Nitrosative and Oxidative Injury to Premyelinating Oligodendrocytes in Periventricular Leukomalacia

ROBIN L. HAYNES, PHD, REBECCA D. FOLKERTH, MD, RACHAEL J. KEEFE, BA, IYUE SUNG, PHD, LUKE I. SWZEDA, PHD, PAUL A. ROSENBERG, MD, JOSEPH J. VOLPE, MD, AND HANNAH C. KINNEY, MD

Abstract. Periventricular leukomalacia (PVL), the major substrate of cerebral palsy in survivors of prematurity, is defined as focal periventricular necrosis and diffuse gliosis in immature cerebral white matter. We propose that nitrosative and/or oxidative stress to premyelinating oligodendrocytes complicating cerebral ischemia in the sick premature infant is a key mechanism of injury interfering with maturation of these cells to myelin-producing oligodendrocytes and subsequent myelination. Using immunocytochemical markers in autopsy brain tissue from 17 PVL cases and 28 non-PVL controls, we found in the PVL cases: 1) selective regionalization of white matter injury, including preferential involvement of the deep compared to intragral white matter; 2) prominent activation of microglia diffusely throughout the white matter; 3) protein nitration and lipid peroxidation in premyelinating oligodendrocytes in the diffuse component; 4) preferential death of premyelinating oligodendrocytes diffusely; and 5) virtual sparing of the overlying cerebral cortex, as demonstrated by markers of activated astrocytes and microglia. These data establish that PVL is primarily a white matter disease that involves injury to premyelinating oligodendrocytes, potentially through activation of microglia and release of reactive oxygen and nitrogen species. Agents that prevent nitrosative and oxidative stress may play a key role in ameliorating PVL in premature infants in the intensive care nursery.

Key Words: Astrocytes; 4-hydroxynonenal-protein adducts; Microglia; Nitric oxide; Nitrotyrosine; Oligodendrocyte precursors.

INTRODUCTION

In the United States alone, approximately 55,000 live newborns are born very prematurely (<1500 gram birth weight) each year (1). Because of modern intensive care, nearly 90% of these infants survive. Approximately 10%, however, develop cerebral palsy (motor deficits) and 25%–50% develop cognitive and behavioral deficits (2). Thus, nearly 5,000 cases of cerebral palsy and 10,000 to 20,000 children with serious learning disabilities result each year. Periventricular leukomalacia (PVL) is the most common substrate of this enormous amount of neurologic disability. The pathogenesis of PVL likely involves multiple factors, particularly ischemia/reperfusion in the critically ill premature infant with impaired regulation of cerebral blood flow (3). Cytokine-induced brain injury associated with maternal or fetal infection may also play an important contributory role (3).

Our over-riding hypothesis is that PVL is due to cerebral ischemia with the production of excessive reactive nitrogen and/or oxygen species in the reperfused tissue, resulting in nitrosative and/or oxidative damage to immature cerebral white matter; these reactive species preferentially injure vulnerable premyelinating (O4 and O1) oligodendrocytes, leading to their loss, subsequent decreased numbers of mature oligodendrocytes, and hypomyelination in long-term survivors. During the peak period of PVL (i.e. 24–32 gestational weeks), O4 and O1 oligodendrocytes dominate human cerebral white matter, with the first appearance of mature, myelin-producing (myelin basic protein [MBP]+) cells around 30 to 35 gestational weeks (4). Recent studies in perinatal rats (5) and rodent cell culture systems (6) indicate that O4 and O1 oligodendrocytes are vulnerable to oxidative stress, suggesting the possibility that the prevention of oxidative injury may be an important treatment modality in PVL. The lack of direct evidence of nitrosative and/or oxidative stress in O4 and O1 cells in human PVL, however, is a critical “missing link” of information that is urgently needed to confirm the relevance of the experimental models to the human disease, and to guide preventive interventions in the premature infant.

The pathology of PVL consists of 2 components, a “focal,” necrotic component in the periventricular region, and a “diffuse” component characterized by reactive gliosis in the surrounding white matter. In this study, we focused more on the diffuse than the focal, necrotic component of PVL because the former is thought to result in widespread impairment in myelination, the likely basis for the global neurological deficits in long-term survivors. Moreover, in modern neonatal intensive care units the diffuse component of PVL is much more common than the focal component by neuroimaging (7). While

From the Departments of Neurology (RLH, RJK, PAR, JJV, HCK) and Neuropathology (RDF, HCK), Children's Hospital and Harvard Medical School, Boston, Massachusetts; New England Research Institutes (IS), Watertown, Massachusetts; Department of Physiology and Biophysics (LIS), Case Western Reserve University School of Medicine, Cleveland, Ohio.

Correspondence to: Robin L. Haynes, PhD, Department of Neurology (Neuroscience), Children’s Hospital, Boston, MA 02115. E-mail: Robin.Haynes@TCH.Harvard.edu

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necrotic foci appear pathologically to be micro-infarcts, thought to be secondary to severe hypoperfusion in the distal end-zones of the long penetrating arteries (8), the less severe but more widespread diffuse component of PVL may reflect other previously unrecognized pathogenetic mechanisms, including protein nitration and oxidative injury to lipids and proteins in partially perfused tissue. We reasoned that the demonstration of protein nitration and lipid peroxidation in O4 and/or O1 cells would carry different implications with respect to the underlying pathophysiology of cerebral white matter damage in PVL, and to potential specific treatment modalities. We sought also to determine whether microglial activation was associated with markers of nitrosative and/or oxidative stress, given recent evidence that microglia are sources of nitric oxide (NO) and peroxynitrite (9). We further tested the hypothesis that O4 and/or O1 cells in the diffuse component of PVL undergo cell death and loss by double-labeling these cells for TUNEL product.

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 classified by the systematic examination of standardized microscopic sections (maximum of 19 sections/case), stained with conventional H&E/Luxol fast blue, from each brain and spinal cord (8). Eleven PVL cases (Cases 1, 5–14; 34–95 postconceptional weeks) and 4 control cases (23–40 postconceptional weeks) had tissue fixed in 4% paraformaldehyde for double-labeling studies. Of note, Case 1 had both formalin- and paraformaldehyde-fixed tissue. Ten of the 14 (71%) PVL cases were born prematurely, and 3 of these 10 infants survived beyond the neonatal period (>44 postconceptional weeks). In the control group, none of the cases analyzed with a postconceptional age >37 weeks were born prematurely.

Single-Labeling Immunocytochemistry in Formalin-Fixed, Paraffin-Embedded Tissue

Standard methods in deparaffinized tissue sections (4 μm) were applied (10). Antibodies specific for the following markers were used: 4-hydroxy-2-nonenal-protein adducts (HNE) (1:100; laboratory of Dr. Luke I. Szweda, or purchased from Calbiotech, San Diego, CA); malondialdehyde–protein adducts (MDA) (1:100; Abcam, Cambridge, United Kingdom); nitrotyrosine protein adducts (NT) (1:100; Upstate, Lake Placid, NY); heme-oxygenase-1 (HO-1) and 2 (HO-2) (both 1:500; Stressgen, Victoria, Canada), glial fibrillary acidic protein (GFAP) (1:9,000, Sternberger Monoclonals, Inc., Lutherville, MD), and CD68 (1:50, Cell Marque, Austin, TX). Stains were optimized using known Alzheimer and ALS tissue as positive controls. CD68-positive control was tonsil. Negative controls omitted the primary antibodies.

Double-Labeling Immunofluorescence for the Demonstration of Oxidative Damage in Specific Cell Types

Freshly collected tissue from 4 PVL and 4 control cases was fixed immediately in 4% paraformaldehyde, and sectioned with a Leica VTS–1000 vibratome (Leica Microsystems, Inc., Bannock, IL) into 40- to 50-μm free-floating sections. Double-labeling experiments were performed sequentially, first with monoclonal antibodies O4 or O1 for detection of oligodendrocytes (1:1,000) (4) or GFAP for detection of astrocytes. The
TABLE 1B
Continued Summary of the Neuropathology of PVL cases

<table>
<thead>
<tr>
<th>Case</th>
<th>Bst</th>
<th>CB</th>
<th>BG</th>
<th>Thal</th>
<th>Hipp</th>
<th>CCH</th>
<th>WMH</th>
<th>SAH</th>
<th>GMH</th>
<th>Diagnosis</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>−</td>
<td>−</td>
<td>A, B</td>
<td>−</td>
<td>A</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>Pottery syndrome; abnormal temporal lobe gyration; malrotation of hippocampi; duplication of dentate gyrus</td>
</tr>
<tr>
<td>2</td>
<td>−</td>
<td>A</td>
<td>C</td>
<td>A, B</td>
<td>A, B</td>
<td>D</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>Congenital diaphragmatic hernia; ECMO treatment</td>
</tr>
<tr>
<td>3</td>
<td>A</td>
<td>−</td>
<td>C</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>Placental abruption</td>
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<tr>
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<td>+</td>
<td>+</td>
<td>−</td>
<td>Breech birth; sepsis; necrotizing enterocolitis</td>
</tr>
<tr>
<td>5</td>
<td>−</td>
<td>−</td>
<td>G</td>
<td>G</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>Osteogenesis imperfecta</td>
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<td>−</td>
<td>C</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>Necrotizing enterocitols; bowel perforation; coagulopathy; sepsis</td>
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<tr>
<td>7</td>
<td>A</td>
<td>I</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>CMV infection in utero; ABO incompatibility; dwarfism/skeletal dysplasia; fetal heart failure</td>
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<tr>
<td>8</td>
<td>−</td>
<td>−</td>
<td>A</td>
<td>−</td>
<td>−</td>
<td>B</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>Urea cycle defect (ornithine transcarbamylase deficiency); hyperammonemia</td>
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<td>NA</td>
<td>J</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>K</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>Breech birth; oligohydramnios; renal sclerosis</td>
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<td>10</td>
<td>A</td>
<td>L</td>
<td>A</td>
<td>A</td>
<td>−</td>
<td>A</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
<td>11</td>
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<td>A</td>
<td>NA</td>
<td>A, M</td>
<td>A</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>Congenital cardiac malformation; ECMO treatment</td>
</tr>
<tr>
<td>12</td>
<td>A</td>
<td>L</td>
<td>A</td>
<td>•</td>
<td>−</td>
<td>B</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>Pulmonary hypertension; pneumonia; ECMO treatment</td>
</tr>
<tr>
<td>13</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>N</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>Breech birth; oligohydramnios; pneumonia/thorax; respiratory failure</td>
</tr>
<tr>
<td>14</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>A</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>Congenital cardiac malformation; ECMO treatment</td>
</tr>
</tbody>
</table>

Legend: +, pathology present; −, pathology absent; A = reactive gliosis; B = neuronal loss; C = ischemic necrosis; D = hemorrhagic infarct; E = apoptosis; F = focal microhemorrhage; G = mineralization; H = perivascular hemorrhage; I = microglial nodules; J = vermis cortical scar; K = organizing infarct; L = Purkinje cell loss; M = microinfarct; N = necrotic focus in hypothalamus; EMCO = extracorporeal membrane oxygenation; NA = not available; Bst, brainstem; CB, cerebellum; BG, basal ganglia; Thal, thalamus; Hipp, hippocampus; CCH, cerebral cortex hemorrhage; WMH, white matter hemorrhage; GMH, germinal matrix hemorrhage.

same sections were subsequently incubated with antibodies to HNE (1:250), MDA (1:100), and NT (1:250). Immunostained sections were visualized with Nikon Eclipse E800 microscope (Nikon, Melville, NY) with image capture using Spot Software (Diagnostics Instruments, Incorporated, Sterling Heights, MI).

Tdt-Mediated Dutp Nick End Labeling (TUNEL) Staining and Double-Labeling Immunofluorescence for Dying O4 and O1

For O4 and TUNEL double labeling, tissue fixed in 4% paraformaldehyde was cut at 40 to 50 μm. Free-floating sections were stained for O4 (see above). Sections were then mounted, allowed to dry, and TUNEL-positive cells were stained using an in situ cell death detection kit from Roche (Indianapolis, IN). For GFAP and TUNEL double labeling, formalin-fixed paraffin-embedded sections were used.

Grading Method and Statistical Analysis

Grading of the single-labeled tissue sections was performed by counting positive cells per high power field (hpf) at ×400 magnification (0.173 mm²), which was selected on the basis of the most intense immunostaining after survey of all fields: 0, no cell staining; 1/2, 0 to 1 immunopositive cell/hpf; 1, 2 to 10 cells/hpf; 2, 11 to 20 immunopositive cells/hpf; and 3, >20 cells/hpf. Two observers scored each case without knowledge of case diagnosis and age. The age-adjusted difference was assessed with age as the covariant.

RESULTS

In the PVL and control tissue sections, we assessed activated microglia/macrophages with an antibody to CD68, and reactive astrocytes with an antibody to GFAP. We used an antibody to nitrotyrosine (NT) to indicate protein nitration, and antibodies to 4-hydroxynonenal (HNE)-protein adducts and malondialdehyde (MDA)-protein adducts to indicate lipid peroxidation, in single- and double-labeling studies in the diffuse component of PVL. In addition, the enzymes heme oxygenase (HO)-1 and −2 were examined to indicate cellular response to oxidative stress. We performed single-labeling immunocytochemistry using markers of nitrosative and oxidative stress and cellular responses in 11 of the 14 PVL cases (Cases 1, 5–14) and 23 controls (without PVL), adjusted for age, in formalin-fixed, paraffin-embedded tissue sections. We performed double labeling studies in brain tissues from 4 PVL and 4 control cases, fixed in 4% paraformaldehyde. We used a semiquantitative grading system (0–3) of the density of immunopositive cells/high
power (×400) microscopic field (hpf, 0.173 mm²) to compare the degree of cellular injury between PVL and control cases in comparable single-labeled tissue sections (Table 2). There was no statistically significant relationship between the selected markers of nitrosative and oxidative stress and postmortem interval (range: 2–132 hours; median: 14 hours) (data not shown), indicating that the differential expression of cellular immunostaining for the selected markers in this study did not represent an artifact of postmortem autolysis.

The Neuropathology of PVL with Cellular Markers for Microglial and Astrocytic Activation

The PVL cases (n = 14) exhibited a spectrum of severity of cerebral white matter pathology. The age at birth, length of survival, and histopathologic characteristics of the PVL lesions of each PVL case are summarized in Table 1. All of the PVL cases, with the exception of three (Cases 2, 8, and 11) were born prematurely. The term deliveries were complicated by congenital diaphragmatic hernia (Case 2), urea cycle defect (Case 8), and congenital heart malformation (Case 11) (Table 1). In 9 cases, focal cystic cavities were grossly visible in the periventricular white matter (Fig. 1A). Microscopically, 7 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; 9 cases had necrotic foci with subacute changes of postmortem autolysis.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Age*</th>
<th>PVL</th>
<th>Control</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFAP</td>
<td>PCA</td>
<td>2.64 (0.17)</td>
<td>1.73 (0.14)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>2.73 (0.22)</td>
<td>1.66 (0.18)</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>PNA</td>
<td>2.68 (0.17)</td>
<td>1.70 (0.14)</td>
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</tr>
<tr>
<td>CD68</td>
<td>PCA</td>
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<tr>
<td></td>
<td>GA</td>
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<td>1.53 (0.23)</td>
<td>0.017</td>
</tr>
<tr>
<td></td>
<td>PNA</td>
<td>2.27 (0.26)</td>
<td>1.15 (0.21)</td>
<td>0.003</td>
</tr>
<tr>
<td>HNE</td>
<td>PCA</td>
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<td>0.92 (0.12)</td>
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<tr>
<td></td>
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<td>0.98 (0.11)</td>
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</tr>
<tr>
<td>MDA</td>
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<tr>
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<tr>
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<tr>
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<td>0.37 (0.24)</td>
<td>&lt;0.001</td>
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<tr>
<td></td>
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<td>1.99 (0.26)</td>
<td>0.38 (0.22)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HO-1</td>
<td>PCA</td>
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<td>1.51 (0.26)</td>
<td>0.252</td>
</tr>
<tr>
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<tr>
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<tr>
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<td>PNA</td>
<td>2.44 (0.27)</td>
<td>1.04 (0.23)</td>
<td>&lt;0.001</td>
</tr>
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</table>

* The age-adjusted mean (± standard error [SEM]) of the degree of oxidative damage (scale: 0–3) for the selected markers of oxidative stress in PVL (n = 11) and control (n = 23) cases using single labeling of glial cells with immunocytochemical techniques in sections of cerebral white matter. The p values are from t tests of the difference between the least-square means for PVL and Control cases. A p value <0.05 is considered significant. Abbreviations:PCA, postconceptional age (gestational plus postnatal age); GA, gestational age; PNA, postnatal age; SEM, standard error of the mean.
FIG. 2. Regional heterogeneity of white matter injury in PVL. A: There is a heterogeneous distribution of reactive glial cells with positive immunostaining for selected markers of cellular injury (MDA shown) in the different components of the cerebral white matter, as demonstrated in a typical PVL case at 35 postconceptional weeks (28 gestational weeks) in the temporal-parietal white matter (level of the temporal horn of the lateral ventricle [Ven]; white matter regions, periventricular [PV], central [Cen], and intragryral [IG]; hippocampus [Hipp]; choroid plexus [CP] (with focal intraplexal hemorrhage) (H&E/Luxol fast blue; ×1). Periventricular necrosis is demonstrated at this level by the rarefaction (pallor) of the periventricular white matter (arrow). B: The IG white matter distant from the periventricular focus of necrosis lacks MDA-immunopositive cells (Grade 0) (×200); central white matter (Cen) is likewise uninvolved (not shown). C: PVL Case 6. In contrast, the MDA-immunopositive glial cells are heavily concentrated (Grade 3) in the periventricular white matter surrounding the focal necrosis (×200).

FIG. 3. Nitrosative damage in white matter in PVL. A: A high density of cells (Grade 3), including larger (arrowhead) and smaller (arrow) cells morphologically consistent with reactive astrocytes and oligodendrocytes, respectively, have nitrotyrosine detected by immunocytochemistry, here illustrated in the diffuse component of PVL in an infant at 44 postconceptional weeks (×400). B: No staining is seen in age-matched control (×400). C, D: Nitrotyrosine (NT) colocalizes (yellow signal) with the marker for premyelinating oligodendrocytes (O4, red; C), and with GFAP (green; D), indicating that both types of glia undergo protein nitration (×600). PVL Case 11 is illustrated.

with cavitation and infiltration of macrophages, indicative of an insult at or beyond 3 to 5 days duration; and 8 cases had chronic changes (glial scarring or mineralization) indicating the site of prior focal necrosis (8). Seven cases had focal lesions of more than one histopathologic age. Of note, the overlying cortical neurons did not appear acutely necrotic, nor was there appreciable chronic neuronal cell loss, with the exception of 2 cases with focal cortical infarctions (Cases 2 and 9) (Table 1). All cases had the diffusely gliotic component of PVL associated with the focally necrotic component, as per the study criteria of PVL. Although macrophages in the subacute necrotic foci were CD68-positive (Fig. 1B, C), as expected, we also found marked microglial/macrophagocytic infiltration, as detected with CD68 immunostaining, throughout the periventricular and central regions of the gliotic white matter in the PVL cases (Fig. 1D; Table 2), with relative sparing of the overlying cerebral cortex (Fig. 1E). The difference in CD68 immunostaining between PVL and control white matter, and between PVL white matter and cortex, was statistically significant (p < 0.005). In the deep white matter surrounding the foci of necrosis there was marked (Grades ≥2) gliosis with reactive (GFAP+) astrocytes, again with striking sparing of the overlying cerebral cortex (Fig. 1F; Table 2). There was marked regional heterogeneity in the density of reactive astrocytes, and of glial cells with positive immunostaining for the selected markers of nitrosative and oxidative stress in the different components of the white matter (Fig. 2A–C).

Immunocytochemistry for Protein Nitration in PVL
Morphologically identified macrophages within the subacute necrotic foci immunostained positively with NT (data not shown). Nitrotyrosine immunoreactivity was also localized to cells morphologically consistent with glial cells in the diffuse component (Fig. 3A) and was increased in PVL when compared to controls (Fig. 3B;
Table 2). The degree of NT immunostaining was statistically significant in PVL cases compared to control cases, adjusted for postconceptional age (PCA), gestational age (GA), or postnatal age (PNA) (Table 2), with increased positive cell density in areas immediately surrounding the focal PVL lesion. To define precisely the specific cell types susceptible to nitrosative stress in PVL, double labeling with markers to O4, O1, or GFAP and to NT was done. In the PVL cases, NT was present in O4-positive oligodendrocytes (Fig. 3C) and O1-positive oligodendrocytes (data not shown) in the diffuse component. Nitrotyrosine also colocalized with GFAP (Fig. 3D), indicating that nitrosative damage occurs in reactive astrocytes, as well as in oligodendrocytes. In control cases, a slight but detectable amount of NT colocalized primarily with GFAP-immunopositive reactive astrocytes and occasionally with O4 and/or O1 cells (data not shown).

Immunocytochemistry for Oxidative Stress-Related Markers in PVL

Antibodies to HNE, MDA, HO-1, and HO-2 were localized to cells morphologically consistent with macrophages within the necrotic foci of PVL (data not shown). In the diffuse white matter lesion, reactive astrocytes were significantly increased in number compared to control cases, adjusted for PCA, GA, or PNA (Table 2). Glial cells, some of which were morphologically consistent with reactive astrocytes (Fig. 4A), within the diffuse lesion were immunopositive for all oxidative stress-related markers analyzed, including HNE (Fig. 4B), MDA (Fig. 2C), HO-1, and HO-2 (Table 2). There was a statistically significant difference in the age-adjusted means of the grade of immunopositive white matter cells between the PVL and control groups for HNE, MDA, and HO-2, regardless of the particular age measure, i.e. whether the cases were adjusted for PCA, GA, or PNA (Table 2). With double labeling, the oxidative stress markers HNE and MDA were present in O4-positive oligodendrocytes (Fig. 4C; HNE shown) and O1-positive oligodendrocytes (data not shown), as well as in astrocytes (Fig. 4D). In control cases, a slight but detectable amount of HNE and MDA colocalized primarily with GFAP-immunopositive reactive astrocytes, and occasionally with O4- and/or O1-positive cells (data not shown).

TUNEL Staining and Loss of Oligodendrocytes in PVL

Dying oligodendrocytes were identified in acute PVL by colocalization of O4 immunoreactive cells with TUNEL-positive nuclei (Fig. 5A, Case 4). In contrast, the GFAP-immunopositive reactive astrocytes, which separately labeled with markers of nitrosative and oxidative damage (Figs. 3D, 4D), were not dying, given the lack of the colocalization of GFAP and TUNEL-positivity (Fig. 5B) and their increase in number, i.e. proliferation. TUNEL staining was performed in 4 PVL cases (Cases 1–4). Although TUNEL-positive oligodendrocytes were identified in other PVL cases, there was an increased amount of TUNEL-positive oligodendrocytes identified in Case 4, shown in Figure 5A. This finding was consistent with a notable amount of pyknotic nuclei seen in H&E sections (data not shown). In 1 case of chronic PVL (Case 1; characterized by glial scarring of and around the necrotic foci), we found a striking qualitative loss of oligodendrocytes (O1 shown) in the diffuse component of PVL, compared to control (Fig. 5C). Interestingly, few TUNEL-positive (i.e. acutely dying) oligodendrocytes were identified in the chronic PVL. Of note, O4 and O1 immunolabeling is technically suboptimal in formalin-fixed, paraffin-embedded tissue sections (4), thereby precluding the evaluation of O4 and O1 cell loss in the extensive archival database studied above with single-label immunocytochemistry.

DISCUSSION

Based upon the use of specific cellular markers with single- and double-labeling immunocytochemistry, this study provides major new insights into the cellular pathology of PVL, with important implications for developing new drugs in patients and in testing hypotheses in animal and cell culture models. The use of markers for reactive astrocytes (GFAP) and activated microglia/macrophages (CD68) in this study establishes that PVL is a primary white matter disease characterized by significant, diffuse astro- and microgliosis, in contrast to a virtually spared overlying cerebral cortex. Thus, the recent demonstration of cortical volume loss by sophisticated neuroimaging in premature infants with PVL (11) is not explained by histopathologic evidence for primary cortical damage in the acute and subacute phases of PVL, using the astrocytic and microglial markers analyzed in this study. Explanations for cortical volume loss must therefore be sought in a more subtle and likely chronic disturbance of cortical neurons that is secondary to injury to axons coursing through cerebral white matter (12), and/or to direct injury to the subplate neurons dispersed throughout the white matter that are critical for development of cortical neurons (13).

The use of markers for glial injury in this study demonstrates clearly that the involvement of the cerebral white matter in the diffuse component of PVL is not uniform. Rather, the deep and central white matter is preferentially involved, with relative sparing of the intragral (subcortical) white matter, and the white matter in the vicinity of periventricular foci of necrosis is involved to a much greater degree than is the white matter distant from these foci. While heterogeneity of white matter damage in PVL has previously been suggested using standard tissue stains (12), the use of injury-specific markers allows for more complete characterization of the...
distribution of damage. This regionalization of white matter damage reinforces the idea that PVL is secondary to ischemia in distal fields of arterial irrigation, i.e. vulnerable “watershed” territories in the deeper (and not superficial) white matter.

The use of the CD68 marker in this study reveals a widespread activation of microglia/macrophages in the diffuse component of PVL, indicating a need for new emphasis on the role of microglia/macrophages in the pathogenesis of PVL. CNS microglia/macrophages are known to be activated by ischemia/hypoxia (14, 15), including up to 1 month after the initial insult (16). Of particular relevance in PVL is the ability of activated microglia/macrophages to release reactive nitrogen species (RNS) (17) and reactive oxygen species (ROS) (18) into the surrounding environment, thus putting oligodendrocytes at risk for nitrosative and oxidative damage (19). Nitric oxide (NO), in particular, is fundamental to nitrosative injury. While NO has normal functions in the brain, including synaptic transmission and the regulation of vascular tone, it also plays a causal role in stroke (20). There are multiple isoforms of nitric oxide synthase (NOS), the synthetic enzyme for NO, including an inducible form in microglia and astrocytes that is a major source of excess NO in pathological conditions (20). Nitric oxide reacts with superoxide anion to form peroxynitrite, a highly reactive molecule that targets tyrosine residues of proteins to form nitrotyrosine residues (21). Excess NO release and subsequent peroxynitrite and nitrotyrosine formation occur in experimental models of ischemia (22), while treatment during reperfusion with inhibitors of inducible NOS reduces damage (23), suggesting a direct role for NO in ischemic injury. In cell culture, premyelinating oligodendrocytes are sensitive to NO-mediated injury and cell death (24, 25). Of interest, the activation of microglia by interferon-γ, β-amyloid, and lipopolysaccharide is neurotoxic to rat neurons through the release of peroxynitrite (9). Our data showing evidence of protein nitration in O4 and O1 cells in the presence of diffuse microglial activation suggest that microglial activation with NO production and formation of...
peroxynitrite may play a key role in O4 and O1 cell injury in PVL.

In addition to nitrosative stress, we demonstrate PVL-related oxidative damage to oligodendrocytes, most of which are premyelinating in the ages affected by PVL (4). Like RNS, ROS are released by activated microglia and upon release, interact with the lipid component of cellular membranes, initiating lipid peroxidation that results in the breakdown of lipid constituents into highly reactive byproducts, including lipid aldehydes, HNE, and MDA (26). These reactive aldehydes then bind to and modify protein creating protein adducts. Lipid peroxidation, as indicated by HNE-protein adducts, occurs in models of ischemia (27). In this regard, a recent study of premature infants found elevated levels of markers of lipid peroxidation and oxidative protein products in cerebrospinal fluid of infants who had PVL documented by magnetic resonance imaging (28). In ischemia, lipid peroxidation and adduct formation may play direct roles in cell toxicity, given that HNE, like NO, is toxic to premyelinating oligodendrocytes in a dose-dependent manner (27, 29). Although RNS and ROS represent different pathophysiological mechanisms of cellular injury and may play a different role in the overall damage in PVL, they may share a similar mode of action with respect to oligodendrocyte injury, i.e. both have been shown to inhibit mitochondrial respiration (20, 30) and induce mitochondrial membrane permeabilization (31, 32).

In this study we demonstrated, via the TUNEL method, that O4 and O1 cells undergo cell death in the diffuse component of PVL. Although we identified dying (likely premyelinating) oligodendrocytes in an acute case of PVL, a striking loss of these cells was found in an advanced case of PVL with secondary scarring of the periventricular necrotic foci, suggesting that O4 and O1 cells die immediately in the acute phase following the ischemic insult and disappear almost completely by the chronic phase. Premyelinating oligodendrocytes are likely damaged at the time of the initial ischemia/perfusion, but also for an extended period thereafter; the latter injury due to subacute tissue reactions (i.e. diffuse microglial activation), which may last weeks. Consequently, this possibility of ongoing inflammatory-related injury extends the “window of opportunity” for drug therapy to protect against O4 and O1 cell damage.

In addition to oligodendrocytes, reactive astrocytes in the diffuse component of PVL immunostain for markers of both nitrosative and oxidative damage. The markers used in this study colocalize to reactive astrocytes in several human neurologic diseases not generally considered ischemic, i.e. amyotrophic lateral sclerosis (33), Alzheimer disease (34), and multiple sclerosis (35). Thus, nitrosative and oxidative damage in reactive astrocytes in human neuropathology suggests RNS and ROS injury as a final common pathway in multiple disease processes, including PVL. Astrocytes may protect surrounding neuronal and oligodendroglial cells under conditions of such stress due to their capacity to release antioxidants into the extracellular fluid (36, 37), and to clear extracellular glutamate (38), which in excessive levels results in excitotoxicity to premyelinating oligodendrocytes (39, 40).

We speculate that reactive astrocytes in diffuse PVL proliferate to act, at least in part, as scavengers of excess oxidants in the environment, thereby protecting O4 and O1 cells from reactive nitrogen and oxygen species. Alternatively, reactive astrocytes may not be protective, but instead contribute to tissue damage in PVL. In this regard, astrocytes upregulate inducible NOS in experimental ischemic injury (41), suggesting that, in addition to activated microglia, they are another source of the free radical NO.

In addition to markers of cellular damage, we showed a statistically significant increase in the density of HO-2-immunopositive glial cells in PVL. The HO family functions as a defense mechanism against free radical injury through degradation of heme, a pro-oxidant, and subsequent production of carbon monoxide, free iron, and biliverdin, which is subsequently converted to bilirubin, a potent antioxidant (42). In contrast to HO-2, HO-1 was not significantly increased in the PVL cases compared to the control. Heme oxygenase-1, shown to be induced by ischemic injury, is also induced by cytokines and prostaglandins, and in response to hemorrhage, sepsis, and pregnancy (43). The lack of a significant difference in HO-1 between PVL and controls suggests that, unlike in other reported settings, HO-1 may not be a specific marker of oxidative stress in developing human brain.

In conclusion, we show a region-specific vulnerability to nitrosative and oxidative damage within the deep periventricular white matter, and a relative sparing of the overlying cerebral cortex. We also show widespread activation of microglia, leading us to speculate that these cells may play a direct role in the white matter damage via release of NO. It is unknown whether nitrosative or oxidative damage in PVL is the primary insult to premyelinating oligodendrocytes, or is secondary to other insults, e.g. excitotoxicity from excessive glutamate release (44), or cytokine toxicity (45) complicating diffuse microglial/macrophagic activation. Irrespective of the precise sequence of events, the burden of nitrosative and oxidative damage to the white matter appears enormous in PVL. Our findings indicate that continued research into the role of reactive nitrogen and oxygen species in the pathogenesis of PVL is urgently needed, with the ultimate goal of the development of drug strategies to prevent this devastating disorder.

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