Cerebral Ischemia or Intrauterine Inflammation Promotes Differentiation of Oligodendroglial Precursors in Preterm Ovine Fetuses: Possible Cellular Basis for White Matter Injury

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White matter injury in premature infants is known to be major cause of long-term neurocognitive disability. but the pathogenic mechanism remains unclear, hampering our ability to develop preventions. Periventricular leukomalacia is a severe form of white matter injury. In the present study, we explored the effects of cerebral ischemia and/or intrauterine inflammation on the development of oligodendroglia in the cerebral white matter using chronically instrumented fetal sheep. Each fetus received one of three insults: hemorrhage, inflammation and their combination. In the hemorrhage group, 40% of the fetoplacental blood volume was acutely withdrawn, and 24 hours after removal, the blood was returned to the fetus. The inflammation group received intravenous granulocyte-colony stimulating factor and intra-amniotic endotoxin and thus suffered from necrotizing funisitis and chorioamnionitis. The inflammatory hemorrhage group underwent acute hemorrhage under the inflammatory state. The sham group received no insults. Importantly, periventricular leukomalacia was not detected in the sham and the inflammation groups. Differentiating oligodendroglia at various developmental stages were identified by immunohistochemical analysis with specific antibodies. No difference in the density of oligodendroglial progenitors was detected among the four groups, whereas oligodendroglial precursors were significantly reduced in the three insult groups, compared to sham control. Moreover, the density of immature oligodendroglia was higher in the inflammation group and the inflammatory hemorrhage group, while the density of mature oligodendroglia was highest in the hemorrhage group. We propose that cerebral ischemia or intrauterine inflammation induces the differentiation of oligodendroglial precursors in preterm fetuses, eventually resulting in their exhaustion.

Keywords: accelerated maturation; immunohistochemical staining; oligodendroglia; periventricular leukomalacia; white matter injury

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Introduction

Despite advances in neonatal intensive care, very low birth weight (< 1,500 g) infants remain highly vulnerable to brain injury; 5-10% of survivors develop cerebral palsy, and 40-50% show intellectual and/or developmental disability (Deng 2010). Periventricular leukomalacia (PVL) is a form of white matter injury (WMI), and is considered to be the main cause of the long-term neurological sequelae frequently identified in premature infants. It has been reported as a serious disease closely related to cerebral palsy and cognitive or behavioral deficits in childhood (Khwaja and Volpe 2008; Volpe 2009; Deng 2010). The detailed pathophysiological mechanisms that cause WMI have not been fully established yet, hampering the development of interventions.

Based on the findings of a number of studies (Riddle et al. 2006; Khwaja and Volpe 2008; Volpe 2009), it is hypothesized that the proliferation and maturation of oligodendroglial (OL) lineage cells is impaired in the cerebral white matter exposed to cerebral ischemia and intrauterine inflammation and that the impairment may occur as a result of the induction of apoptosis in vulnerable OL precursors by neurotoxic factors, such as inflammatory cytokines released by reactive astrocytes and activated microglia. In fact, a profound decrease in the amount of cerebral white matter is observed by magnetic resonance imaging (MRI) in neonates with either focal or diffuse WMI at around 18

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months (de Vries et al. 2002; Volpe 2008). However, only a few investigations have been carried out to determine the processes that OL lineage cells undergo following exposure to ischemia and/or inflammation (Billiards et al. 2008; Riddle et al. 2012).

We have previously developed two ovine model systems for studying fetal WMI: a PVL model with acute blood withdrawal and a PVL model of intrauterine inflammation (Matsuda et al. 2006; Saito et al. 2009). In the PVL model with acute blood withdrawal, about 40% of the fetoplacental blood volume was acutely withdrawn to generate ischemia-reperfusion injury in the deep cerebral white matter (Matsuda et al. 2006). This model thus mimics the placental abruption or placenta previa that is associated with fetal cerebral WMI. In the PVL model with intrauterine inflammation, intravenous granulocyte-colony stimulating factor (G-CSF) and intra-amniotic endotoxin were used to induce PVL lesions and multiple hemorrhagic necrotic lesions in the subcortical white matter (Saito et al. 2009). This experimental system thus models fetal cerebral WMI induced by strong and chronic inflammation in utero, such as that seen in association with necrotizing funisitis and chorioamnionitis.

In the present study, we examined changes in OL lineage cells according to the developmental stage in the cerebral white matter at the sub-acute stage of PVL following exposure to acute hemorrhagic insult and/or intrauterine inflammation. Specific primary antibodies produced differential staining of the OL lineage cells according to the developmental stage (OL progenitors, OL precursors, immature OL and mature OL), and the developmental rate in each stage was clarified based on the density of the cells at each stage (Back et al. 2007; Welin et al. 2007), and the staining characteristics among the sham, hemorrhage, inflammation, and inflammatory hemorrhage groups were compared. The aim of the present study was to analyze the influence of cerebral ischemia and/or intrauterine inflammation on the proliferation and development of OL lineage cells and to clarify whether developmental disabilities such as accelerated maturation and growth retardation were induced or not.

Materials and Methods

This study was performed between April 2008 and June 2010 following review and the approval (No. 20HpA-4, 21MdA-5 and 22MdA-19) from the Animal Care and Use Committee of Tohoku University Graduate School of Medicine. As the experimental materials, we used fetal brain tissues obtained through following more or less the same protocols as reported from our previous two experiments (Matsuda et al. 2006; Saito et al. 2009). The hemorrhage group tissue samples were obtained from PVL induced by acute blood withdrawal (Matsuda et al. 2006) and those in the other three groups, namely the sham group, the inflammation group and the inflammatory hemorrhage group, tissues were obtained from PVL related to intrauterine inflammation (Table 1 and Fig. 1) (Saito et al. 2009). The characteristics of the immunohistological findings were statistically

compared among the four groups.

Animal preparation

Suffolk ewes with timed pregnancies underwent surgery on 102-107 days of gestation (term = 147 days). The ewes in the hemorrhage group were anesthetized with intrathecal tetracaine hydrochloride and intravenous ketamine hydrochloride (Matsuda et al. 2006). Animals in the remaining three groups were intubated, ventilated, and anesthetized with 1.5% to 2% isoflurane during all the procedures (Saito et al. 2009).

After laparotomy and hysterotomy, three electrodes were fixed to the fetal chest wall and polyvinyl catheters were inserted into the fetal superior vena cava, inferior vena cava, distal abdominal aorta and amniotic cavity, respectively. All electrodes and catheters were exteriorized through a small incision in the flank of each ewe.

After surgery, the ewes were unrestrained and housed in individual cages, with free access to water and food throughout the study period. A recovery period of at least two days was allowed before starting the experiments; during that time, antibiotics were administered to the mother, fetus and amniotic cavity.

Estimation of fetoplacental blood volume

An isovolemic exchange transfusion was performed on each fetus using heparinized fresh plasma from another fetal sheep to determine the volume of blood removal for the hemorrhagic insult based on the actual fetoplacental blood volume without anesthesia before the insult. The hematocrit values were measured before and after the exchange transfusion. The fetoplacental blood volume was calculated using the following formula: V = v/ln (hematocrit before transfusion/hematocrit after transfusion), where V represents the fetoplacental blood volume and v represents the exchange blood volume (Matsuda et al. 1999, 2006; Saito et al. 2009). After the exchange transfusion, the separated erythrocytes were suspended in sodium chloride solution adjusted to the same volume as the exchange volume and returned to the fetuses over 5 hours to recover from the anemia.

Experimental protocol for hemorrhage group

Systemic fetal hypotension was induced through acute hemorrhage (n = 7); namely, 35-40% of the fetoplacental blood volume was withdrawn at a constant rate from the inferior vena cava catheter over a period of 20 minutes, and 24 hours after removal, the blood was returned to the fetuses over a period of 5 hours. Following this, 5 of the 7 fetuses that showed PVL were selected for the hemorrhage group (n = 5) in the present study (Table 1 and Fig. 1) (Matsuda et al. 2006). A gestational age of 110 days in the sheep is approximately equivalent to a human gestational age of around 30 weeks, with regard to both chronological stage and development of myelination (Matsuda et al. 1999, 2006).

Experimental protocol for inflammatory insult groups

Following estimation of the fetoplacental blood volume, the fetuses (n = 15) were randomly divided into two groups: a sham group (n = 5) and an inflammatory insult group (n = 10). All the fetuses in the inflammatory insult group received daily intravenous infusions of 40 μ g of G-CSF (Neutrogin® R; Chugai Co. Ltd, Tokyo, Japan) solubilized in 2 mL of saline from 105-109 days of gestation. G-CSF has been shown to increase polymorphonuclear leukocytes (PMNLs) counts in the circulating blood (Watanabe et al. 2007).

Table 1. Comparison of basic fetal characteristics.

	Group					
Variable	ShamHemorrhage $(n = 5)$ $(n = 5)$		Inflammation $(n = 5)$	Inflammatory hemorrhage $(n = 4)$		
Gestational days of:						
Initial operation	103 ± 0.0	107 ± 1.3	102 ± 0.2	103 ± 0.2		
Cesarean section	113 ± 0.0	116 ± 1.3	113 ± 0.0	113 ± 0.0		
Gestational days of:						
Endotoxin injection	_		107 ± 0.0	107 ± 0.0		
Hemodynamic insults	_	110 ± 1.3		108 ± 0.0		
Withdrawn blood volume (%)	_	40.3 ± 0.4		39.4 ± 0.6		
Weight:						
Body (kg)	2.14 ± 0.18	2.39 ± 0.18	2.04 ± 0.13	1.89 ± 0.10		
Brain (g)	34.2 ± 1.9	35.6 ± 1.6	31.2 ± 0.5	30.8 ± 1.1		
Brain/Body weight ratio	1.64 ± 0.06	1.60 ± 0.12	1.56 ± 0.12	1.64 ± 0.04		
Histopathology:						
Necrotizing Funisitis (%)	0	0	100	100		
PVL (%)	0	100	0	100		





The four groups of the current study were derived from the two animal experiments previously conducted in our laboratory. The sheep fetuses in the hemorrhage group (n = 5) were selected from those in which PVL was induced in the acute hemorrhage experiment. The other three groups were derived from the experiments of PVL induction by inflammation. The fetuses in the sham group (n = 5) did not receive inflammatory or hemodynamic insult, and thus no PVL was induced. Those in the inflammation group (n = 5) were subjected only to the inflammatory insult and did not show PVL. Those in the inflammatory hemorrhage group were subjected to both inflammatory and hemorrhagic insults and in consequence, were selected from the fetuses in which PVL was induced (n = 4).

Fetuses were then exposed to 20 mg of endotoxin (*Escherichia coli* 055:B5 endotoxin; Sigma Chemical Co., St. Louis, MO) solubilized in 5 mL of saline administered into the amniotic cavity once on 107 days of gestation to activate the PMNLs and induce inflammation around the amniotic cavity (Watanabe et al. 2007). In the sham group, no inflammatory insult was performed and as a result, none of these fetuses had PVL (Table 1 and Fig. 1) (Saito et al. 2009).

At 24 hours after the endotoxin infusion on 108 days of gestation, the fetuses in the inflammatory insult group (n = 10) were randomly divided into two groups (n = 5, each): an inflammation group and an inflammatory hemorrhage group. The following experiments were then conducted as described previously (Saito et al. 2009). In the inflammatory hemorrhage group, the acute blood withdrawal was performed using a procedure similar to the experiment on PVL induced by acute hemorrhage; 24 hours after the acute hemorrhage, the blood was returned to the fetuses over a period of 5 hours. In the inflammation group, no hemodynamic insult was performed. As a result, 4 of the 5 fetuses in the inflammatory hemorrhage group had PVL and thus comprised the inflammatory hemorrhage group (n = 4)in the present study, but none of the fetuses in the inflammation group had PVL (Table 1 and Fig.1) (Saito et al. 2009).

Histopathological analysis

On 113-116 days of gestation, cesarean sections were performed and the fetuses were weighed. The fetal membrane, umbilical cord and placentomes were placed in 10% formalin solution for fixation. Under anesthesia, the fetal brains were perfused with 10% neutralized buffered formalin for fixation, removed and weighed. The cerebral hemispheres were cut into four standardized coronal sections at the level of the frontal lobe, the anterior basal ganglia, the mamillary bodies and the occipital lobe. Multiple additional sections of the cerebellum, midbrain, pons and medulla oblongata were also obtained. After macroscopically observing each section, a histopathologic evaluation was performed using 4-µm sections stained with hematoxylin and eosin.

Using the criteria proposed by Banker and Larroche (1962), PVL was defined as the presence of scattered round neuroaxonal swelling and focal coagulation necrosis with infiltration by microglia/ macrophages localized within the deep white matter around the lateral ventricles. Necrotizing funisitis was defined using the criteria of Navarro and Blanc (1974). The same observer (Y.K.) performed all histopathologic assessments in a blinded fashion.

Single-labeling immunocytochemistry

Single-labeled immunohistochemical staining with antibodies and lectin (Table 2) were performed using formalin-fixed coronal section samples at the level of the anterior basal ganglia. A streptavidinbiotin peroxidase method (SAB-PO kit, mouse and rabbit, 03AM0769, NICHIREI, Japan) was used for the immunohistochemical staining. To enhance antigenicity, autoclave techniques were used in the sections for chondroitin sulfate proteoglycan 4 (NG2) (10 mM citrate buffer for 15 minutes). Sections for the O4 stain were incubated for 15 minutes with proteinase K (0.4 mg/ml, S3020, DAKO, USA). Sections stained for 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) were incubated for 30 minutes at 37°C with 0.1% trypsin in phosphate buffered saline (PBS) (0.05M Tris-HCl buffer/ Trypsin/CaCl₂). The sections for proteolipid protein (PLP) and glial fibrillary acidic protein (GFAP) were not pretreated. Endogenous peroxidase in each section was removed with 0.3% H₂O₂ in methanol for 10 minutes and each section was incubated with normal serum for 10 minutes to block adhesion of protein. Primary antibodies were diluted in PBS and the sections with each primary antibody were incubated for 2 hours at 37°C. Secondary antibodies were incubated for 10 minutes and a further 5 minutes with the enzyme reagent Sections were visualized using 3,3-diamionbenzidine (DAB). Hematoxylin counterstain was applied to each section (Duncan et al. 2002).

Activated microglia were detected with tomato lectin histochemistry. Sections were incubated for 30 minutes at 37°C with 0.1% trypsin in PBS. The sections with the tomato lectin diluted in PBS were incubated for 2 hours at 37°C and for a further 5 minutes with the enzyme reagents. Sections were visualized using DAB, and a hematoxylin counterstain was applied to each section (Welin et al. 2007)

Apoptotic cells were identified with the TUNEL technique (in situ cell death detection Kit, 1684817, Roche Diagnostics, USA). Sections were incubated for 15 minutes with proteinase K and incubated with the TUNEL reaction mixture for 60 minutes at 37°C. Sections were incubated with TUNEL-POD for 30 min at 37°C. Sections were visualized using DAB and a hematoxylin counterstain was applied to each section (Welin et al. 2007).

Analysis of cell density and distribution

In each fetal brain section, images of the total 10 sampling areas (the sum of the 5 periventricular and 5 subcortical samples) from white matter without any PVL lesions were randomly selected (Fig. 2A) and captured at 400× magnification (DP70[®], Olympus, Tokyo, Japan) (Fig. 2B) (Welin et al. 2007). To evaluate the proliferation and development of OL lineage cells, we analyzed stainable cellular density. Immunostained areas positive for OL lineage cells, activated microglia and reactive astrocytes in each picture were automatically extracted based on a signal threshold function for removal of background noise, using Win ROOF software, version 6.1 (MITANI corporation, Tokyo, Japan). Subsequently, immunostainability of the

Table 2. Antibodies and lectin used in this study.									
Antibodies/lectin	Objective	Dilution	Catalog No.	Source					
NG2 (anti-mouse)	OL progenitor	1:10	MAB5520	CHEMICON international, USA					
O4 (anti-mouse)	OL precursor to immature OL	1:100	MAB345	CHEMICON international, USA					
CNPase (anti-mouse)	Immature OL to mature OL	1:500	MAB326R	CHEMICON international, USA					
PLP (anti-mouse)	Mature OL	1:1,000	MAB388-100UG	CHEMICON international, USA					
Tomato lectin (biotinylated)	Activated microglia	1:100	BK-3000	VECTOR laboratories, USA					
GFAP (anti-rabbit)	Reactive astrocytes	1:500	M0761	DAKO, USA					

To identify and classify the OL lineage cells (OL progenitors, OL precursors, immature OL and mature OL), the following antibodies, respectively, were used; antibody to NG2 (chondroitin sulfate proteoglycan 4) for OL progenitors, antibody to O4 for precursors to immature OL, antibody to CNPase (2',3'-cyclic nucleotide 3'-phosphodiesterase) for immature to mature OL, and antibody to PLP (proteolipid protein) for mature OL (Back et al. 2007; Volpe et al. 2011). Activated microglia and reactive astrocytes were identified with the tomato lectin and GFAP (glial fibrillary acidic protein) antibodies, respectively.

Table 2.	Antibodies	and	lectin	used	in	this	stuc





Fig. 2. Selection and process of sampling for analysis of the immunohistochemically stained images. In the left coronal section at the level of the anterior basal ganglia in each fetal brain, images of the total 10 sampling areas (the sum of the 5 periventricular and 5 subcortical samples) from all over the white matter without any PVL lesion were randomly selected.

(A) A representative image of the fetal brain derived from the inflammatory hemorrhage group. Closed triangles indicate focal PVL, and open squares indicate the sampling regions. Five subcortical images were sampled within the white matter near the cortex, and five periventricular images were near the lateral ventricle. All sampled regions were confirmed to be out of the histologic changes induced by focal PVL, and did not contain necrotic tissue, axonal swelling and apparent infiltration of reactive astrocyte or activated microglia/macrophage, at least at 400× magnification in the hematoxylin and eosin stain.

(B) A representative picture of immunohistochemical staining of the fetal brain with the O4 antibody. The tissue section was prepared from the inflammatory hemorrhage group. Arrows indicate the O4-positive cells.

extracted areas was transformed into numerical values of the integrated density, defined as a sum of signal intensity in the positivestaining color of all pixels divided by a total number of pixels in each picture and then expressed as values relative to those in the sham group.

Sections stained with TUNEL were also analyzed using Win ROOF version 6.1. The number of TUNEL-positive cells were counted and divided by the number of total cells, and the ratio was expressed as percentage. The ratio in the sham group was considered to be a baseline value, reflected physiologic apoptosis of OL precursors at the time of the myelination spurt.

The preparation, stain and analysis of the sampling sections were performed at one time by one blinded observer (R.K.).

Statistical analysis

All values were expressed as the mean \pm SEM. Differences in continuous variables between the 4 groups were assessed using the two-way Kruskal-Wallis test; if a significant difference was suggested, the Dunnett's multiple comparison test was performed for comparison with the sham group. Differences in continuous variables between two groups were assessed using the Wilcoxon signed-rank test. All *p* values were two-tailed, and a value of *p* < 0.05 was considered significant.

Results

Basic fetal characteristics

Table 1 shows the results of the statistical analysis of the basic fetal characteristics of the sham, hemorrhage, inflammation and inflammatory hemorrhage groups. No significant differences among the four groups were found with regard to the gestational days of the initial operation and cesarean section, fetal brain and body weight and brain/ body weight ratio. No significant differences in the gestational days of the hemorrhagic insult and the withdrawn

blood volume were noted between the hemorrhage and inflammatory hemorrhage groups. All the fetuses in the inflammation and inflammatory hemorrhage groups showed necrotizing funisitis, in which numerous PMNLs had infiltrated from the vascular space toward the amniotic cavity, and had accumulated and degenerated within the epithelial layers (Watanabe et al. 2007; Saito et al. 2009). No fetuses in the sham and hemorrhage groups had funisitis. All the fetuses in the hemorrhage and inflammatory hemorrhage groups showed PVL though there were no fetuses with PVL in the sham and inflammation groups. PVL observed in the hemorrhage group was characterized predominantly by axonal swelling (5/5 fetuses) and/or focal necrosis with activated microglia/macrophages (4/5 fetuses) within the deep white matter around the lateral ventricles (Matsuda et al. 2006). PVL found in the inflammatory hemorrhage group was characterized by not only focal coagulation necrosis (4/4 fetuses), but also multiple petechial hemorrhages (1/4 fetuses) in the subcortical white matter, which always surrounded a necrotic core lesion adjacent to a small vessel (Saito et al. 2009).

Distinct density values of the OL lineage cells in fetal brain depending on the insults

Fig. 3 shows the results of the statistical analysis of the immunohistochemistry of the sham, hemorrhage, inflammation and inflammatory hemorrhage groups. Regarding the integrated density of NG2-positive OL progenitor cells, no significant differences were observed among the hemorrhage (0.93 ± 0.15), inflammation (0.90 ± 0.06) and inflammatory hemorrhage (0.77 ± 0.07) groups (Fig. 3A). In contrast, the density values of O4-positive OL precursor cells were significantly lower in the hemorrhage (0.18 ± 0.23),



Fig. 3. Comparison of the cell density in the immunohistochemical stains among the 4 groups. The cell density in the immunohistochemical stains is shown and compared among the sham (*white bar*), hemorrhage (*gray bar*), inflammation (*dark bar*) and inflammatory hemorrhage group (*black bar*). The cell density of hemorrhage, inflammation and inflammatory hemorrhage group were expressed as values relative to those in the sham group. The OL lineage cells were identified and classified using single-labeled immunocytochemistry with the following antibodies: NG2 for OL progenitor (A), O4 for OL precursor (B), CNPase for immature OL (C) and PLP for mature OL (D). In addition, GFAP was used for reactive astrocytes (E), tomato lectin for activated microglia (F), and the TUNEL method for apoptotic cells (G). The vertical axis indicates the relative value of the integrated density to that of the sham group. All data are expressed as the mean ± SEM. **P* < 0.05 (Dunnett's test).</p>

inflammation (0.60 ± 0.06) and inflammatory hemorrhage (0.62 ± 0.08) groups than the value in the sham group (Fig. 3B). The density values of CNPase-positive immature OL cells were significantly higher in the inflammation (2.85 ± 0.32) and inflammatory hemorrhage (3.66 ± 0.53) groups

than the value in the sham group, although no significant change in the hemorrhage group (0.61 ± 0.11) was found compared with the sham group (Fig. 3C). Unexpectedly, the density value of PLP-positive mature OL cells was remarkably higher only in the hemorrhage group (4.01 ± 0.11)

0.64) than that in the sham group (Fig. 3D); namely, no significant difference was noted in the inflammation (0.93 \pm 0.09) or inflammatory hemorrhage group (1.90 \pm 0.37).

Increased density of reactive astroglia in fetuses with PVL

The density values of GFAP-positive reactive astroglia were significantly higher in the hemorrhage (4.37 ± 0.54) and inflammatory hemorrhage (2.24 ± 0.22) groups than the value in the sham group, although no significant change in the inflammation group (1.57 ± 0.15) was found (Fig. 3E).

Increased density of activated microglia in fetuses with hemorrhagic insult

The density value of activated microglia was significantly higher in the hemorrhage group (1.26 ± 0.14) than the value in the sham group (Fig. 3F). Unexpectedly, however, the density value was significantly lower in the inflammatory hemorrhage group (0.53 ± 0.05) . There was no significant change in the inflammation group (1.02 ± 0.07) , compared with the sham group (Fig. 3F).

Decreased number of apoptotic cells in fetuses with each insult

Unexpectedly, the density values of apoptotic cells

were significantly lower in the hemorrhage (0.27 ± 0.11) , inflammation (0.08 ± 0.06) and inflammatory hemorrhage (0.13 ± 0.07) groups than the density in the sham group (Fig. 3G). On the other hand, there was no significant difference in the number of total cells in the background among the four groups.

Discussion

The most noteworthy points shown in the results of this study were: i) that the density of OL precursors (O4+) in the cerebral white matter was significantly lower in preterm ovine fetuses 6 days after hemorrhage and/or inflammation than in the sham group (Fig. 3B); and ii) at the same time the density of mature OL (CNPase+ or PLP+) was markedly higher (Fig. 3C, D). If hemorrhage and/or inflammation induced apoptosis of vulnerable OL precursors and thus inhibited the proliferation of the entire OL lineage in the cerebral white matter as in the previous hypothesis (Riddle et al. 2006; Khwaja and Volpe 2008; Volpe 2009), the density of mature OL, which are at the developmental stages following OL precursors, should decrease as well as the density of OL precursors. On the contrary, however, the density of mature OL markedly increased, and this phenomenon cannot be fully explained by the previous hypothesis.



Fig. 4. Schematic diagram illustrating of the current hypothesis.

The normal development of the OL lineage cells is indicated in the upper panel of the figure. OL precursors are at the helm: do they proliferate or differentiate? It is quite important for brain growth and development that an appropriate balance is maintained between the proliferation and differentiation of OL lineage cells.

The former hypothesis is shown in the middle panel. Cerebral ischemia or intrauterine inflammation simply induces apoptosis of the fragile OL precursors, consequently inhibiting the proliferation of the entire OL lineage cells, resulting subsequently in a profound volume loss of the cerebral white matter.

The new hypothesis based on the current experiments is shown in the lower panel. Cerebral ischemia and/or intrauterine inflammation inhibit the proliferation of OL precursors and also induce the differentiation of OL precursors. As a result, myelination of the entire OL lineage cells may be eventually impaired because of exhaustion of the OL precursors, which leads to the profound decrease in the white matter volume. In addition, the number of TUNEL-positive apoptotic cells in the cerebral white matter was significantly lower in the fetuses after hemorrhage and/or inflammation than in the sham group (Fig. 3G), a finding that is also inconsistent with the previous hypothesis that apoptosis is strongly induced in OL precursors.

To explain the results of this study (Fig. 4), we propose a new hypothesis that the stress of hemorrhagic ischemia and/or intrauterine inflammation may induce the differentiation of OL precursors. OL precursors are at the helm as to whether the entire OL lineage proliferates or differentiates. Therefore, if priority is given to differentiation, rather than proliferation, of OL precursors in response to anti-stress hormones such as glucocorticoids, and differentiation into immature and mature OL is strongly induced, OL precursors must be exhausted for a while as a consequence. At the same time, this would be reflected as a decrease in the number of TUNEL-positive apoptotic cells, because, naturally, OL precursors are likely to undergo apoptosis most frequently at this stage (Barres et al. 1992; Buss et al 2006).

The effects of glucocorticoids on OL lineage cells are complicated, but their important role has recently been elucidated (Jauregui-Huerta et al. 2010). In general, glucocorticoids inhibit the proliferation of OL precursors, promote differentiation into postmitotic OL (Barres et al. 1994), initiate myelination (Kumar et al. 1989) and reinforce the biosynthesis of myelin components (Chan et al. 1998). On the other hand, in an experiment where betamethasone was administered to pregnant ewes, it was discovered that the higher the dose of betamethasone at earlier gestation, the lower the immunostainability of myelin basic proteins in the fetal cerebral white matter (namely, myelination was inhibited) (Antonow-Schlorke et al. 2009). Therefore, if stress-responsive glucocorticoids induce differentiation of OL precursors to markedly inhibit their proliferation in a particular time window, myelination may be seriously delayed eventually, even if myelination is promoted transiently. Consequently, glucocorticoids caused the decease in the white matter volume, as observed in MRI of diffuse WMI at around 18 months of corrected age. Such a viewpoint may be one of the suggestive clues to consider the pathogenic mechanism of diffuse WMI and to develop its animal model in the next step.

Significant changes in the density of reactive astrocytes (GFAP) and activated microglia (tomato lectin+) were observed in fetuses with PVL in the hemorrhage and inflammatory hemorrhage groups compared to the sham group fetuses, but no obvious changes were found in the inflammation group fetuses (Fig. 3E, F). It has long been well known that reactive astrocytes and activated microglia are observed in the white matter surrounding focal WMI such as PVL (Banker and Larroche 1962; Volpe 2009), and the increase in the density of these cells is consistent with this finding. However, it is difficult to explain why the density of activated microglia decreased in the inflammatory hemorrhage group based on the results of this study. One out of 4 fetuses had multiple small hemorrhagic necrosis in the inflammatory hemorrhage group, and therefore, activated microglia may have been exhausted in the cerebral white matter as a result of the recruitment of a large amount of these cells in the necrotic foci. On the other hand, the phenotype of microglia might have shifted from activated to resting state in the white matter because tomato lectin histochemistry could not detect resting but activated microglia.

No significant changes were observed in the density of reactive astrocytes or activated microglia in the inflammation group, which is consistent in part with the fact that PVL was not induced in the inflammation group. This finding suggests that the enhanced differentiation of OL precursors may be one of the essential causes of diffuse WMI associated with intrauterine inflammation.

Based on the above investigations, we propose that cerebral ischemia or intrauterine inflammation not only causes focal WMI but also promotes the differentiation of OL precursors in preterm ovine fetuses. As a result, OL precursors transiently decrease in their number, and myelination by postmitotic OL proceeds. However, when the serious decrease in OL precursors is prolonged, myelination of the entire OL lineage cells may eventually be impaired to induce diffuse WMI. Further studies are required to clarify the real nature of the enhanced maturation processes, including the stress responsiveness of OL precursors, the changes in the ultrastructure of the myelin sheath, and MRI of the whole brain.

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Conflict of Interest

The authors declare no conflict of interest.

References

- Antonow-Schlorke, I., Helgert, A., Gey, C., Coksaygan, T., Schubert, H., Nathanielsz, P.W., Witte, O.W. & Schwab, M. (2009) Adverse effects of antenatal glucocorticoids on cerebral myelination in sheep. *Obstet. Gynecol.*, **113**, 142-151.
- Back, S.A., Riddle, A. & McClure, M.M. (2007) Maturationdependent vulnerability of perinatal white matter in premature birth. *Stroke*, **38** (2 Suppl), 724-730.
- Banker, B.Q. & Larroche, J.C. (1962) Periventricular leukomalacia of infancy. A form of neonatal axonic encephalopathy. *Arch. Neurol.*, 7, 386-410.
- Barres, B.A., Hart, I.K., Coles, H.S., Burne, J.F., Voyvodic, J.T., Richardson, W.D. & Raff, M.C. (1992) Cell death and control of cell survival in the oligodendrocyte lineage. *Cell*, 70, 31-46.
- Barres, B.A., Lazar, M.A. & Raff, M.C. (1994) A novel role for thyroid hormone, glucocorticoids and retinoic acid in timing oligodendrocyte development. *Development*, **120**, 1097-1108.
- Billiards, S.S., Haynes, R.L., Folkerth, R.D., Borenstein, N.S., Trachtenberg, F.L., Rowitch, D.H., Ligon, K.L., Volpe, J.J. & Kinney, H.C. (2008) Myelin abnormalities without oligodendrocyte loss in periventricular leukomalacia. *Brain Pathol.*,

18, 153-163.

- Buss, R.R., Sun, W. & Oppenheim, R.W. (2006) Adaptive roles of programmed cell death during nervous system development. *Annu. Rev. Neurosci.*, 29, 1-35.
- Chan, J.R., Phillips, L.J. 2nd. & Glaser, M. (1998) Glucocorticoids and progestins signal the initiation and enhance the rate of myelin formation. *Proc. Natl. Acad. Sci. USA*, **95**, 10459-10464.
- Deng, W. (2010) Neurobiology of injury to the developing brain. Nat. Rev. Neurol., 6, 328-336.
- de Vries, L.S., Groenendaal, F. & Meiners, L.C. (2002) Ischemic lesions in the preterm brain. In *MRI of the Neonatal Brain: Periventricular leukomalacia*, 1st ed., edited by Rutherford, M. WB Saunders, Philadelphia, PA, pp. 155-164.
- Duncan, J.R., Cock, M.L., Scheerlinck, J.P., Westcott, K.T., McLean, C., Harding, R. & Rees, S.M. (2002) White matter injury after repeated endotoxin exposure in the preterm ovine fetus. *Pediatr: Res.*, **52**, 941-949.
- Jauregui-Huerta, F., Ruvalcaba-Delgadillo, Y., Gonzalez-Castañeda, R., Garcia-Estrada, J., Gonzalez-Perez, O. & Luquin, S. (2010) Responses of glial cells to stress and glucocorticoids. *Curr. Immunol. Rev.*, 6, 195-204.
- Khwaja, O. & Volpe, J.J. (2008) Pathogenesis of cerebral white matter injury of prematurity. Arch. Dis. Child Fetal Neonatal Ed., 93, F153-F161.
- Kumar, S., Cole, R., Chiappelli, F. & de Vellis, J. (1989) Differential regulation of oligodendrocyte markers by glucocorticoids: post-transcriptional regulation of both proteolipid protein and myelin basic protein and transcriptional regulation of glycerol phosphate dehydrogenase. *Proc. Natl. Acad. Sci. USA*, 86, 6807-6811.
- Matsuda, T., Okuyama, K., Cho, K., Hoshi, N., Matsumoto, Y., Kobayashi, Y. & Fujimoto, S. (1999) Induction of antenatal periventricular leukomalacia by hemorrhagic hypotension in the chronically instrumented fetal sheep. *Am. J. Obstet. Gynecol.*, 181, 725-730.
- Matsuda, T., Okuyama, K., Cho, K., Okajima, S., Kobayashi, Y., Hoshi, Y. & Kobayashi, K. (2006) Cerebral hemodynamics during the induction of antenatal periventricular leukomalacia by hemorrhagic hypotension in chronically instrumented fetal

sheep. Am. J. Obstet. Gynecol., 194, 1057-1063.

- Navarro, C. & Blanc, W.A. (1974) Subacute necrotizing funisitis. J. Pediatr., 85, 689-697.
- Riddle, A., Luo, N.L., Manese, M., Beardsley, D.J., Green, L., Rorvik, D.A., Kelly, K.A., Barlow, C.H., Kelly, J.J., Hohimer, A.R. & Back, S.A. (2006) Spatial heterogeneity in oligodendrocyte lineage maturation and not cerebral blood flow predicts fetal ovine periventricular white matter injury. J. Neurosci., 26, 3045-3055.
- Riddle, A., Maire, J., Gong, X., Chen, K.X., Kroenke, C.D., Hohimer, A.R. & Back, S.A. (2012) Differential susceptibility to axonopathy in necrotic and non-necrotic perinatal white matter injury. *Stroke*, 43, 178-184.
- Saito, M., Matsuda, T., Okuyama, K., Kobayashi, Y., Kitanishi, R., Hanita, T. & Okamura, K. (2009) Effect of intrauterine inflammation on fetal cerebral hemodynamics and whitematter injury in chronically instrumented fetal sheep. Am. J. Obstet. Gynecol., 200, 663.e1-663.e11.
- Volpe, J.J. (2008) Neuronal proliferation, migration, organization, and myelination. In *Neurology of the Newborn:Human brain development*, 5th ed., edited by Volpe, J.J. WB Saunders, Philadelphia, PA, pp. 99-100.
- Volpe, J.J. (2009) The encephalopathy of prematurity: brain injury and impaired brain development inextricably intertwined. *Semin. Pediatr. Neurol.*, 16, 167-178.
- Volpe, J.J., Kinney, H.C., Jensen, F.E. & Rosenberg, P.A. (2011) The developing oligodendrocyte: key cellular target in brain injury in the premature infant. *Int. J. Dev. Neurosci.*, 29, 423-440.
- Watanabe, T., Matsuda, T., Hanita, T., Okuyama, K., Cho, K., Kobayashi, K. & Kobayashi, Y. (2007) Induction of necrotizing funisitis by fetal administration of intravenous granulocyte-colony stimulating factor and intra-amniotic endotoxin in premature fetal sheep. *Pediatr. Res.*, 62, 670-673.
- Welin, A.K., Svedin, P., Lapatto, R., Sultan, B., Hagberg, H., Gressens, P., Kjellmer, I. & Mallard, C. (2007) Melatonin reduces inflammation and cell death in white matter in the mid-gestation fetal sheep following umbilical cord occlusion. *Pediatr. Res.*, 61, 153-158.