The occurrence of diffuse axonal injury in the brain: associated with the accumulation and clearance of myelin debris

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Abstract

The accumulation of myelin debris may be a major contributor to the inflammatory response after diffuse axonal injury. In this study, we examined the accumulation and clearance of myelin debris in a rat model of diffuse axonal injury. Oil Red O staining was performed on sections from the cerebral cortex, hippocampus and brain stem to identify the myelin debris. Seven days after diffuse axonal injury, many Oil Red O-stained particles were observed in the cerebral cortex, hippocampus and brain stem. In the cerebral cortex and hippocampus, the amount of myelin debris peaked at 14 days after injury, and decreased significantly at 28 days. In the brain stem, the amount of myelin debris peaked at 7 days after injury, and decreased significantly at 14 and 28 days. In the cortex and hippocampus, some myelin debris could still be observed at 28 days after diffuse axonal injury. Our findings suggest that myelin debris may persist in the rat central nervous system after diffuse axonal injury, which would hinder recovery.

Key Words: nerve regeneration; neurodegeneration; diffuse axonal injury; myelin debris; neuroinflammation; traumatic brain injury; head trauma; central nervous system; inflammation; axon; prognosis; NSFC grants; neural regeneration

Introduction

Diffuse axonal injury, characterized by widespread swelling and deformation of axons, often occurs in patients with severe traumatic brain injury and is associated with high mortality and morbidity rates. Among diffuse axonal injury patients, 43% do not survive, 9% suffer from a persistent vegetative state or major handicap, and less than 50% are able to lead independent lives (Chelly et al., 2011). Although diffuse axonal injury has devastating consequences, research on diffuse axonal injury in the central nervous system is limited, and the mechanisms underlying diffuse axonal injury are still unclear.

In diffuse axonal injury, axonal degeneration results from the stretching and shearing caused by mechanical force during the sudden movement of the brain tissue during the traumatic event. The axon lesion is, therefore, traditionally considered to occur immediately after the trauma. However, more recent studies revealed that much of the axonal degeneration develops progressively after traumatic brain injury (Park et al., 2008; Che et al., 2009). During traumatic brain injury, the stretching and shearing of axons lead to the damage to the axonal cytoskeleton and impairment of axoplasmic transport. These changes eventually cause the swelling and tearing of the axons (Povlishock et al., 1995). Effort has been made to identify possible therapeutic targets for axonal damage. However, so far no effective treatment has been established (Langham et al., 2000; Pillai et al., 2003). There is clearly a need for a better understanding of the pathogenesis of diffuse axonal injury so that new therapeutics can be developed.

In recent years, neuroinflammation has become a focus of diffuse axonal injury research (Schmidt et al., 2005; Lenz et al., 2007; Lin and Wen, 2013). Inflammation and degeneration of brain white matter may continue several years after traumatic brain injury (Johnson et al., 2013a; Smith et al., 2013). Myelin debris accumulated after axonal damage during diffuse axonal injury is an important mediator of inflammation (Fancy et al., 2010). Unlike the peripheral nervous system, an effective clearance mechanism for myelin debris is not present in the central nervous system. However, very few studies have been performed to investigate the accumulation and clearance of myelin debris in different brain regions after diffuse axonal injury. It is hypothesized that the myelin debris accumulated during diffuse axonal injury may persist at the damage site, resulting in chronic inflammation. In this study, we investigated the accumulation and clearance of Oil Red O-stained myelin debris at different time points following head injury in a rat model of diffuse axonal injury.
Materials and Methods

Animals
A total of 30 male clean Sprague-Dawley rats, purchased from the Experimental Animal Research Center of Zhejiang Academy of Medical Sciences, China (SCXK (Zhe) 2008-0033), were used in this study. According to Marmarou’s method, rats of approximately 2 months old, weighing between 300 and 350 g, were subjected to head trauma (Marmarou et al., 1994). All experimental protocols were approved by the Institutional Animal Care and Use Committee and were in accordance with the Guide for the Care and Use of Laboratory Animals issued by the U.S. National Institutes of Health.

Reagents
To prepare 0.15% Oil Red O staining solution, 0.15 g Oil Red O (Sigma, St. Louis, MO, USA) was dissolved in 100 mL isopropanol. The solution was heated until Oil Red O was completely dissolved. The 0.15% Oil Red O staining solution was then filtered and stored in a sealed bottle until use. To prepare 10% formaldehyde-calcium fixative, 1 g CaCl₂ and 10 mL formaldehyde was added to 90 mL distilled water. Hematoxylin staining solution was purchased from Fude Biotechnology (Hangzhou, Zhejiang Province, China). All other chemical reagents including chloral hydrate, paraformaldehyde, 100% ethanol, isopropanol and glycrogelatin were purchased from Huadong Medical Technology Co., Ltd. (Hangzhou, Zhejiang Province, China).

Rat models of diffuse axonal injury
Twenty-five rats were used to produce the diffuse axonal injury model (diffuse axonal injury group); five rats were used as the control (control group). The experimental diffuse axonal injury rat model was produced according to a method previously developed and characterized by Marmarou et al., 1994, but with less impact strength. The device used for diffuse axonal injury model production included a 2.8-cm-diameter, 2-m-long steel tube, a 2-cm-diameter, 288-g cylindrical weight, a 2.5-cm-wide, 2-mm-thick steel plate, and a sponge cushion pad. Briefly, the rats were anesthetized by intraperitoneal injection of 10% chloral hydrate and perfused with 4% paraformaldehyde. The rat brains were snap frozen with liquid nitrogen and stored at −80°C before use. The brain samples were embedded in optimal cutting temperature medium, and coronal sections (10 µm thick) of the dorsal neocortex, dentate gyrus of the hippocampus, and mid-brain and medulla oblongata regions were made. The section temperature was maintained at −16–20°C. For each rat, five sections were randomly selected from each site, randomly labeled, and stored at −80°C until further processing.

The frozen sections were first fixed in 10% formaldehyde-calcium fixative for 10 minutes and washed with distilled water once. For Oil Red O staining, the sections were then incubated for at least 30 seconds twice with 0.15% Oil Red O, followed by extensive washing with 60% dimethylcarbinol, and rinsed in H₂O. After that, nuclei were counterstained with hematoxylin for 3 minutes, and then washed with distilled water for 1 minute. Sections were coverslipped with a glycrogelatin-based aqueous mounting medium (Vallieres et al., 2006).

Image analysis and statistics
Images of three randomly selected fields from each slide were taken under a light microscope (Olympus EX71, Tokyo, Japan) at 200 × magnification to estimate the Oil Red O-stained staining in the neocortex, hippocampal dentate gyrus, and midbrain regions. The total number of Oil Red O-stained particles in each image was determined with Image-Pro Plus 16.0 software (Version 6.0.0.260; Media Cybernetics Inc., Silver Spring, MD, USA).

Statistical analysis
All data were presented as the mean ± SEM. All data were analyzed with SPSS 17.0 software (SPSS, Chicago, IL, USA). Statistical differences between the control and other groups were compared by one-way analysis of variance. Intragroup and intergroup differences were examined by the least significant difference method. P < 0.05 was considered statistically significant.

Results
Quantitative analysis of experimental animals
Three Sprague-Dawley rats in the diffuse axonal injury group died during production of the diffuse axonal injury model, and one rat died 21 days post injury. At least five randomly selected diffuse axonal injury rats were processed at 1, 7 and 14 days, and the remaining six rats were processed at 28 days. All five rats in the control group survived during the sham operation and were processed.

Morphology of the cerebral cortex, hippocampus and brain stem in rats with diffuse axonal injury
The accumulation and clearance of myelin debris in rats with diffuse axonal injury were monitored using Oil Red O staining. Oil Red O staining is a highly specific staining method for myelin debris (Ma et al., 2002; Vallieres et al., 2006; Barrette et al., 2008). Oil Red O staining was not observed in the cortex, hippocampus or brain stem region among rats in the...
In the diffuse axonal injury group, almost no Oil Red O staining was observed in the cortex, hippocampus or brain stem 1 day after injury. Seven days post injury, an apparent accumulation of Oil Red O-stained particles was observed in the cortex, hippocampus and brain stem of diffuse axonal injury rats. Most of the Oil Red O-stained particles were distributed as clusters in the diffuse axonal injury brain tissue. Moreover, the Oil Red O-stained particles were still present in the cortex and brain stem 28 days post injury (Figure 1).

The amount of myelin debris was quantified by measuring the total number of Oil Red O-stained particles in the brain sections (Table 1). In the cortex, the accumulation of Oil Red O-stained particles was obvious 7 days post injury. The number of Oil Red O-stained particles at 14 days was significantly higher than at 7 days post injury ($P < 0.01$). At 28 days post injury, the number of Oil Red O-stained particles was significantly lower than at 14 days post injury. No significant difference in Oil Red O staining in the cortex was observed between 7 and 28 days after diffuse axonal injury ($P > 0.05$) (Table 1). A similar temporal pattern of Oil Red O staining was found in the hippocampus with the number of stained particles peaking at 14 days after diffuse axonal injury. However, at 28 days post injury, the number of Oil Red O-stained particles was significantly lower than at 7 days ($P < 0.01$) (Table 1). In contrast, in the brain stem, the number of Oil Red O-stained particles peaked at 7 days after diffuse axonal injury, and decreased dramatically at 14 and 28 days post injury ($P < 0.01$) (Table 1). However, no significant difference was observed between 14 and 28 days ($P > 0.05$) (Table 1).

Our results show that the accumulation and clearance of myelin debris differ between the various brain regions examined. The amount of myelin debris peaked at 14 and 7 days after diffuse axonal injury in the cortex and brain stem,

**Table 1** Quantification (n/200-fold field of view) of Oil Red O-stained particles in brain sections

<table>
<thead>
<tr>
<th>Brain section</th>
<th>Days after injury</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>7</td>
</tr>
<tr>
<td>Cortex</td>
<td>48.00±16.70</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>11.67±3.06</td>
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<tr>
<td>Brain stem</td>
<td>51.67±6.81</td>
</tr>
</tbody>
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$^aP < 0.01$, vs. 7 days; $^bP < 0.01$, vs. 14 days. Data are represented as the mean ± SEM. One-way analysis of variance and the least significant difference test were used.

Figure 1 Morphology of the cerebral cortex, hippocampus and brain stem of rats with diffuse axonal injury and control rats (Oil Red O staining, × 200, bars: 50 µm).

The time course and differences in number of Oil Red O-stained particles in the cortex, hippocampus and brain stem are shown. The dense red-stained particles indicate myelin debris (arrows in B). Representative images from randomly selected fields are shown (see Materials and Methods). In the images of the control group (A, E, I), there is almost no myelin debris in the brain. Seven days post injury, much myelin debris can be observed in the cortex, hippocampus and brain stem (B, F, J). Fourteen days post injury, the amount of myelin debris increased in the cortex and hippocampus (C, G, F). Some myelin debris can be observed in the brain 28 days post injury (D, H, L). D: days after injury.
respectively. At 28 days post injury, a large amount of myelin debris was still present in these two brain regions. In contrast, although the amount of myelin debris in the hippocampus continued to increase significantly until 14 days post injury, most of the myelin debris was cleared at 28 days.

Discussion
This study surveyed the accumulation and clearance of Oil Red O-stained myelin debris in the cortex, hippocampus and brain stem regions at 1, 7, 14 and 28 days after brain injury using a diffuse axonal injury rat model. As expected, the brain in the control group was free of myelin debris. There was no myelin debris accumulation in the brain 1 day post injury. Myelin debris accumulation in the cortex, hippocampus and brain stem was apparent, and the amount peaked at 7–14 days post injury. The amount of myelin debris was decreased by 28 days post injury. However, a significant amount of myelin debris was still seen in the cortex and brain stem 28 days post injury. This indicates that there is a delayed accumulation and incomplete clearance of myelin debris in diffuse axonal injury rats.

Diffuse axonal injury is one of the most common consequences of closed head injury, occurring in approximately half of all severe traumatic brain injury cases (Johnson et al., 2013b). The inflammatory response after traumatic brain injury is considered one of the major causes of secondary brain injury. Numerous studies have investigated the role of neuroinflammation in focal brain injury (Woodroffe et al., 1991; Csuka et al., 2000; Harting et al., 2008; Kadhim et al., 2008). However, little is known about inflammation in the context of diffuse axonal injury, although the topic is currently under investigation (Lin and Wen, 2013). Several factors, including myelin debris, have been proposed as potential inducers of chronic inflammation in the brain, which leads to progressive secondary neuronal damage and the inhibition of damage repair. Therefore, comprehensive long-term studies using animal models are required to clarify the effects of diffuse axonal injury in the central nervous system. Our study is one of the first long-term studies on myelin debris in the diffuse axonal injury rat model. A major finding of our study is that myelin debris accumulates and persists in the brain for a protracted period of time after diffuse axonal injury.

Normal axon fibers are covered by a myelin sheath. As axonal damage progresses, demyelination often follows and myelin debris is produced. In the peripheral nervous system, axonal degeneration triggers an inflammatory response, which is followed by the activation of Schwann cells, in a process called Wallerian degeneration (Gaudet et al., 2011). Macrophages and other immune cells are then recruited to the site of damage by Schwann cells to clear the myelin debris after injury. The process is swift and efficient; therefore, myelin debris accumulation often does not happen following peripheral neuronal injury (David et al., 2012; Dubovy et al., 2013). In contrast, the clearance of the myelin debris in the central nervous system is often limited because of the weak phagocytic capabilities of activated microglial cells. Although macrophage infiltration can occur following blood-brain barrier damage after brain injury, the number of infiltrated peripheral immune cells is too low to contribute significantly to myelin debris clearance. Kelley et al. (2007) reported that myelin debris can be observed under an electron microscope 7–28 days following diffuse axonal injury. Consistent with their observations, we found that a significant amount of myelin debris was present in the cortex, hippocampus and brain stem regions 7–28 days following diffuse axonal injury. In addition, we observed that myelin debris accumulation in the brain was not obvious immediately after injury.

Myelin debris is an important mediator of the inflammatory response in the nervous system. Its regulatory functions in inflammation have been well documented in peripheral nerve injury and multiple sclerosis (Gaudet et al., 2011; Zindler and Zipp, 2011; David et al., 2012; Rawji and Yong, 2013). Furthermore, as a byproduct of axonal degeneration, myelin debris is considered one of the inducers of secondary neuronal damage, which has been documented in multiple sclerosis (Fancy et al., 2010; Sun et al., 2010; Clarnet et al., 2012). Therefore, it is possible that the accumulated myelin debris in diffuse axonal injury leads to chronic inflammatory responses in the central nervous system and subsequent damage to the brain. The persistent presence of the myelin debris and the long-term neuroinflammation in the cortex and brain stem might be important contributors to the poor prognosis in diffuse axonal injury patients. In addition, the presence of the myelin debris itself inhibits regeneration and myelin repair as well, leading to limited recovery. Myelin debris is known to inhibit oligodendroglial maturation and regeneration in the central nervous system (Fawcett et al., 2012; Plemel et al., 2013). A recent study showed that oligodendrocyte precursor cells attached to myelin debris are strongly inhibited from maturing (Plemel et al., 2013). The inhibition of oligodendrocyte precursor cell maturation may contribute to the failure of remyelination and neuronal recovery, because oligodendrocytes are actively involved in these processes. Moreover, other signaling molecules, such as MAG and Nogo, associated with the myelin debris may prevent regeneration in the central nervous system (Fawcett et al., 2012). Both the inflammatory response and the inhibition of myelin regeneration are disadvantageous for recovery from diffuse axonal injury and lead to poor outcome.

In summary, the accumulation and clearance of myelin debris at different time points post diffuse axonal injury in different brain regions were evaluated. Our results show a delayed accumulation and incomplete clearance of myelin debris in the injured brain. As an important mediator of inflammation and inhibitor of regeneration, the long-term presence of myelin debris after diffuse axonal injury may lead to persistent secondary immune damage to the brain. However, there are several limitations of this study, such as the limited observation period. Further studies are required to examine the effects of the long-term presence of myelin debris after diffuse axonal injury, and whether it may lead to persistent secondary immune damage to the brain.
Author contributions: Wen L designed and performed research, and wrote the paper. Xu J, Liu WC and Zhan TX performed the research. Wang H and Huang X analyzed the data. Yang XF designed the research and provided technical or material support. Zhan RY provided technical or material support, and supervised the research. All authors approved the final version of the paper.

Conflicts of interest: None declared.

References


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