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Histopathological Correlates of MRI-Defined Chronic Perinatal White Matter Injury

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Abstract

Objective—Although MRI is the optimal imaging modality to define cerebral white-matter injury (WMI) in preterm survivors, the histopathological features of MRI-defined chronic lesions are poorly defined. We hypothesized that chronic WMI is related to a combination of delayed oligodendrocyte (OL) lineage cell death and arrested maturation of pre-oligodendrocytes (preOLs). We determined whether *ex vivo* MRI can distinguish distinct microglial and astroglial responses related to WMI progression and arrested preOL differentiation.

Methods—We employed a preterm fetal sheep model of global cerebral ischemia where acute WMI results in selective preOL degeneration. We developed novel algorithms to register histopathologically defined lesions with contrast- and diffusion-weighted high-field *ex vivo* MRI data.

Results—Despite mild delayed preOL degeneration, preOL density recovered to control levels by 7 days after ischemia and was ~2 fold greater at 14 days. However, pre-myelinating OLs were significantly diminished at 7 and 14 days. WMI evolved to mostly gliotic lesions where arrested preOL differentiation was directly proportional to the magnitude of astrogliosis. A reduction in cerebral WM volume was accompanied by four classes of MRI-defined lesions. Each lesion type displayed unique astroglial and microglial responses that corresponded to distinct forms of necrotic or non-necrotic injury. High-field MRI defined two novel hypo-intense signal abnormalities on T₂-weighted images that coincided with microscopic necrosis or identified astrogliosis with high sensitivity and specificity.

Interpretation—These studies support the potential of high-field MRI for early identification of microscopic necrosis and gliosis with preOL maturation arrest, a common form of WMI in preterm survivors.

Cerebral white matter injury (WMI) is the most common cause of chronic neurological disability in children with cerebral palsy.¹ Survivors of premature birth are at particular risk for WMI, which results in disrupted WM maturation and chronic myelination disturbances.² Advances in neonatal neuro-imaging have identified a pronounced shift in the features of WMI defined by conventional (T₁- and T₂-weighted) and diffusion-weighted MRI. Whereas, the cystic-necrotic lesions of periventricular leukomalacia (PVL) were previously the most common, the incidence of PVL has markedly declined,³ and a new form of chronic WMI has emerged as defined by MRI, dominated by focal or diffuse non-destructive lesions.^{4–7} However, controversy exists regarding the extent to which necrotic injury contributes to chronic human WMI.² Although there is a significant incidence of microscopic lesions seen at autopsy, these lesions are not readily detected by MRI in clinical studies.⁸ Hence, the extent to which necrotic injury contributes to the overall burden of WMI is unclear. Moreover, there appear to be many cases of WMI where necrosis is not seen in association with diffuse white matter gliosis. The clinic-pathological significance of these lesions is unclear, although such lesions coincide with preOL maturation arrest.⁹

Critically ill preterm neonates appear susceptible to WMI after episodes of oxidative stress that selectively target susceptible pre-oligodendrocytes (preOLs)^{10, 11} The spatial distribution of WMI is related to the relative density of susceptible preOLs and more resistant myelinating OLs.¹² However, preOL degeneration in rodents was followed by rapid regeneration of preOLs that failed to differentiate in the astroglial scar of chronic lesions.⁹ Growing evidence supports that arrested maturation of the OL lineage at the preOL stage is a central feature of myelination failure in both human perinatal WMI¹³ as well as adult demyelinating lesions and traumatic spinal cord injury.^{14, 15}

Although MRI is the optimal imaging modality to define WMI in preterm survivors,^{7, 16–18} the histopathological features of particular MRI signal abnormalities have received limited study.^{19–21} To determine whether MRI can distinguish distinct cellular responses related to WMI progression and preOL maturation arrest, we developed novel algorithms to register histopathologically defined lesions with contrast- and diffusion-weighted high-field MRI data. We analyzed WMI in a clinically relevant large pre-clinical model, preterm fetal sheep that sustained global cerebral ischemia.^{12, 22} In this model, fetal sheep display cerebral hemodynamics and brain maturation similar to human^{23–25} and develop acute WMI with preOL loss that closely resembles human.^{12, 26}

Chronic WMI evolved to mostly gliotic lesions in which preOL arrest was directly proportional to the magnitude of astrogliosis. A reduction in cerebral WM volume was accompanied by four classes of MRI-defined lesions. Each type of lesion displayed unique astroglial and microglial responses that corresponded to distinct forms of necrotic or non-necrotic injury. A new hypo-intense signal abnormality was identified on T₂-weighted images that identified early gliotic lesions without necrosis. In addition, high-field T₂w-imaging identified microcyts that have been difficult to demonstrate at lower field. Although there was a high incidence of microcysts, these lesions were infrequently observed in large regions of WMI and were not an essential feature of diffuse astrogliosis that was associated with preOL maturation arrest in our model. To our knowledge, lesions with these characteristics have not been previously reported, potentially as a result of limited study of perinatal WMI with static magnetic field strengths of greater than 3T. These studies support the potential of high-field MRI for early identification of gliosis with preOL maturation arrest, the major form of WMI in human preterm survivors.

Materials and Methods

Animal surgical procedures

Surgery was performed on time-bred sheep of mixed western breed between 88–91d of gestation (term 145d) as previously described.¹² For detailed methods on surgical procedures, physiological monitoring and blood analyses, see supplemental methods.

Cerebral Hypoperfusion Studies

Ischemia of 37 min duration was performed on the second or third post-operative day as previously reported.¹² Briefly, sustained cerebral hypoperfusion was initiated by bilateral carotid artery occlusion after inflation and reestablished by deflation of the carotid occluders.

Tissue handling

The ewe and fetuses were sacrificed (barbiturate overdose, Euthasol) at 1 (control, n=8; ischemia, n=8) or 2 (Control, n=6; ischemia, n=6) weeks following completion of the occlusion protocol. One 1-week experimental animal that developed extensive cystic necrotic injury was excluded from the study, making the final number studied 7. Fetal brains were immersion fixed at 4° C in 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4 for 3 d and then stored in PBS for at least 60 days.

Tissue preparation

Fixed fetal brains were cut into five equivalent coronal blocks in proportion to the distance between the frontal and parietal poles (6–10 mm). All frontal blocks studied spanned from the genu of the corpus callosum to the optic chiasm.

Ex vivo magnetic resonance imaging

Tissue was embedded alongside a twin control tissue block from the same level in 0.5% agarose and immersed in PBS within a 4 cm diameter plexiglass tube. A custom single-turn solenoidal coil (5 cm diameter, 5 cm length) was utilized for radiofrequency transmission and reception. Experiments were performed using an 11.7 T magnet interfaced with a 9 cm inner diameter magnetic field gradient coil (Bruker, Rheinstetten, Germany). Procedures generally followed the previously published strategy that used diffusion tensor imaging (DTI) to characterize postmortem tissue from other species.^{27, 28} Detailed scanning and image segmentation procedures are provided in supplemental methods.

Immunohistochemical studies

After MRI, frontal tissue blocks were cryoprotected by sequential equilibration in 15% and 30% sucrose solutions over 3d. Tissue was rapidly frozen using a liquid nitrogen interface for optimal preservation of O4 and O1 staining. Tissue blocks were serially sectioned at 50 µm using a CM 1950 cryostat (Leica Microsystems Inc., Bannockburn, IL). The detailed immunohistochemical protocols to visualize specific cell types were performed as previously described.^{9, 12, 29} Immunohistochemical procedures including antibodies and dilutions used are provided in supplemental methods.

Quantification of the density of GFAP, O4- and O1-labeled cells

The total density of O4- and O1-labeled cells in the corona radiata was determined by a blinded individual in a minimum of three serial adjacent sections for each case as previously described (see supplemental methods for detailed procedures).¹²

Quantification of GFAP stained area fraction

GFAP staining in each O4/O1-counted field was photographed using a 40x objective with fixed image acquisition settings. GFAP-labeled area was determined by a blinded individual in an unbiased fashion as previously described.³⁰ For detailed protocols, see supplemental methods.

Quantification of activated caspase-3

The density of AC3 in WM lesions followed the protocol outlined above for O4/O1 double-labeling studies (see supplemental materials for more detailed methods).

Registration of histopathological and MRI data

From each tissue block, 50 μ m serial sections at 600 μ m intervals (~12 sections per block) were triple-labeled with anti-Iba1, anti-GFAP, and anti-NeuN. NeuN montages from each block (scaled to match MRI resolution, see above) were aligned to create a 3D volume using the TurboReg plugin³¹ (ImageJ). The MRI from each block was aligned with the 3D NeuN stack using FSL (Analysis Group, FMRIB, Oxford, UK).^{32–34} Precise 2D alignment between NeuN slices and the corresponding 3D-aligned MRI slices was obtained using a custom program written based on the Insight Segmentation and Registration Toolkit (NIH; www.itk.org).³⁵ For detailed protocols, see supplemental methods.

Quantification of GFAP and IBA-1 ROI

MRI-derived segmentations were rescaled to match the native histo-pathological resolution and overlaid onto individual GFAP and IBA-1 histo-pathological montages (ImageJ). GFAP and IBA-1 expression in the MRI-defined ROIs was quantified at 5x using the unbiased foreground extraction method previously described for GFAP, above.

Definition of the sensitivity and specificity of high-field MRI

All GFAP montages were analyzed by a blinded individual and image segmentations of gliotic WM areas containing hypertrophic glial processes and increased GFAP density were created. MRI-derived segmentations were rescaled to match the native histopathological resolution and overlaid onto individual GFAP montages (ImageJ). Areas of astrogliosis were defined as the gold standard for brain injury and were aligned and analyzed in 3D. All ROI that were connected in the z plane were classified as a lesion. Overlap between GFAP- and MRI-defined segmentations was analyzed across all registered sections in the injured cohort. Sections without a GFAP ROI were defined as negative. GFAP lesions that did not overlap with MRI ROI were considered false-negative. MRI ROI that were unconnected to GFAP ROI were defined as false-positive. Lesions were divided into two groups, those with GFAP-defined lesions greater and less than 2.5 mm³ for sensitivity analyses. Sensitivity was defined as the proportion of GFAP lesions that were correctly identified by MRI. Specificity was defined as the proportion of negatives that were correctly identified by MRI.

Statistical analysis

Data analysis was performed using Prism 4 statistical software (GraphPad Software Inc., La Jolla, CA) except where noted. Data were expressed as means $\pm$ 1 SEM unless otherwise noted. Comparisons between brain weight data, cell counts and lesion detection were performed using unpaired two-tailed t-tests. Blood gas, regional MRI, lesion-class MRI and MRI lesion-defined histo-pathological descriptive indices were analyzed using ANOVA with *post hoc* inference testing done with Tukey's multiple comparison test. Analysis of the association between GFAP and OL markers was performed using Pearson correlations on triplicate data from each animal. $P < 0.05$ was considered statistically significant.

Results

Ischemia at 0.65 gestation generates three types of chronic progressive WMI

We analyzed 0.65 gestation fetal sheep that had equivalent physiological responses to global ischemia (Supp. Table 1) at 1 (n=7) or 2 (n=6) weeks recovery. At 1 week recovery, there was no difference in brain weight between the two groups (ischemia; 19.4 ± 2.3 g vs. control; 20.4 ± 2.1 g; mean $\pm$ SD). However, at 2 weeks recovery the ischemic group showed a significant reduction in brain weight (ischemia; 25.3 ± 2.8 g vs. control; 31.1 ± 1.8 g; $p < 0.01$) that was accompanied by cerebral white matter atrophy with enlarged lateral ventricles (Supp. Fig. 1). No animals showed signs of intracerebral or intraventricular hemorrhage.

Three types of cerebral WMI were identified. In 1 and 2 week survivors, the most frequent were diffuse lesions with pronounced astrogliosis (Fig. 1A vs. B). Consistent with prior studies, GFAP-labeled astrocytes had a hypertrophic reactive morphology (Supp. Fig 2A vs. B) and a trend for increasing density at 1 week that was significant at 2 weeks (Supp. Fig 2C).^{36, 37} Iba1-labeled microglia and macrophages displayed reactive features (e.g., amoeboid morphology), but were not markedly increased in density (Fig. 1A vs. 1B and insets), and neurofilament protein-labeled axons appeared normal (Fig. 1F). Less frequently observed were apparent focal necrotic lesions where the core contained numerous microglia/macrophages (Fig. 1C) while the periphery typically had more prominent GFAP-labeling. A minority of these lesions had dystrophic-appearing axons (Fig. 1G, arrows) and axonal spheroids (Fig. 1G, inset). Two week survivors rarely exhibited microcystic lesions (< 1 mm across) that were rich in microglia, but contained no astrocytes or axons (Fig. 1D, H).

PreOLs accumulate in diffuse gliotic lesions

In diffuse gliotic lesions, we next analyzed the response of the two major successive OL lineage stages (i.e., preOLs and immature (pre-myelinating) OLs) that predominate in preterm human cerebral WM.²⁹ Despite the initial reduction in the total density of these two OL lineages stages by 1 d after ischemia (Fig 2A, upper panel), density recovered to control levels at 1 week, and was significantly increased by 2 weeks. This acute injury response data is from a prior study and illustrates the trajectory of OL maturation.¹² Unexpectedly, a marked increase in density of preOLs at 2 weeks accounted for this pronounced expansion in total OL lineage cells (Fig. 2A middle panel). However, ischemia resulted in a significant reduction in immature OL density at 1 and 2 weeks relative to control (Fig. 2A, lower panel). Indeed, immature OLs failed to increase by 2 weeks after ischemia, despite a 50% increase in controls. Figure 2B, D illustrates a diffuse lesion enriched in reactive astrocytes (GFAP, red) that rarely contained immature OLs or early myelin (O1, green) in contrast to adjacent less gliotic WM. Rather, these gliotic lesions were rich in preOLs (Fig. 2C, E; O4, red). Hence, regions of diffuse astrogliosis coincided with apparent hypo-myelinated lesions that showed an arrest in OL lineage progression at the preOL stage.

In order to determine the associations between preOL and immature OL densities and the degree of gliosis, we quantified GFAP-labeled astrocytes at 1 and 2 weeks in both control and ischemia groups. At 1 week, immature OL density was significantly negatively associated with the GFAP area fraction (Fig. 2F), consistent with onset of arrested OL lineage maturation by 1 week (Fig. 2F upper panel, $r^2=0.32$, $p<0.001$). By 2 weeks, preOL accumulation was significantly positively associated with GFAP area fraction (Fig. 2F lower panel, $r^2=0.68$, $p<0.00001$). Thus, progressive preOL accumulation and maturation arrest occur in areas of increasing gliosis, consistent with the notion that astrogliosis is a surrogate marker of preOL maturation arrest in diffuse lesions.

Diffuse WMI is accompanied by early OL progenitor proliferation and delayed preOL death

Expansion of the preOL population also coincided with a progressive increase in the density of preOLs that displayed morphological features of degeneration (Fig. 3A). By 2 weeks, preOL degeneration was more than two-fold higher than at 1 week (Fig. 3B). Rarely, degenerating cells were labeled with AC3 (Fig. 3C). However, increased preOL degeneration was not accompanied by increased staining for AC3 in either the 1 or 2 week lesions (Fig 3D). The magnitude of preOL degeneration was strongly associated with the extent of astrogliosis as defined by the GFAP area fraction ($r^2=0.39$, $p<0.001$). We next determined whether the accumulation of preOLs in diffuse gliotic lesions was related to increased proliferation of preOLs or the OL progenitors that generate preOLs. PDGFR α -positive OL progenitors were infrequently labeled with Ki67 in controls (Fig. 3E), but were increased in lesions (Fig. 3F). By contrast, preOLs rarely co-localized with Ki67 (not shown). Chronic cerebral lesions were, thus, characterized by astrogliosis that coincided with preOL maturation arrest, OL progenitor expansion and persistent preOL degeneration.

Chronic WMI results in progressive cerebral growth retardation

We employed ex vivo MRI to determine if the progressive failure in OL maturation in chronic lesions was accompanied by volumetric changes in total cerebral WM. Based upon the T₂w-images (with confirmation from ADC and FA images), maps were generated that classified WM image voxels (Fig. 4A). At 1 week, there were no differences in WM volume between control and lesion groups (Fig. 4B). However, at 2 weeks, WM volume was significantly lower in the ischemic group, at levels similar to 1-week controls. By contrast, WM volume in the control animals increased by 30% between 1 and 2 weeks (Fig. 4B). Thus, chronic injury inhibited the normal maturational increase in WM volume.

High field strength MRI signal abnormalities associated with WMI

We analyzed MRI data for changes in T₂w image intensity, ADC and FA within WM. Between 1 and 2 weeks, T₂w intensity decreased in controls (Fig. 4C), consistent with a process of normal WM maturation. ADC was significantly elevated at 1 week but returned to control levels by 2 weeks (Fig. 4D), consistent with transient edema. There was a non-significant trend toward increasing FA from 1 to 2 weeks (Fig. 4E), but no changes in FA due to ischemia. Consistent with observations in preterm human, FA was elevated in more rapidly maturing WM tracts, such as the posterior limb of the internal capsule (Supp. Fig. 3).³⁸

Several types of high field strength MRI signal abnormalities were identified across a spectrum of WMI that ranged from moderate (Fig. 5B, E) to more severe (Fig. 5C, F). At 1 week, T₂w image intensity identified diffuse hypo-intense (D-hypo) abnormalities (Fig. 5B, C) in the deep and sub-gyral WM as well as less frequent focal hyper-intense (F-hyper) lesions that usually localized to superficial gyral WM (Fig. 5C). At 2 weeks, T₂w hypo-intensities (Fig. 5F; D-hypo) were still observed but were less pronounced. However, diffuse WM hyper-intensities (D-hyper) were detected in the deep WM at 2 weeks (Fig. 5E, F). Small focal T₂w hypo-intensities (F-hypo) were detected in the deep WM of some animals (Fig. 5E; F-hypo). F-hyper lesions were still detected in locations similar to week 1 (Fig. 5F; F-hyper). At 1 week, D-hypo and D-hyper lesions were significantly different from control by T₂w (Fig. 5G). At 2 weeks, only D-hyper lesions did not differ significantly from control (Fig. 5J). Diffusion characteristics were not altered within most of the defined lesion types. ADC was elevated in F-hyper lesions at both 1 and 2 weeks (Fig 5H, K), and FA was significantly reduced in these lesions at 2 weeks (Fig. 5L). However, diffusion imaging was insensitive to the diffuse lesions in the deep WM (D-hypo and D-hyper, Supp. Fig. 4).

Diffuse deep WM lesions were the largest and most commonly observed (Table 1), comprising 88% of total lesion volume at 1 week and 83% at 2 weeks. In contrast, focal gyral WM lesions (F-hyper) were markedly smaller, less frequently observed and represented only 12% of total lesion volume at 1 week and 16% at 2 weeks. Focal hypo-intensities (F-hypo) in the deep WM were infrequently observed and constituted less than 2% of lesion volume at 2 weeks. Thus, diffuse deep WM lesions represented the most common lesion observed as well as the greatest contributor to total lesion burden at both 1 and 2 weeks.

Registration algorithms define histopathological features of high-field MRI abnormalities

We next sought to determine if the different types of WM lesions identified by histopathology (Fig. 1) correspond to definable MRI abnormalities (Fig. 5). We quantified the astrocyte marker GFAP and the microglial/macrophage marker Iba1 within each MRI-defined lesion analyzed for T₂w signal abnormalities. Figure 6 demonstrates selected aspects of our protocol (Suppl. Fig. 5) for the registration of diffuse hypo-intense (D-hypo) WM lesions defined by MRI with GFAP staining. Histopathological images (Fig. 6A) were aligned with MRI data (Fig. 6B), allowing MRI-defined ROIs (Fig. 6C) to be superimposed on histopathological injury markers (Fig. 6D, E) at high resolution (Fig. 6F).

Microscopic necrosis has not been readily identifiable by neuroimaging of human preterm survivors.² We found that F-hypo lesions, which occurred infrequently (Table 1), corresponded to areas of microscopic necrosis defined by microcysts. Figure 7 shows two focal hypo-intense (F-hypo) lesions (Fig. 7B, D) that correspond to two microcysts intensely labeled with Iba1 (Fig. 7C, E) but with reduced labeling for GFAP (Fig. 7F). This unusual example of two microcysts occurring in close proximity demonstrates the ability of our registration method to resolve these discreet lesions. Hence, it is feasible to register both diffuse WM lesions at the sub mm level in a large brain region (Fig. 6) and discrete focal lesions less than 500 microns across (Fig. 7) that are identifiable by MRI and histopathology.

Figure 8 provides a quantitative analysis of the astroglial and microglial responses in non-necrotic (Fig. 8A, C, E) and apparent necrotic lesions (Fig. 8B, D, F) defined by histopathology and MRI. At 1 week, robust GFAP-defined astrogliosis occurred in both D-hypo lesions and F-hyper lesions (Fig. 8A, B). In D-hypo lesions (Fig. 8A), astrogliosis was accompanied by a non-significant trend toward elevation of Iba1, consistent with moderate microglial activation in non-necrotic lesions. By contrast, F-hyper lesions (Fig. 8B) showed a significant increase in Iba1, consistent with lesions with early necrosis. At 2 weeks, astrogliosis remained significantly elevated only in D-hypo lesions and Iba1 remained similar to week 1 (Fig. 8C). Similarly, an increase in both GFAP and Iba1 also occurred in D-hyper lesions but neither reached significance (Fig. 8E). In F-hyper lesions, GFAP dramatically decreased from 1 to 2 weeks and Iba1 markedly increased to over 300% of control levels (Fig. 8D), consistent with evolving necrosis. Similarly, F-hypo lesions, which coincide with microcysts (Fig. 7), showed robust elevation of Iba1 at 2 weeks but had minimal astrogliosis (Fig. 8F). Hence, at both 1 and 2 weeks, D-hypo and D-hyper identified non-necrotic astrogliosis with marginal microglial activation, whereas F-hyper and F-hypo identified apparent evolving necrosis. Thus, MRI was able to distinguish three distinct histopathological classes of lesions.

Sensitivity and specificity of high-field MRI

Since gliotic lesions were most commonly observed, we defined the sensitivity and specificity of non-necrotic T₂w signal abnormalities (D-hypo and D-hyper) in astrogliotic lesions. MRI lesions were present in all animals with astrogliosis at 1 and 2 weeks. At 1

week, D-hypo lesions detected 13/19 astrogliotic lesions. However, MRI was much more sensitive to large lesions ($> 2.5 \text{ mm}^3$) than small lesions ($< 2.5 \text{ mm}^3$) with a sensitivity of 100% (12/12) and 14% (1/7), respectively ($p < 0.001$, two-tailed t test), such that the effective limit of detection for these lesions was 2.5 mm^3 . Large lesions comprised the majority of the total lesion volume (377 mm^3 large vs. 6 mm^3 small). GFAP-defined ROIs that constituted 84% of the total lesion volume were detected by MRI. MRI identified three small false-positive lesions and its specificity was 92% (3/39 negative observations). At 2 weeks, D-hypo or D-hyper lesions detected 9/21 astrogliotic lesions. Large lesions were also more readily detected than small lesions with a sensitivity of 75% (6/8) and 23% (3/12), respectively ($p < 0.05$). Large lesions still dominated total lesion volume (186 mm^3 large vs. 20 mm^3 small). GFAP-defined ROIs that constituted 66% of the total lesion volume were detected by MRI. Only one small false-positive lesion was identified and specificity was 97% (1/29 negative observations).

Discussion

Progress to develop treatments for perinatal WMI has been hampered by the lack of surrogate markers for serial assessment of perinatal WMI progression. We analyzed a pre-clinical model of chronic cerebral WMI in preterm fetal sheep where novel registration algorithms were applied to define the potential of high-field MRI to distinguish several distinct types of histopathologically-defined injury. This study yielded the following novel findings: (1) A spectrum of chronic WMI was generated similar to that commonly observed in human preterm survivors.¹³ Thus, WMI with focal or diffuse gliosis predominated and apparent cystic necrotic PVL-like lesions were infrequently observed. (2) Despite the fact that preOL degeneration predominates in early WMI,^{11, 12} diffuse gliotic lesions contained an expanded population of preOLs that failed to differentiate to OLs. There was, thus, a net increase in total preOLs in lesions, that fully compensated for minimal delayed preOL degeneration. (3) PreOL maturation arrest was directly associated with the degree of gliosis, which supported that diffuse astrogliosis is a surrogate marker for lesions with arrested preOL differentiation. (4) Consistent with volumetric MRI studies in human preterm survivors,³⁹ chronic WMI was accompanied by a significant reduction in white matter growth. (5) Novel registration algorithms demonstrated that high-field MRI distinguished three major types of chronic WMI. (6) High-field MRI was up to 100% sensitive and 92% specific for histopathologically-defined astrogliotic lesions larger than 2.5 mm^3 .

The propensity for myelination failure is a central pathological feature that distinguishes chronic WMI from other forms of cerebral palsy. We previously proposed that myelination failure in chronic human WMI is related to targeted deletion of a susceptible pool of preOLs required to generate mature OLs.⁴⁰ Our results support a more complex mechanism whereby a combination of proliferative, degenerative and arrested maturational processes result in a net expansion in the pool of preOLs with potential to generate OLs. Similar to the rat,⁹ expansion of the preOL pool was driven by proliferation of PDGFr $^+$ OL progenitors. However, delayed preOL death in rats was more severe than in fetal sheep. In rats, chronic gray and white matter injury generated preOL death that was ~50% of that observed acutely and involved widespread activation of caspase-3.⁹ By contrast, more moderate and relatively selective acute WMI is generated in our fetal sheep preparation.¹² Although preOL death was still increasing two weeks after ischemia, it nevertheless, accounted for only ~15% of acute death and occurred independently of caspase-3 activation. Taken together, our rodent and sheep data support a mechanism where preOL survival outweighs death with a persistent net expansion in the preOL pool.

WMI was characterized by a chronic progressive process that resulted in blunting of WM growth. In fact, the magnitude of preOL arrest and astrogliosis were significantly associated

across a wide range of injury responses in 2 week survivors. Further studies are needed to define the mechanism of preOL arrest in astrogliotic lesions. In demyelinating lesions, traumatic spinal cord injury, and ischemic lesions, robust expression of hyaluronan or its receptor CD44 has been detected.^{14, 15, 41} Arrest of preOL maturation is stimulated both *in vitro* and *in vivo* by hyaluronan derived from reactive astrocytes.^{14, 42} Chronic myelination failure may, thus, arise from one or more inhibitory factors that block progression of preOLs to mature myelinating OLs. It is presently unclear, however, whether preOLs in chronic lesions would retain their myelinogenic potential if extrinsic inhibitory signals were removed. Additional factors such as delayed axonal degeneration may also contribute to chronic myelination failure.

Although performed in postmortem fixed tissue, there are a number of studies that indicate that both diffusion and T₂-mediated MRI contrast observed in these studies is likely to be preserved *in vivo*. Physiological effects *in vivo* can influence diffusion parameters, but diffusion characteristics due to tissue microstructure observed *ex vivo* are likely to be observed *in vivo*. Several studies have quantitatively characterized the relationship between *in vivo* and *ex vivo* MRI of fixed postmortem tissue.^{43–47} In particular, absolute ADC values decrease by a factor of ~2.7 but ADC contrast between neighboring regions are preserved after death.^{47, 48} Similarly, T₂ values decrease after death and fixation, but retain the *in vivo* pattern of image contrast between gray matter and WM,^{49–52} as well as between multiple sclerosis (MS) lesions and normal WM.^{53–55} T₂ hypointense MRI artifacts have been identified in tissue preserved for years in formalin.⁵⁶ Tissue in our study was preserved in paraformaldehyde and stored long-term in saline and we do not believe the image contrast pattern observed due to this effect resembles the large T₂w abnormalities seen in fetal WM.

Many previous studies that co-analyzed MRI and histopathological data relied on visual level matching, which did not permit quantitation. An alternative approach employed placement of fiduciary markers, which required prior surgical intervention and tissue destruction.^{57, 58} A recent mouse atlas study employed three-dimensional reconstruction of histology for association with MRI data.⁵⁹ We improved upon this approach to address the unique challenges related to analysis of a gyrencephalic fetal brain that sustains more distortion during tissue preparation. We achieved sub-mm registration that permitted direct measurement of cellular elements within MRI-defined regions.

Our studies provide new insight into the pathogenesis of chronic WMI associated with preOL maturation arrest. It has been proposed that the dominant lesion in the majority of cases of diffuse WMI contains microscopic areas of necrosis defined as noncystic PVL (PVL with microcysts) that are not readily detected by MRI, and that diffuse gliosis without microcysts is of uncertain pathological significance.² This microscopic necrosis has further been proposed as the dominant lesion that coincides with preOL maturation arrest.⁶⁰ Given the small dimensions of microcysts, they may be underestimated by histopathological surveys. Our most commonly observed MRI lesions were the D-hypo and D-hyper signal abnormalities. These corresponded to lesions with diffuse gliosis without necrosis (Table 2). Consistent with the pronounced decline in cystic PVL in human,^{60, 61} we also observed few necrotic PVL lesions that were enriched in reactive microglia and identified by focal hyperintensities on T₂w imaging (Table 1). Small discrete microcysts enriched in macrophages were visualized by T₂w as small focal hypo-intense lesions. Thus, high-field T₂w imaging detected microcysts not well visualized by neuroimaging in preterm survivors.² Microcysts were not detected at 1 week, but by 2 weeks, appeared to evolve to discrete lesions visualized by MRI. Although, as in human,⁸ these lesions were commonly observed (50% of animals) they constituted only 1.5% of total lesion volume. Thus, microcysts were infrequent, even in animals with extensive WMI, and diffuse astrogliosis frequently

occurred without microcysts. Our findings support that preOL maturation arrest occurs in areas of diffuse WM gliosis with or without microscopic areas of necrosis.

Our ability to detect diffuse gliosis was clearly time- and modality-dependent, with greatest sensitivity at 1 week in T₂w images. These lesions were highly sensitive for large, potentially clinically-relevant, gliotic lesions, and captured the majority of the lesion at 1 week. The sensitivity, however, declined by 2 weeks and MRI underestimated both the number and size of gliotic lesions. We also detected no change in diffusion characteristics at 1–2 weeks. The latter finding may be consistent with prior studies where sensitivity of diffusion characteristics to identify diffuse WMI was observed in preterm infants with WMI that were studied at ~3 weeks or later after birth.^{62, 63} Other predominantly necrotic lesions displayed diffusion abnormalities that were increased at 2 weeks after injury as previously reported for patients with PVL.⁷ At 1–2 weeks, hypo-intense lesions were identified as novel T₂w lesions with markedly low image intensity that were most prominent at 1 week.

The detection of T₂ hypo-intense lesions may be limited by the timing of imaging after injury and magnetic field strength. While we observed the greatest sensitivity to detect deep WM lesions 1 week after injury in the immature fetal sheep (~28–30 week human equivalence), prior studies have imaged preterm survivors either within weeks after birth or at term equivalence at 1–3 T.^{7, 16–18} The detection of small lesions, such as microcysts, *in vivo* may also be hampered by the relatively limited imaging resolutions achievable with most clinically-available MRI systems. Further, magnetic field strength-dependent factors that influence transverse relaxation may impart increased sensitivity to high field strength MRI systems, such as the 11.7 T system used herein, relative to current clinically accessible magnetic field strengths of 1.5–3 T. For example, we did not observe well-defined T₂w hypo-intense lesions at 3 T within a subset of the tissue samples that had hypo-intense lesions used for this study (unpublished observations). Prior studies of MS lesions have demonstrated T₂ hypo-intensities related to T₂* contrast at 7 T.⁶⁴ Thus, imaging modalities that maximize sensitivity to magnetic susceptibility, such as T₂*-weighting, may enhance the contrast of the lesions at lower field strengths. The biochemical source and the sensitivity of alternate MRI modalities for these lesions are currently under investigation.

Hence, current clinical MRI field strength may be a limiting factor to detect diffuse gliosis, microscopic necrosis and possibly other types of WMI. These data underscore the need for future studies to determine the clinical-translational utility of high-field MRI for improved diagnosis of perinatal WMI. Our large preclinical animal model provides unique experimental access to questions directed at the mechanisms of myelination failure in advanced lesions as well as definition of the optimal field strength and modality to resolve evolving lesions by MRI. Such studies will be critically important to define the potential windows after injury when myelination failure might be ameliorated. One such potential therapeutic strategy may be to reverse preOL maturation arrest by agents that alter the composition of the glial scar to promote myelination.⁶⁵

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Three major lesion types in chronic WM lesions. A, Control white matter contained resting astrocytes (GFAP) and microglia/macrophages (Iba1) with a resting morphology (inset, Iba1; red, nuclei; blue). B, Diffuse WM lesions had pronounced astrogliosis and a lesser population of microglia/macrophages with a reactive morphology (inset). C, Necrotic foci were observed less frequently that contained numerous reactive microglia/macrophages with amoeboid morphology (inset) and reduced astrocyte staining. D, At 2 weeks, microcysts were infrequently observed that were distinguished by their small size (< 1mm), well-defined borders, intense microglia/macrophage activation and diminished astrocyte staining. E, F Neurofilament staining (SMI 312) reveals normal appearing axons in control white

matter (E) and in diffuse gliotic lesions (F, arrows). G, Necrotic foci contained disrupted axons (arrows) and axonal spheroids (inset). H, A microcyst (arrows) surrounded by normal-appearing axons. Insets: A–D ($40\text{ }\mu\text{m} \times 40\text{ }\mu\text{m}$); Iba1:red, Hoechst: blue. Scale bars: A–D; $100\text{ }\mu\text{m}$, E–H; $20\text{ }\mu\text{m}$.

Figure 2.

OL lineage maturation arrest in diffuse gliotic lesions. A, After initial depletion (1 d), total OL lineage cells (O4-labeled preOLs and immature OLs) recovered to control levels by 7 d and were significantly increased by 14 d. PreOLs (O4⁺O1⁻) expanded significantly vs. control by 14 d, while immature OLs (O4⁺O1⁺) remained significantly reduced at both 7 and 14 d. Data are presented as mean $\pm$ SEM. B, D, A gliotic lesion with increased GFAP had rare immature OLs or early myelin (O1, green). C, E, The same lesion was rich in preOLs (O4, red). F, Linear regression analysis of the association between the degree of astrogliosis and preOL maturation at 7 and 14 d. Importantly, at 14 d, increased preOL density was highly significantly associated with increased gliosis, indicating that gliosis is a strong positive predictor of preOL maturation arrest in chronic diffuse gliotic lesions. The solid line denotes the regression lines and the dashed lines indicate 95% confidence intervals. Scale bars: B, C; 100 μ m; D, E; 25 μ m. [‡] Previously reported;¹² * $p < 0.05$.

Figure 3.

Chronic WM injury is accompanied by delayed preOL death and OL progenitor proliferation. A, Degenerating preOLs were observed 1 and 2 weeks after ischemia by morphological criteria including increased cytoplasmic labeling, process degeneration and nuclear condensation (arrowheads) as compared to healthy preOLs (arrows). This degeneration was significant at 1 and 2 weeks (B). C, Apoptotic (activated caspase-3 positive) preOLs were observed in the WM (arrow), however they were a minority of degenerating cells (D). E, Early OL progenitors (PDGFR α ⁺) in control WM were occasionally positive for Ki67 (arrows). F, Ki67 staining was increased in early OL progenitors 2 weeks after ischemia (arrows) and occasional non-OL lineage cells were also

positive for Ki67 (arrowheads). Triple-labeling studies with PDGFR α (red), Ki67 (green), O4 (orange) confirmed that the Ki67-positive cells were O4-negative early OL progenitors. Counterstained with hoechst (blue). Inset; (40 μ m $\times$ 40 μ m). Scale bars: 20 μ m * p<0.05

Figure 4.

High-field MRI analysis of chronic WM injury. A, Coronal hemisections at the level of the corpus callosum and caudate at 1 week are shown. A WM segmentation was generated based on the T₂w image and used to analyze WM volume, ADC and FA maps. B, WM volume was unchanged at 1 week. At 2 weeks, control WM expanded ~ 30%, but this growth was significantly retarded in the ischemia group. C, Analysis of T₂w image intensities revealed a reduction in WM intensity between 1 and 2 weeks but no significant differences due to ischemia. D, ADC was significantly increased in the 1 week ischemia group, but no other differences were found. E, WM FA values tended to increase between the 1 and 2 week groups but did not reach significance ($p=0.07$) and no overall differences were found due to ischemia. Abbreviations: CC; corpus callosum, Cd; caudate, CI; claustrum, Pu; putamen, SVZ; subventricular zone, V; ventricle. * $p<0.05$

Figure 5.

High-field strength MRI signal abnormalities were associated with chronic WM lesions. A, A representative 1 week control T₂w image. B, A T₂w image of an ischemic animal shows prominent diffuse WM hypo-intensities (D-hypo). C, T₂w image shows the most severely observed focal gyral WM hyper-intensity (F-hyper) as well as D-hypo lesions. D, A representative 2-week control T₂w image with notable decrease in WM T₂w image intensity vs. 1-week control. E, Two weeks after ischemia, new types of WM signal abnormalities are apparent. These include small focal WM hypo-intensities (F-hypo) and diffuse apparent hyper-intensities (D-hyper) in the PVWM. F, The new lesion types are observed in addition to D-hypo and F-hyper lesions, although they are less prominent than at 1 week. G, One

week D-hypo and F-hyper lesion T₂w image intensities differ significantly from control. H, ADC is significantly increased only in F-hyper lesions at 1 week. I, FA in the lesions did not differ from control by 1 week. J, At 2 weeks, T₂w intensities were significantly different from control for D-hypo, F-hyper and F-hypo lesions. K, ADC was significantly increased only in F-hyper lesions. Interestingly, ADC trended down in D-hyper lesions (p=0.07). L, At 2 weeks, F-hyper lesions displayed significantly reduced FA values. * p<0.05

Figure 6.

Independent histopathological-MRI registration allows quantification of cellular gliosis. A, The NeuN montage with tissue, but no lesion, contrast from a 1 week ischemia survivor used for registration with MRI. B, T₂w image of the MRI slice registered to A with apparent WM signal abnormalities. C, MRI-based ROI map corresponding to panels A and B. D, The GFAP montage that was co-acquired with A. Inset boxes in B–D correspond to panel E. E, GFAP montage with superimposed MRI-defined ROI (dashed line) indicating the area of GFAP quantification. Inset corresponds to panel F. F, High-resolution histopathological data with cellular detail was acquired and analyzed for GFAP quantification.

Figure 7.

Registration algorithm allows sub-mm alignment of MRI and histopathological features of the WM. A, The NeuN montage with tissue, but no lesion, contrast from a 2 week ischemia survivor used for registration with MRI. B, The T₂w image registered of A with apparent WM signal abnormalities including two small WM signal abnormalities in the box. C, The Iba1 montage that was co-acquired with A. Two small microglial/macrophage foci are apparent in the box. Inset boxes in B and C correspond to panels D–E. D, Registered T₂w image of inset in B. Note the pair of abnormal WM hypo-intensities. E, F, Registered Iba1 and GFAP images respectively. Two lesions ~300 µm in diameter with intense microglial/macrophage infiltration and reduced astroglial staining (arrows) consistent with microcysts are aligned with the signal abnormalities in D.

Figure 8.

Quantification of GFAP and Iba1 within MRI-defined WM signal abnormalities. A, At 1 week, D-hypo lesions had significantly elevated GFAP, consistent with diffuse gliotic injury. B, At 1 week, F-hyper lesions had significantly elevated GFAP and Iba1. C, At 2 weeks, GFAP remained elevated in D-hypo lesions and Iba1 was also elevated. D, At 2 weeks, GFAP was no longer elevated in F-hyper lesions and Iba1 was markedly increased vs. age and region matched control, consistent with progressive necrotic injury. E, D-hyper lesions tended to have increased GFAP and Iba1, but were not significantly different. F, F-hypo lesions had markedly increased Iba1 labeling and no change in GFAP labeling vs. control, as seen in microcysts. * $p < 0.05$

Table 1

Features of MRI-defined lesions.

MRI Type	Survival	Total lesion number	Mean lesion volume/animal $\pm$ S.D. (mm ³)	% Lesion volume at 1 or 2 weeks	% animals with lesion
D-hypo	1 week	23	68.4 $\pm$ 64.4	88.6	100 (7/7)
D-hypo	2 week	12	40.5 $\pm$ 12.4	50.6	83 (5/6)
D-hyper	2 week	5	22.4 $\pm$ 7.6	32.0	67 (4/6)
F-hyper	1 week	12	13.3 $\pm$ 13.1	11.4	71 (5/7)
F-hyper	2 week	6	6.2 $\pm$ 2.8	15.9	67 (4/6)
F-hypo	2 week	8	0.8 $\pm$ 1.1	1.5	50 (3/6)

Table 2

Summary of histopathological and MRI characteristics of cerebral WM lesions.

Path. Diagnosis	Survival	MRI Type	Location	Histopathological Features		MRI Characteristics		
				MG/Mø	AS	T ₂ w	ADC	FA
Diffuse non-cystic gliosis	1 week	D-hypo	Deep & Sub-gyral WM	+	+++*	↓*	NC	NC
	2 week	D-hypo	Deep & Sub-gyral WM	+	+++*	↓*	NC	NC
		D-hyper	Deep WM	+	+	↑	NC	NC
Focal necrosis	1 week	F-hyper	Gyral WM	++*	+++*	↑*	↑*	↓
	2 week	F-hyper	Gyral WM	+++*	+	↑*	↑*	↓*
	2 week	F-hypo	PVWM	+++*	-	↓*	↑	↓

MG, microglia; Mø, macrophage; AS, astrocyte; NC, no change vs. control; ↑, increased relative to control; ↓, decreased relative to control;

* values significantly different from control

MG/Mø (Iba1 % Control): +, 100–175%; ++, 175–250%; +++, > 250%.

AS (GFAP % control): -, < 100%; +, 100–125%; ++, 125–150%; +++, > 150%;