cortical plate at a time of intense dendritic differentiation of deep cortical neurons (28–30 gestational weeks) (36). The fate of subplate neurons in PVL has been open to conjecture (37), and potential effects of free radicals on the subplate, as in the cortical plate itself, include cell death and sublethal injury interfering with afferent ingrowth and dendritic arborization. Subplate neurons are known to be selectively vulnerable to hypoxia-ischemia in rodents (38), and they can be ablated in the developing cat using kainate excitotoxicity (32), but injury to subplate neurons in the human by free radicals has, to our knowledge, not been demonstrated.

Differences Between Cerebral Cortical and White Matter Injury Due to Free Radicals

The absolute increase in marker scores in the white matter compared with the cortex in PVL raises the question of whether “more” free radical injury might be occurring in the white matter. The possible explanation for this difference could include the relative hypovascularization of the white matter (as an end-arterial zone) compared with the cerebral cortex (39). In addition, oligodendrocytes are a rich source of iron and are postulated to play a part in the Fenton reaction that creates increased hydroxyl radicals in the setting of ischemia and reperfusion (11, 40). The immaturity of antioxidant systems in the white matter also likely contributes to a disparity in the vulnerability of the white matter to oxidative and nitrative injury (41–43). This contrasts to the relatively early expression of antioxidant enzymes in the developing cortex (44) (R. D. Folkther, unpublished observations). In addition, white matter astrocytes and subplate neurons, as well as white matter microglia, may be sources of NO and superoxide and, thus, nitrative and oxidative injury (29).

Comparisons With Animal Models

The pattern of oxidative modification of cerebral neurons in our cohort resembles that described in a fetal sheep model of hypoxic-ischemic brain injury (45). In that model, HNE immunostaining was found in the cerebral cortex along with other gray and white matter sites in the experimental group that had undergone umbilical cord occlusion; this staining was completely absent in controls. Specifically, HNE adducts were observed in Layers II to V of the parietal cortex; Cajal-Retzius and subplate neurons were not described. In a rat cerebrocortical slice system, NT was detected in neurons by immunohistochemistry after hypoxia and N-methyl-D-aspartate exposure and was attenuated by the N-methyl-D-aspartate antagonist MK-801 (46). That study illustrates the convergence of hypoxic-ischemic and excitotoxic factors in the pathogenesis of free radical injury in the brain.

Potential Mechanisms of Cortical Injury and Volume Loss

How might subtle free radical injury to the developing cortex contribute to cortical volume loss (i.e. thinning on quantitative magnetic resonance)? The 2 main postulated mechanisms requiring investigation include 1) neuronal cell loss (in cortex, subplate, or both) via apoptotic and/or necrotic cell death; and 2) sublethal injury to cortical and/or subplate neurons, affecting the subcellular synaptodendritic compartment, that is, loss of neuropil. We believe that the finding of variable immunostaining of the background neuropil indicates free radical exposure, adduct formation, and potential sublethal injury in the cortex, which may not necessarily lead to gliosis or overt neuronal loss. In animal models of cerebrovascular disorders, loss of presynaptic markers SNAP-25 and synaptophysin (47) and changes in nerve terminals and dendritic spines (48) have been described. Such changes can be caused directly by the action of oxygen free radicals on the neuronal cytoskeleton, as illustrated in vitro by the disruption of neuronal microtubules and microfilaments, and modification of vimentin intermediate filaments and cellular tubulin, leading to disruption of neurite outgrowth (49, 50). Future studies directly in postmortem human tissue, many ongoing in our laboratory at this time, will be necessary to determine the relative contributions of cell death and sublethal neuronal injury to gray matter volume loss.

CONCLUSION

In summary, subtle but significant modification of cerebral cortical neurons by free radicals occurs in the setting of periventricular white matter injury and lends support to the notion of shared mechanisms of gray and white matter injury in “perinatal panencephalopathy.” Although many issues remain to be elucidated, these findings suggest a basis, at least in part, for the loss of cerebral cortical volume in survivors of prematurity and their cognitive difficulties upon reaching school age. They also indicate the need for preventive strategies targeted at sources of free radical injury in the gray matter and white matter in the newborn.
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REFERENCES

6. Nosarti C, Al-Asady MHS, Franfou S, Stewart AL, Rifkin L, Murray RM. Adolescents who were born very preterm have decreased brain volume. Brain 2002;125:1616–23
44. Takikawa M, Kato S, Esumi H, et al. Temporospatial relationship between the expressions of superoxide dismutase and nitric oxide synthase...