Early pathological alterations of lower lumbar cords detected by ultrahigh-field MRI in a mouse multiple sclerosis model

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Magnetic resonance imaging (MRI) is widely employed for the diagnosis of multiple sclerosis (MS). However, sometimes, the lesions found by MRI do not correlate with the neurological impairments observed in MS patients. We recently showed autoreactive T cells accumulate in the fifth lumbar cord (L5) to pass the blood–brain barrier and cause inflammation in the central nervous system of experimental autoimmune encephalomyelitis (EAE) mice, an MS model. We here investigated this early event using ultrahigh-field MRI. T2-weighted image signals, which conform to the water content, increased in L4 and L5 during the development of EAE. At the same time, the sizes of L4 and L5 changed. Moreover, angiographic images of MRI showed branch positions of the blood vessels in the lower lumbar cords were significantly altered. Interestingly, EAE mice showed occluded and thickened vessels, particularly during the peak phase, followed by reperfusion in the remission phase. Additionally, demyelination regions of some MS patients had increased lactic acid content, suggesting the presence of ischemic events. These results suggest that inflammation-mediated alterations in the lower lumbar cord change the homeostasis of the spinal cord and demonstrate that ultrahigh-field MRI enables the detection of previously invisible pathological alterations in EAE.

Keywords: EAE, lumbar cords, MRI, spinal cords

Introduction

A recent genome-wide association study examining nearly 35 000 subjects including ~15 000 patients indicated that multiple sclerosis (MS) is a T, (CD4+ T cell)-mediated autoimmune disease (1). Autoreactive CD4+ T cells, particularly IL-17-expressing T,17 cells, are believed to play a role in the development of inflammation in the central nervous system (CNS) during MS. Indeed, MS patients suffer from inflammatory lesions consisting of various immune cells including CD4+ T cells, B cells and macrophages followed by a loss of neurological functions in various regions of the CNS. Magnetic resonance imaging (MRI) is a popular clinical tool for diagnosing MS and is routinely used for the in vivo detection of the corresponding lesions (2, 3). In fact, the pathological hallmark of MS, demyelinated lesions in the CNS white matter, can be visualized as focal signal changes detected by MRI (4). However, the size and number of the MRI lesions have had only modest correlations with neurological impairments in MS (5). Furthermore, such alterations reflect relatively advanced pathogenesis and not earlier symptoms like the breakdown of the blood–brain barrier (BBB) or edema accumulation (6).

We recently showed that pathogenic CD4+ T cells including T,17 cells in the blood migrate to the CNS via the dorsal blood vessels of the fifth lumbar cord (L5) in experimental autoimmune encephalomyelitis (EAE) mice (7). Type 1 collagen+ cells around these vessels express chemokines including CCL20, a T,17 chemoattractant, in a manner dependent on the inflammation amplifier, an inflammation-related NF-xB...
feedback loop that can be activated by regional neural stimulation. In fact, soleus muscle-mediated neural activation was seen to enhance inflammation amplifier activation via sympathetic neurons in the L5 cord (7). We, thus, proposed the gate theory to describe how regional neural activation enables immune cells to enter not only the CNS but also other organs from the blood and argued that this theory might provide novel therapeutic targets for inflammatory diseases and disorders (7). These results indicate that the lower level of the lumbar cord is the initial site of inflammation of EAE, which would explain why previous reports observed no alterations in lesions in the upper levels or brain of EAE mice by MRI (8–12). In the present report, we analyzed the earliest events of the disease using ultrahigh-field MRI. The T2-weighted image (T2WI) signal, which can be used to measure water content around the lower spinal cord, particularly at L4 and L5, correlated with the development of MS. The size of the lower lumbar cords also changed. Moreover, the diffusion-weighted imaging (DWI) signal, which represents the fiber density and myelin sheath in white matter, was altered. Additionally, branch positions of the vessels in the lower lumbar cords were significantly altered. EAE mice also showed occluded and thickened vessels particularly during the peak phase, followed by reperfusion in the remission phase. Consistent with this result, the demyelination regions of some MS patients had increased lactic acid content, suggesting the presence of ischemic events. These results suggest that inflammation, including edema in the lower lumbar cord, changes the homeostasis of the spinal cord during disease development, and that highly sensitive MRI can identify previously invisible pathologies in EAE mice.

Methods

Experimental model

Adult male C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan). Mice were housed in a controlled vivarium environment (24°C; 12:12 light:dark cycle) and fed a standard pellet diet and water ad libitum. All experimental procedures involving animals and their care were carried out in accordance with the Osaka University Guidelines for the Animal Experimentation and National Institutes of Health Guide for the Care and Use of Laboratory Animals.

EAE induction was performed as described previously (7, 13). Briefly, C57BL/6 mice were injected with a myelin oligodendrocyte glycoprotein (MOG) peptide in complete Freund’s adjuvant followed by intravenous injection of pertussis toxin. CD4+ T cells from the affected mice were sorted, and a passive transfer EAE model was induced in wild-type C57BL/6 mice via intravenous injection of 1.5 × 10^7 pathogenic CD4+ T cells. All affected mice (n = 10) were weighed and assessed for neurological symptoms using a defined clinical scoring method everyday (13). For the MRI measurements, mice were anesthetized with a mixture of air and 2.8% isoflurane (Abbott Laboratories, Abbott Park, IL, USA) and then placed in an MRI-compatible animal cradle. The isoflurane concentration and a constant respiration rate were maintained at 1.5 ± 0.4% and 60 ± 10 breaths min⁻¹, respectively, throughout the MRI sessions.

MR imaging

MRI was conducted using an 11.7 T vertical bore Bruker Avance II imaging system (Bruker Biospin, Ettlingen, Germany) and a home-made 12-mm diameter transmit/receive surface radio frequency coil. Respiratory signals were monitored with a physiological monitoring system (SA Instruments, Inc., Stony Brook, NY, USA). MRI was performed 5, 7, 9, 12 and 14 days after pathogenic T-cell transfer (Fig. 1). Each measurement of the MRI corresponds to the pre-clinical (day 5), onset (day 7), peak (day 9), partial remission (day 12) or late remission phase (day 14). An axial T2-weighted rapid acquisition with relaxation enhancement sequence (T2WI; repetition time/echo time (TR/TE) = 5000/38 ms, number of averages (NA) = 16, field of view (FOV) = 12.8 × 9.6 mm, matrix size = 256 × 192, slice thickness = 300 µm and acquisition time = 16 min), DWI sequence with a transverse motion-probing gradient (MPG; anterior–posterior single direction) (b value = 1600, TR/TE = 7500/20 ms, NA = 2, FOV = 12.8 × 9.6 mm, matrix size = 256 × 192, slice thickness = 300 µm and acquisition time = 36 min) and compensated 2D time-of-flight MR angiography (TR/TE = 15/1.5 ms, NA = 4, FOV = 12.8 × 9.6 mm, matrix size = 256 × 192, slice thickness = 300 µm and acquisition time = 32 min) were acquired at the level of the third to sixth lumbar vertebra. MR images were viewed and processed using OsiriX imaging software (Pixmeo SARL, Geneva, Switzerland) (14).

Proton magnetic resonance spectroscopy (1H-MRS) was performed at 3.0 T (General Electric: Signa Excite 3T) as described previously (15).

1H-MRS in humans

MS patients, including a 64-year-old male whose data we show in this article, were diagnosed at Iwate Medical University Hospital and Osaka University Hospital, and healthy control...
subjects were observed by 1H-MRS. Informed consent was obtained from the subjects, and the study was approved by the Ethics Committees of Iwate Medical University Hospital and Osaka University Hospital.

Statistical analysis
To determine differences in the disease progression, the T2WI signal intensity, swelling of the spinal cord and alterations in the dorsal blood vessels between each level of the spinal cord were measured, and an analysis of variance on ranks and a Bonferroni’s all-pairwise multiple comparison test (KaleidaGraph 4.1; Hulinks, Japan) were used. For all comparisons, \( P < 0.05 \) was considered statistically significant.

Results
EAE induction after transfer of pathogenic CD4+ T cells
We employed a passive transfer method for EAE induction, as described previously (7, 13). Pathogenic CD4+ T cells were isolated from wild-type C57BL/6 mice immunized with a MOG peptide plus adjuvants (see Methods) and then cultured in vitro with irradiated splenic antigen-presenting cells in the presence of the same MOG peptide plus IL-23 to enrich the IL-17 secreting pathogenic CD4+ cells. The resulting pathogenic CD4+ T cells, which contained T\(_h\)17 plus T\(_h\)1 cells, were intravenously transferred into wild-type C57BL/6 mice at day 0. Mean clinical scores and body weights are shown from day 0 to day 14 after the pathogenic CD4+ T-cell transfer (Fig. 1). MRI measurements were performed to cover the disease progression adequately including the pre-clinical phase (pre, day 5 after the pathogenic CD4+ T-cell transfer), onset (day 7), peak (days 9–10), partial remission (day 12) and late remission (after day 14). Observations in normal C57BL/6 mice and EAE mice in the pre-clinical phase were very similar. We also investigated the MRI data of normal control mice receiving a saline transfer for comparison with the EAE mice at the pre-clinical stage, finding no alterations in the control mice. Therefore, MRI can only detect swelling once clinical symptoms have enhanced after the onset stage. In other words, it is difficult to show differences among the lumbar cords in mice at the pre-clinical stage using a MRI device of low sensitivity.

T2WI signals and the size of the spinal cord increased with the development of EAE
We investigated the entire spinal cord and found no detectable alterations in its higher levels or the brain even during the peak phase (data not shown). In contrast, definitive changes in the low lumbar levels were observed. The T2WI signal, which mainly reflects water content (edema, etc.), was increased at each level of the lumbar cord in the peak phase, especially at L4 and L5 (Fig. 2, inside the dotted yellow polygons), and decreased in the remission phase (Fig. 3A). Additionally, the rates of the T2WI signal increases were highest in lower cords including L4 and L5 (Fig. 3B), although each lumbar level showed significant increases in the signal compared with the pre-clinical phase, and the peak signal in L3 was later than it was for the other lumbar cords (Fig. 3B). Interestingly, more T-cell accumulation in L5 correlates with a stronger T2WI signal (Fig. 3C). Moreover, the fluid volume of the cerebrospinal fluid (CSF), which represents the hyperintense signal surrounding the spinal cord (the region between the yellow and blue polygons), was thinner at the peak phase than at the pre-clinical and remission phases (Fig. 2).

In addition, the lumbar spinal cord was markedly enlarged until the peak phase of EAE development and gradually decreased thereafter (Fig. 4). The size of the alterations was most notable in L5 and L6 even during the onset phase of EAE development (Fig. 4B).

Fig. 2. MRI images of lesions in the spinal cord (L3–L6) at different stages of EAE. The images show T2WI signals. The spinal bodies at each stage are shown inside the dotted yellow polygons. The regions between the yellow and blue dotted polygons represent the CSF. Scale bar equals 500 \( \mu \)m. The top of each image shows the dorsal side.
The T2WI signal increased 20–30% at each level of the spinal cord in the peak phase compared with the pre-clinical phase (Fig. 3B). Although the signal dipped at the late remission phase, it still sustained a 10% increase. Consistent with this increased intensity, we found that a certain number of immune cells including pathologic CD4+ T cells are present in L5 after the late remission phase (data not shown). Moreover, we employed pathogenic CD4+ T cells derived from IL-17A-deficient mice to show the experimental link between MRI, particularly the T2WI signal, and previous data. The MRI images obtained after a transfer of IL-17A-deficient pathogenic CD4+ T cells agreed with the data from wild-type healthy mice (data not shown). Thus, the intensity of the T2WI signal is well correlated with the clinical score, which was based on the inflammation-mediated edema. Moreover, the results suggest there remains some pathological condition in the lumbar levels even after the late remission phase.

**DWI signals of the spinal cord increased with the development of EAE**

We also investigated the DWI signal, which represents high fiber density and the myelin sheath in white matter, using ultra-high-field MRI (Fig. 5). A high DWI in the pre-clinical phase indicated a distinct marginal layer around the spinal cord (Fig. 5A–C). The signal decreased particularly in the dorsal marginal layers of L4 and L5 during the onset phase (Fig. 5E and F, arrows) and further decreased in L3 and L4 during the peak phase (Fig. 5G and H, arrows), but recovered in L5, although the clinical scores remained high (Figs 1 and 5I). During remission, the signal approached the pre-clinical phase.

**Fig. 3.** Quantitative T2WI signals in the spinal cord during disease development. (A) Temporal T2WI signal changes are shown (mean ± SEM) for each level of the spinal cord. The T2WI signal increased during EAE development. (B) T2WI signal intensities at each stage normalized by the signal at the pre-critical stage. (C) A plot of T-cell numbers versus change in T2WI during EAE development is shown. Significant changes in the signal intensity in comparison with the pre-critical stage (pre) are indicated by *P < 0.05 and **P < 0.01.
level (Figs 1 and 5J–L) and returned there by late remission (Fig. 5M–O). These results suggest that changes in the DWI signal might precede clinical scores and, therefore, act as an effective marker for disease prognosis.

**Localizations of branching vessels in the lower lumbar cord changed with EAE development**

We also used ultrahigh-field MRI to acquire angiographic images. Because we demonstrated that pathogenic CD4+ T cells accumulate around the dorsal blood vessels of L5 during EAE development, we investigated morphological alterations there. We analyzed the horizontal 1-mm thick slice maximum intensity projection (MIP) because this analysis displayed the bifurcation of the dorsal spinal vessels (Fig. 6A). We found a clear bifurcation between L3 and L4 (and sometimes between L4 and L5; see Fig. 8) in almost all mice (Fig. 6B, arrows). We used this bifurcation as a landmark to investigate the vascular distribution around the dorsal spinal region in the lower cord during the disease. We first defined the position of the bifurcation in the pre-clinical stage as the baseline (Fig. 6B, blue dotted line) and found that the position of the bifurcation gradually shifted caudally with EAE development (Fig. 6B). At the peak phase, a significant dislocation of over 2 mm between the pre-clinical and onset phases was observed (Fig. 7). Interestingly, EAE mice showed at least partially occluded and thickened vessels during the peak phase that localized with each other (Fig. 8). Reperfusion occurred in the remission phase, suggesting a reperfusion injury in EAE pathogenesis. Consistent with this observation, apoptosis and the concentration of free radicals increased in L5 at the peak phase, and the development of EAE was ameliorated by the addition of a scavenger, edaravone (data not shown). Moreover, we showed that the demyelination regions of some MS patients, where inflammation occurs, had increased lactic acid content (Fig. 9 and data not shown), suggesting the presence of ischemic events. Additionally, the
dislocation of the bifurcation remitted toward the baseline in the EAE remission phase but remained significantly different (Fig. 7). These results support the idea that the localizations of the branching vessels in the lower lumbar cord are well correlated with the clinical score, and that some pathological status might exist at the lumbar levels even after late remission.

Discussion

We recently reported that an initial site for autoreactive T<sub>17</sub> cells to breach the BBB is the dorsal vessels of L5 in the CNS. Mechanistic analysis demonstrated that sympathetic activation via sensory activation of anti-gravity soleus muscles secretes norepinephrine there (7). Consistent with that study, we found here that inflammation-mediated responses including edema formation and vessel alterations are present in the lumbar cords. Along with an enlarged spinal cord, the edema formation became more significant with EAE development, particularly in lower lumbar levels like L5, suggesting inflammation was induced by an accumulation of immune cells there. These MRI data clearly support our theory that L5 dorsal vessels are the starting point of inflammation, which then spreads to other levels of the spinal cord.

We have previously demonstrated that the initial gateways of the pathogenic CD4<sup>+</sup> T cells toward the CNS are L5 dorsal vessels. However, we also found that CCL20 expression eventually reaches other regions, particularly the anterior lumbar cords including L3 and L4 at 5 days after the pathogenic CD4<sup>+</sup> T-cell transfer, suggesting the range of the BBB compromised after pathogenic T-cell accumulation in the CNS via L5 dorsal vessels increases with time (please see Figure S2C in Arima et al., (7)). In the present work, we did not find any alterations in the lumbar cords including L5 at 5 days after the pathogenic CD4<sup>+</sup> T-cell transfer (please see ‘pre’ data in Figs 3 and 4) but did find alterations at later time points (please see ‘onset and peak’ data in Figs 3 and 4). These results strongly suggest the MRI used here has lower sensitivity than direct measurements of CCL20 in the vessels used in our previous study. Moreover, the similar increases in the T2WI signal and size alterations for L5 and other lumbar levels like L3 and L4 argues that all lumbar cords have a certain level of inflammation at later time points following the transfer.

We also found decreases of the DWI signal in the dorsal sides of L4 and L5 during the onset phase, further decreases in those of L3 and L4 during the peak phase, and even more in L3 alone during partial remission. These results correlate with the disease development process spreading from L5 to other lumbar levels. Indeed, pathogenic CD4<sup>+</sup> T cells first accumulate in the dorsal vessels of L5 (7) and then extend to the upper levels including L3 and L4 (Y. Arima, manuscript in preparation). Because DWI signals preceded the clinical scores, they may make an effective marker for disease diagnosis.

Interestingly, the spinal cord was significantly enlarged at L5 and L6 compared with L3 and L4 from the onset phase.
It is known that L5 and L6 spinal nerve roots consist of the cauda equina, which is rich in CSF space. Consistent with this observation, a clear CSF space, indicated by a strong T2WI signal, was seen around the spinal cord in normal animals and EAE mice during the pre-clinical phase. However, the signal indicated that the space was smaller in the upper levels of the spinal canal including L3 and L4 than in lower levels like L5 and L6. The different volumes of CSF may explain the

Fig. 8. Angiographic images of the dorsal spinal artery in an EAE mouse using the MIP. Temporal alterations of the dorsal spinal artery in the coronal plane. The level of the spinal cord was registered using anatomical signals of the interspinal disks. Occluded vessels (pink dot circle) and thickened vessels (arrows) were found during the peak phase. Dislocation of the bifurcation from the baseline (blue dotted line) was visualized. Scale bar equals 1 mm.

Fig. 9. 1H-MR spectra (TR/TE = 3000/30 ms) obtained from a healthy control (above) and a MS patient (below) matched for age and gender. Note the pronounced increases of choline and lactate in the MS patient compared with the control subject.
clear enlargement of the L5 and L6 spinal bodies after EAE induction, which is consistent with other MRI studies (8, 9). Moreover, the position of the bifurcation gradually shifted caudally with EAE development. We hypothesize that enlargement of the lower spinal levels caused this shift by dragging the lumbar vessels to the lower side because there were some vessels that did not shift with the disease development (see side vessels in Fig. 6B). It is possible that a product of the local damage contributes to the edema in the lumbar cords. It is also possible that the edema between L3 and L5 detected by the MRI analysis is triggered by the expression of CCL20 in their dorsal vessels followed by accumulations of pathogenetic CD4+ T cells there. Furthermore, we show that CCL20 expression eventually reaches other regions, particularly the anterior lumbar cords including L3 and L4 at 5 days after the pathogenetic CD4+ T-cell transfer, suggesting that the BBB is compromised in several regions after pathogenetic T-cell accumulation in the CNS (please see Figure S2C in Arima et al. (7)).

Vessels in the lumbar levels were at least partially occluded in the peak phase in EAE animals, and reperfusion occurred in the remission phases, indicating perhaps that there exists a reperfusion injury particularly downstream of the occluded vessels. Consistent with this observation, we found that apoptosis and the concentration of free radicals increased in L5 in the peak phase, and that the addition of a free radical scavenger, edaravone, ameliorated the EAE development as well as alterations detected by the MRI (Supplementary Figure 1, available at International Immunology Online, and data not shown). Similar results were obtained in an immunization-type EAE model that considered free radicals in microglia but not the reperfusion injury (16). Moreover, we found that demyelination regions of some MS patients had increased lactic acid content, suggesting the presence of ischemic events, which is consistent with previous reports describing elevated lactate concentration in MS regions including those of patients with early MS (17–20). Our results suggest that the pathogenesis at least in a subgroup of MS patients might be related to ischemic events caused by reperfusion injuries. Consistent with this notion, Lassmann showed that markers for hypoxia-like tissue injury also increase in MS lesions (21).

We attempted to confirm the alterations of the branch positions of the blood vessels in the lower lumbar cords by classical histology but could not (data not shown). One reason may be because the spinal cords shrank in paraformaldehyde solution, which could explain why such changes have not been observed before.

One limitation of MRI for the investigation of the lower lumbar cords of mice is the structure’s width, which is too thin to visualize. We specially modified our MRI device to achieve greater sensitivity by narrowing the diameter and shortening the length of the probe coil. The shape of the coil is also well suited for murine spinal cord imaging. In addition, a passive EAE model lowers the background inflammation level and heightens the sensitivity to detect detailed temporal and spatial alterations during EAE development.

Supplementary data
Supplementary data are available at International Immunology Online.


