Prenatal Ischemia and White Matter Damage in Rats

Paul Olivier, MS, Olivier Baud, MD, PhD, Philippe Evrard, MD, Pierre Gressens, MD, PhD, and Catherine Verney, PharmD, PhD

Abstract

Ischemia/reperfusion injury to the developing brain is a major cause of neurologic abnormalities in preterm infants. To investigate the underlying mechanisms, we modified a previously described rat model of unilateral uterine-artery ligation on the 17th embryonic day (E17). Growth retardation was taken as an index of in utero ischemia, and pups born with a birth weight more than 2 standard deviations below that of controls were compared with the same-litter, normal-growth control pups born from the nonligated horn. Prenatal ischemia probably associated with hypoxia and followed by reperfusion at birth induced white matter damage at a developmental stage corresponding to extreme prematurity in humans. On P0 (day of birth), growth retarded pups exhibited lesions in the cingular white matter and internal capsule with increased counts of activated microglial cells for 2 weeks compared with controls. Astrogliosis was detected in the injured white matter. On P3, increased apoptotic cell death was seen in O4-positive preoligodendrocytes, which were abnormally scarce on P7. Defective myelination, as assessed by myelin-binding-protein labeling, was detected until adulthood. The diffuse white matter damage in growth-retarded rats replicated the main features of white matter damage in human preterm infants.

Key Words: Astrogliosis, Ischemia/reperfusion injury, Microglial activation, Myelination, Oligodendrocyte, Periventricular leukomalacia, Prematurity.

INTRODUCTION

Despite substantial improvements in neonatal intensive care, approximately 15% of very premature infants who survive the neonatal period later exhibit the motor deficits characterizing cerebral palsy (1). Perinatal brain injury variably involves the gray and white matter, depending on the stage of cerebral developmental and vessel maturation (2). In preterm infants, the most common form of white matter damage is periventricular leukomalacia (PVL), which is the main pathologic substratum of cerebral palsy. Preventing PVL is a major public health challenge. A number of factors present in isolation or in combination may cause white matter damage in humans. They include ischemia–reperfusion (3) and infection/inflammation as part of the fetal inflammatory response syndrome (4, 5). In most rat models of hypoperfusion-induced white matter damage, cerebral ischemia/hypoxia is caused in 7- to 9-day-old animals by permanently ligating one or both carotid arteries; transient hypoxia is then used in some models (4–9). The developmental stage of the pups is roughly similar to 36 to 42 weeks of postconceptual age in humans, and the white matter damage is considered secondary to gray matter damage (10, 11). These models mimic human perinatal hypoxic/ischemic insults, in which white matter damage is associated with cortical damage (7). Few rat models replicating white matter damage in preterm infants have been developed (12–14). The features of white matter damage induced by carotid ligations performed within 3 days after birth suggest that younger pup ages may better replicate human PVL. Technical limitations have precluded the introduction in these models of a reperfusion phase, which usually occurs in humans when preterm infants receive resuscitation.

Because the rat pup brain at birth is thought to correspond to the human brain around 22 to 24 weeks of gestational age, we used an animal model involving prenatal ischemia by ligation of one uterine artery on embryonic day 17 (E17) followed by a return to normal oxygenation at delivery simulating the reperfusion phase (15). Uterine artery ligation diminishes placental blood flow, thereby inducing fetal ischemia and possibly hypoxia. Pups born from the ligated horn exhibit growth restriction (GR) at birth (16–20). The severity of GR reflects the severity of prenatal ischemia and probably hypoxia in the pups from the ligated horn. Birth after prenatal ischemia/hypoxia may induce oxidative stress related to reperfusion/reoxygenation. This study shows that GR pups exhibit white matter damage after birth associated with inflammation and increased cell death, followed by astrogliosis and deficient myelination that persists into adulthood. These abnormalities replicate the main features of white matter damage seen in human preterm infants.

MATERIALS AND METHODS

Animal Model

Unilateral uterine artery ligation was performed in pregnant Sprague-Dawley rats on E17 under anesthesia with
intraperitoneal injection of 350 mg/kg of chloral hydrate (Merck, Darmstadt, Germany). Because there was no means of marking pups according to the horn they developed in, we considered that pups with GR at birth had been subjected to ischemia and probably hypoxia related to uterine artery ligation (i.e. developed in the ligated horn) (18). We defined GR as a birth weight more than 2 standard deviations (SDs) below the mean control value in 54 pups from 5 litters of normal Sprague-Dawley rats born at our animal facility (6.24 ± 0.37 g), that is, below 5.50 g. This control value was not significantly different from the mean birth weight in 32 pups from sham-operated dams (6.34 ± 0.17 g). GR pups were compared with same-litter controls having a birth weight greater than the mean control value in the 54 pups minus 1 SD (5.87 g) (Table 1). Pups with birth weights between 5.50 and 5.87 g were excluded to minimize the risk of misclassifying pups regarding exposure to prenatal ischemia and probable hypoxia. In addition to newborn pups, we studied pups obtained by cesarean section on E21, classifying pups from the ligated horn as having GR and those from the intact horn as controls (Table 1). The pups were weighed and perfused as described subsequently.

All experiments were carried out in compliance with the ethical principles developed by our research institution (Institut National de la Sante et de la Recherche Medicale [INSERM]) and with the National Research Council Guide for the Care and Use of Laboratory Animals.

### Brain Sections

Four to 8 pups from each group (GR group and controls) were studied on E21 and after birth on P0, P3, P7, P10, P14, P21, and P60. The animals were perfused transcardially with 4% paraformaldehyde in phosphate buffer (0.12 M, pH 7.4) under anesthesia with inhaled isoflurane (Abbott France, Rungis, France). The brains were postfixed in the same fixative for 3 hours, cryoprotected in sucrose, and frozen in liquid nitrogen-cooled isopentane. Coronal sections 10 µm in thickness were cut serially through the septum (see Figs. 15–36 in reference (21)); 4 sections from at least 4 animals in each group (GR group and controls) were evaluated as mentioned in the following section on the quantification of immunoreactive cells.

### Immunocytochemical Staining

The sections were incubated overnight in the primary antibodies (Table 2) diluted in phosphate-buffered saline (PBS)/0.25% Triton X-100/0.2% gelatin (PBS-TX-gel). The primary antibodies were visualized using the streptavidin–biotin peroxidase complex (Amersham, Buckinghamshire, UK) (22) with the substrate H2O2 (0.005%) in TRIS (0.05 M) in the presence of the chromogen diaminobenzidine 0.02% and of 0.6% nickel ammonium sulfate. Double labeling was performed using anti-rabbit fluorescein-labeled whole antibody (Amersham) or Texas Red anti-mouse IgM (Vector, Burlingame, CA) fluorescent markers.

### TUNEL Staining

On P3, cell death in white matter was detected using TUNEL staining as previously described (23). On each section, labeled nuclei were counted in the cingulum and corpus callosum of a hemisphere section through the septum (see Figures 15 to 36 in reference (21)); 4 sections from at least 4 animals in each group (GR group and controls) were evaluated as mentioned in the following section on the quantification of immunoreactive cells.

### Quantification of Immunoreactive Cells

Quantitative analysis of immunoreactive (IR) cells was performed in the white matter of the cingulum (see Plates 15–36 in [21]) and in the internal and external capsules (see Plates 49–65 in [21]) on E21, P0, P3, P7, P14, P21, and P60. A blinded procedure was used to count labeled cells at 400× magnification in 0.065 mm² of 4 sections from at least 4 animals in each group (GR group and controls). Blood vessel density was assessed by counting the number of intersections between Glut1-IR vessel walls with a grid at 400× magnification in an area of 0.065 mm². The thickness of the genu of the corpus callosum and of the cerebral cortex on sections labeled with MBP was evaluated at the level of the motor cortex (see Plates 30–37 in [21]) and visual cortex (see Plates 70–82 in [21]) at 200× magnification using an optic ruler. All these data were collected by 2 investigators working independently who were blinded to group assignment.

### Statistical Analysis

All data are reported as mean ± standard deviation. The data were evaluated by analysis of variance with age (from E21–P60) and group (GR group and controls) as the factors. Bonferroni posttest was used for multiple pairwise comparisons (Prism 4.0 for Windows; SAS Institute Inc., Cary, NC). When evaluating data from a single age, we used the non-parametric Mann-Whitney U test.

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### TABLE 1. Effect of Prenatal Ischemia (probably with hypoxia) on the Body Weight of Rat Pups

<table>
<thead>
<tr>
<th>Age of Rats</th>
<th>E21</th>
<th>P0</th>
<th>P3</th>
<th>P7</th>
<th>P14</th>
<th>P21</th>
<th>P60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n: 18</td>
<td>5.59 ± 0.16</td>
<td>6.16 ± 0.33</td>
<td>8.83 ± 0.52</td>
<td>16.96 ± 1.02</td>
<td>35.69 ± 2.91</td>
<td>61.11 ± 7.87</td>
<td>283.40 ± 48.84 g</td>
</tr>
<tr>
<td>GR rats</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n: 10*</td>
<td>4.11 ± 0.23</td>
<td>4.48 ± 0.51</td>
<td>6.27 ± 1.12</td>
<td>11.59 ± 2.03</td>
<td>27.71 ± 3.31</td>
<td>52.78 ± 5.86</td>
<td>188.50 ± 25.89 g</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard deviation. The controls were from the same litter, as indicated in the “Materials and Methods” section. 
* p < 0.05, † p < 0.001 for comparisons between GR animals and same-litter controls.
RESULTS

Growth-Restricted Animals Did Not Recover Normal Weight

The body weight of individual pups was measured on P0, P3, P7, P10, P14, P21, and P60 (Table 1). Mean weight on P0 was 4.48 ± 0.51 g for the GR pups and 6.16 ± 0.33 g for the control pups (p < 0.001). Subsequently, weight remained lower in the GR pups than in the controls, even in adulthood (P60: 188.50 ± 25.89 g vs 283.40 ± 48.84 g, p < 0.001).

Specific White Matter Lesions in Growth-Restricted Pups

On hematoxylin and eosin-stained sections from GR animals, no damage was detected in the cortical plate. In contrast, the cingulum showed diffuse lesions in GR animals on P0 (100%, n = 8) with a moth-eaten appearance and macrophage invasion (Fig. 1B). The surface of tissue microcysts has been measured in the cingular cortex and the underlying white matter (Fig. 1A, B) in the GR and control groups. Damaged tissue has been detected in the cingular white matter but not in the cingular cortex in the GR group as compared with controls (Figs. 1B, 2A). On P3, these cingular lesions in GR pups were even more diffuse. On P7, the cingular white matter displayed a similar common aspect in the GR group and controls. Interestingly, no differences in white matter appearance were found on E21 between GR fetuses and controls.

Increased Cell Death in the White Matter of Growth-Restricted Pups

Animals with GR showed a significant increase in TUNEL-positive nuclei in the white matter underlying the cerebral cortex on P3 compared with controls despite the small number of detected cells (Fig. 2B). Also, the number of cleaved-caspase 3-IR cells was increased in the white matter of GR pups on P3 (Fig. 2C). This feature was observed predominantly in the cingular white matter compared with the corpus callosum.
FIGURE 2. (A) Assessments of white matter lesions. The extent of tissue damage measured as the percentage of unstained areas (indicating lost tissue) was significantly different in the cingular white matter in the growth-restricted (GR) group compared with the control group, whereas no difference were measured in the cortex. (B) Quantitative analysis of the number of TUNEL-positive cells per hemisphere in the white matter in GR groups and control (cingulum and corpus callosum, Figs. 15–36 [21]) on P3. (C) Quantitative analysis of the number of cleaved-caspase 3-positive cells per hemisphere in the same area on P3. Bars represent the mean ± standard deviation. The asterisks indicate significant differences between the GR group and the control group (*, p < 0.05, **, p < 0.01, and ***, p < 0.001 by the nonparametric Mann-Whitney U test). (D) Quantitative increase of blood vessel density in the cingular white matter in GR group at various ages (**, p < 0.01, and ***, p < 0.001 by 2-way analysis of variance with the Bonferroni correction).

FIGURE 3. (A, B) Increased density of activated OX42-IR microglial cells in the cingulum of a growth-restricted (GR) pup (B) compared with a control (A) on P7. Bars = 250 μm. (C, D) High-power view of resident microglia in a control (C) and activated macrophages in a GR pup on P7 (D). Bars = 100 μm. (E, F) Activated OX42-positive macrophages were still present in the cingulum on P14 in a GR pup (F), whereas resident microglia were detected in a control (E). Bars = 500 μm. V, ventricle.
Transient Perinatal Angiogenesis Followed Prenatal Growth Restriction

Transient hypervascularization visualized by increases in Glut1-IR vessel walls (Fig. 1C, D) was found in the white matter (cingulum) at the septal level from E21 to P7 in the GR group compared with the controls (Fig. 2D). Vessel walls density in the GR group returned to control levels by P14.

Protracted Inflammatory Response in the White Matter in Growth-Restricted Pups

During normal rat development, activated and resident OX42-IR cells are found in the cingulum (Fig. 3A–D), external capsule, and internal capsule during the first postnatal week (23). The number of activated OX42-IR cells (Fig. 3D) at these sites was assessed from E21 to P60. From P0 to P7,

<table>
<thead>
<tr>
<th>Age (Days)</th>
<th>Control Group</th>
<th>Group with GR</th>
</tr>
</thead>
<tbody>
<tr>
<td>E21</td>
<td>5 ± 2</td>
<td>8 ± 3</td>
</tr>
<tr>
<td>P0</td>
<td>10 ± 1</td>
<td>15 ± 3</td>
</tr>
<tr>
<td>P3</td>
<td>12 ± 2</td>
<td>17 ± 4</td>
</tr>
<tr>
<td>P7</td>
<td>15 ± 3</td>
<td>20 ± 5</td>
</tr>
<tr>
<td>P14</td>
<td>20 ± 5</td>
<td>25 ± 6</td>
</tr>
<tr>
<td>P21</td>
<td>25 ± 7</td>
<td>30 ± 8</td>
</tr>
</tbody>
</table>

Asterisks indicate significant differences between the GR and control groups (**, p < 0.01, and ***, p < 0.001 by 2-way analysis of variance with the Bonferroni correction).

![Bar chart showing quantitative increase of the number of OX42-positive cells at various ages in the growth-restricted (GR) group compared with the control group in the cingulum white matter (A) and internal capsule (B). Bars represent the mean ± standard deviation. Asterisks indicate significant differences between the GR and control groups (**, p < 0.01, and ***, p < 0.001 by 2-way analysis of variance with the Bonferroni correction).](chart)

GFAP immunostaining is far stronger in the cingulum and corpus callosum of a growth-restricted (GR) pup (B) compared with a control (A) on P14. Bars = 400 μm. (C, D) High-power view of activated astrocytes in GR rats (D) compared with control astrocytes (C) and on P14. Bars = 50 μm. (E, F) Note the stronger GFAP immunostaining in the internal capsule (asterisks) on P21 in a GR (F) compared with a control animal (E). Bars = 400 μm. V, ventricle. Asterisks indicate counting areas (see Fig. 6).
activated OX42-IR cells were more numerous in the GR group than in the control group in the cingulum (Figs. 3A, B, 4A) and internal capsule (Fig. 4B), whereas no differences were detected in the external capsule. The number was increased about 2-fold from P0 to P7 in the GR group. On P14, 50% of GR animals (n = 8) still had increased activated microglial cell counts compared to the controls (Fig. 3E, F).

**White Matter Lesions Resolved in Protracted Astrogliosis**

From P7 to P21, activated astrocytes displaying an increased number of processes and larger cell bodies were detected in the cingular white matter of GR animals (Fig. 5A–D). The number of GFAP-IR astrocytes was significantly increased in the GR group compared with controls in the cingulum (Fig. 6A) and internal capsule (Figs. 5E, F, 6B) but not in the external capsule (Fig. 6C). On P60, no such differences in astrogliosis were detected between the groups.

**Persistent Myelination Deficiency in Growth-Restricted Animals**

On P7, loss of O4-IR preoligodendrocytes was found in the cingulum of GR pups (Figs. 7A, B, 8A). A decrease in the number of discrete bundles of MBP-IR myelin fascicles was noted in the GR group on P7, most notably in the corpus callosum and contiguous cortex (Fig. 7C, D). Severe MBP-IR fiber deficiency was found in the corpus callosum, cingulum, and overlying cortex throughout development and into adulthood (P60) in the GR group. On P60, 6 of the 9 GR animals showed MBP-IR fiber deficiency in the main brain fascicles, most notably the corpus callosum and internal capsule (Fig. 7E, F). Moreover, dramatic myelination deficiency was noted in 2 of the 9 GR pups. On P60, examination of MBP-immunolabeled sections showed a significant decrease in thickness of the genu of the corpus callosum on sections through the motor cortex (323 ± 621.27 μm in GR pups vs 408.3 ± 26.52 μm in controls, p < 0.001) (Fig. 8B) and visual cortex (400.5 ± 56.46 μm vs 313.3 ± 35.60 μm, p < 0.05, data not shown). No difference was found on P60 regarding thickness of the motor cortex (1863 ± 77.73 μm in GR pups vs 1894 ± 65.75 μm in controls) or the visual cortex (1273 ± 132.4 μm in GR pups vs 1263 ± 76.89 μm in controls).

**DISCUSSION**

Our results showed that pups subjected to chronic prenatal ischemia with probable hypoxia induced by uterine artery ligation exhibited diffuse white matter lesions and macrophage overactivation immediately after birth followed by astrogliosis during the first 2 postnatal weeks. One of the most striking events after white matter damage in this model was defect of axonal myelination until adulthood associated with a decreased density of preoligodendrocytes in the cingulum detected on P7 in GR pups.
Prenatal Ischemia/Hypoxia Is a Relevant Model for Preterm White Matter Damage

Unilateral uterine artery ligation induced ischemia and probable hypoxia responsible for GR of fetuses developing in the ligated uterine horn (18). Assessing prenatal hypoxia by measuring arterial blood gas concentrations in the fetuses was not feasible. However, the GR and increased blood vessel density in the fetuses on the ligated side argue strongly in favor of severe ischemia and hypoxia. This feature was detected as early as E21, before lesions in cingular white matter were observed after birth. We speculate that the white matter lesions resulted from the combination of ischemia before birth and reperfusion at birth. Pre- and postnatal GR in our model may also partly explain the white matter damage observed after birth. Indeed, in a recent study, preterm infants with severe intrauterine growth restriction were 4 to 6 times more likely to have cerebral palsy than those with moderate intrauterine growth restriction (25).

In our model, the combination of white matter lesions, inflammation, astrogliosis, and deficient myelination closely resembles the white matter damage observed in the developing human brain and in animal models of white matter disease (7, 23, 26, 27). In particular, our model shares similarities with another model recently developed at our laboratory, in which pregnant dams are subjected to chronic hypoxia during most of the gestational period (23). However, several differences exist between the 2 models. First, antenatal GR was more severe after uterine artery ligation than after chronic hypoxia; therefore, some pups failed to show catchup growth and remained smaller than the controls in adulthood. Second, the white matter lesions in pups with GR after ligation were more diffuse, extending to the cingulum, corpus callosum, and internal capsule. Third, myelination was abnormal until adulthood in the ligation model, whereas microglial activation or astrogliosis were comparable in the 2 models. These data indicate that white matter damage was more marked after severe ischemia in late gestation than after chronic hypoxia during gestation.

Major Influence of Inflammation Associated With Oxidative Stress at Birth in the Genesis of White Matter Damage

Data from several animal models combining hypoxic/ischemic injury and infection/inflammation support a role for
inflammation in the pathogenesis of perinatal white matter damage (7). In our model, the damaged white matter was characterized by increased numbers of activated ameboid microglia during the first 2 postnatal weeks. Under physiological conditions, microglial activation was detected in the white matter of the cingular axonal bundle and internal capsule during the first postnatal week (24). Evidence supporting a role for microglia was also obtained from another model of white matter damage induced by intracerebral injection of an excitotoxic compound in mice without infection (26). However, in this last model, inflammation occurred after the excitotoxic insult. Features observed in our GR model and in the chronic gestational hypoxia model suggest that reperfusion/reoxygenation process at birth may induce a “double hit” insult consisting in both oxidative stress and microglial activation leading to white matter damage (28). However, the mechanism underlying microglial activation in response to oxidative stress in these 2 models remains unclear and further experimental studies are needed to test this hypothesis.

Permanent Myelination Deficiencies in the Rats After Prenatal Uterine Ligation

Chronic myelination deficiency is the main pathologic feature of white matter damage and subsequent cerebral palsy in humans (1), suggesting a pathogenic role for developing oligodendrocytes. In pups with GR induced by ischemia and possibly hypoxia, the permanent myelination deficiency observed from P7 to adulthood was associated with a decreased density of preoligodendrocytes in the developing white matter. As mentioned here, chronic prenatal ischemia and possible hypoxia, the permanent myelination deficiency observed from P7 to adulthood was associated with a decreased density of preoligodendrocytes in the developing white matter. However, the relative role of oxidative stress, excitotoxic cascade, or energy failure in preoligodendrocyte cell death remains to be investigated in our in vivo models.

In conclusion, in the present study, neonatal white matter damage is virtually confined to the white matter and is accompanied with inflammation, cell death, and astrogliosis leading to prolonged deficit of axonal myelination. These features closely mimic white matter disease observed in extremely preterm human neonates. At this critical early stage, a prominent reduction in cortical grey matter volume compared with full-term infants has been recently associated with white matter injury. A combination of white and grey matter injury could account for the cognitive and behavioral deficits observed in 25% to 50% of very premature infants weighing less than 1500 g at birth (36–38). Consequently, our model holds promise as a tool for investigating the pathophysiology of white matter disease and its subsequent consequence on the development of neuronal circuitry.

REFERENCES

FIGURE 8. (A) Quantitative analysis of the number of O4-positive cells according to age in the growth-restricted (GR) group and control group in the cingular white matter on P7. (B) Quantitative analysis of the thickness of the genu of the corpus callosum (using MBP) at the motor cortex level in control and GR animals on P60. Bars represent the mean ± standard deviation. Asterisks indicate significant differences between the GR group and the control group (***, p < 0.001 by the nonparametric Mann-Whitney U test).