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Multi-parametric MRI Reveals Dynamic Changes in Molecular Signatures of Injured Spinal Cord in Monkeys

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Abstract

Purpose—To monitor the spontaneous recovery of cervical spinal cord injury (SCI) using longitudinal multi-parametric MRI methods.

Methods—Quantitative MRI imaging including diffusion tensor imaging (DTI), magnetization transfer (MT), and chemical exchange saturation transfer (CEST) was conducted in anesthetized monkeys at 9.4 T. The structural, cellular and molecular features of the spinal cord were examined before and at different time-points after a dorsal column lesion in each monkey.

Results—Images with MT contrast enhanced visualization of the grey and white matter boundaries and the lesion, and permitted differentiation of core and rim compartments within an abnormal volume (AV). At early weeks after SCI, both core and rim exhibited low cellular density and low protein content, with high levels of exchanging hydroxyl, amine, and amide protons, as evidenced by increased apparent diffusion coefficient (ADC) value, decreased fractional anisotropy (FA), decreased magnetization transfer ratio (MTR), decreased nuclear Overhauser effect (NOE), and large CEST effects. Over time, while cellular density and fiber density increased, amide, amine, and hydroxyl levels dropped significantly, but at differing rates. Histology confirmed the nature of the AV to be a cyst.

Conclusion—Multi-parametric MRI offers a novel method to quantify the spontaneous changes in structure and cellular and molecular compositions of SC during spontaneous recovery from injury.

INTRODUCTION

Spinal cord injuries (SCIs) have devastating consequences for patients due to severe impairment of motor and/or sensory functions (1). Over time, patients with SCIs can regain some lost functions, although the mechanisms mediating recovery are not completely understood (2-5). Evidence in the literature suggests that functional recovery depends upon

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local nerve fiber sprouting and regeneration around the injured spinal cord as well as reactivation and/or reorganization of neural responses in the brain (6-9). Within the spinal cord, injured tissue may undergo spontaneous recovery from damage and show various changes over time (9,10). The initial trauma may lead to the death of cells (including neurons and glia cells), and sometimes may disrupt the functioning of descending and ascending axonal white matter tracts. The creation of tissue cavities and formation of cysts can further interrupt proper functions of these tracts. From minutes to weeks after the initial insult, the injured spinal cord and surrounding tissue may experience secondary damage, including vascular changes, free radical formation and lipid peroxidation, apoptosis of neurons, oligodendrocytes and microglia, glutamate excitotoxicity (increased release of extracellular glutamate), and an inflammatory response. In the chronic phase (weeks to years after the initial insult), white matter (WM) demyelination, gray matter (GM) dissolution, connective tissue deposition and reactive gliosis that leads to glial scar formation, may each occur. A glial scar acts like a physical barrier, preventing axons to grow through it. In approximately 25% of SCI patients the glial scar surrounds a cystic cavity (6,9). In this context, the ability to detect changes in structural, molecular and cellular properties of the damaged spinal cord as early as possible and to monitor these changes over time, would provide crucial information for understanding the spontaneous recovery of SCIs and a biomarker for evaluating the effectiveness of inventions designed to promote recovery. Thus, the goal of this study is to explore the ability of a multi-parametric MRI approach for early detection and monitoring of changes in the structural, cellular and molecular properties of injured spinal cord tissue over time in a non-human primate model of SCI.

Conventional MRI methods, such as T₁-weighted (T1W), T₂-weighted (T2W), and diffusion-weighted imaging (DWI), have previously been used to study spinal cord pathology and recovery after injury in animals and humans (11-18). In this study, we implemented diffusion tensor imaging (DTI), magnetization transfer (MT), and chemical exchange saturation transfer (CEST) imaging, to characterize and quantify dynamic changes of injured spinal tissue. Each measurement provides complementary information about the progression and recovery. For example, the apparent diffusion coefficient (ADC) and fractional anisotropy (FA) values derived from DTI imaging provide information about cell density, microstructure, and WM fiber density (19-21). These measures are believed to be more specific than conventional methods for characterizing spinal cord pathology (13,22), for reviews see (23,24). By measuring the water signal decreases caused by the transfer of longitudinal magnetization, MT detects water's non-specific interactions with immobile proteins and lipids (25,26). Thus, MT is capable of detecting changes in macromolecular content in a damaged or diseased spinal cord (27-33). By exploiting the effects of specific exchangeable protons from metabolites, mobile proteins, and peptides, such as -NH₂, -NH, and -OH groups (34,35), CEST imaging can be used to detect concentration changes of different molecules such as amide (36-38), glycogen (35,39,40), and several specific brain metabolites that are associated with neurotransmitters degradation (41,42) as well as tissue pH (43-45). A recent study by Kogan and colleagues (46) documented the effectiveness and potential of CEST imaging-mediated evaluation of molecular signatures of human spinal cord tissues. The present study aims to explore the full potential of multi-parametric MRI for

monitoring structural, molecular and cellular properties changes of damaged spinal cord tissues.

METHODS

Animal preparation

Six adult (6-8 years old) male squirrel monkeys (*Saimiri sciureus*) were included in the study. Normal control data were collected from all of them. A subset of three monkeys, identified herein as SM-P, SM-G, and SM-T, underwent a unilateral dorsal column lesion at high cervical spinal cord (C4–C5) level. Detailed descriptions of the surgical procedures can be found in previous publications (47,48). The monkeys were scanned before SCI and at different intervals after SCI based on its lesion size, behavioral deficit, and progression. The timeline for imaging after SCI is shown in supplementary Table 1. During the MRI scans, the monkey was anesthetized with isoflurane (0.5–1.5%), and received mechanical ventilation with its head and body stabilized in an MR compatible frame. Vital signs were monitored and maintained throughout the imaging sessions, which typically last 6 hours. All the procedures were approved by the IACUC (Institutional Animal Care and Use Committee) at Vanderbilt University.

In vivo MRI data acquisition

All MR images were acquired using a 9.4-T Agilent MRI spectrometer with a saddle-shaped transmit-receive surface coil ($2.5 \times 3 \text{ cm}^2$ in size) positioned around the neck. The image field of view (FOV) was centered at level C4, where the lesion had been targeted. Gradient echo high-resolution T₁-weighted (T1W) images (TR/TE = 70/3.24 ms, FOV = $40 \times 40 \text{ mm}^2$, matrix size = 128×128 , slice thickness (ST) = 0.5 mm) were acquired to visualize the entire cervical spinal cord.

MT contrast (MTC) images were acquired using a gradient echo sequence (TR/TE = 220/3.24, matrix size = 128×128 , ST = 0.5 mm for coronal slices, 0.75 mm for sagittal slices, and 1.0 mm for axial slices) that incorporated a Gaussian radio frequency (RF) saturation pulse (offset, 5000 Hz). Power calibration and quantitative measurements were obtained for a selected single coronal slice located in the posterior portion of the spinal cord where the dorsal column resides. To ensure that the same region was sampled for quantitative comparisons across imaging sessions, high-resolution sagittal and axial MTC images were referenced for the selection of the coronal slice (defined as 0.5 mm from the posterior WM outer edge at the level of the lesion).

The magnetization transfer ratio (MTR) was calculated using a 2D RF-spoiled gradient echo sequence (TR = 24 ms, TE = 3.3 ms, flip angle = 7° , FOV = $40 \times 40 \text{ mm}^2$, matrix size = 128×128 , ST = 1 mm, number of excitation (NEX) = 64). Off-resonant RF irradiation was produced with slice-selective Gaussian RF pulses (RF offset, 5000 Hz; duration, 10 ms; saturation flip angle $\theta_{\text{sat}} 820^\circ$). Control images (S_0) were acquired without MT pulses.

We acquired CEST data using a continuous wave (CW) CEST sequence with a 5.0-s rectangular irradiation pulse followed by multishot spin-echo echo-planar imaging (EPI, 2 shots, TR = 7.5 s, TE = 17.6 ms, matrix size = 64×64 , ST = 1 mm, and NEX = 2). Z-

spectra were acquired with RF offsets from -2000 Hz to 2000 Hz (with an interval of 80 Hz). An irradiation power (B_{cw}) of 1.0 μ T was applied. Scans with an RF offset of 100,000 Hz served as controls. Respiration gating was applied to minimize artifacts from motion of the spine associated with breathing.

A respiratory-gated, spin-echo EPI DTI protocol was employed with the following parameters: TR = 3 s, TE = 35 ms, duration of gradient pulse/diffusion time (δ/γ) = 4 ms/12 ms, amplitude = 19.96 gauss/cm, two b-values (0 and 1000 s/mm²), 6 diffusion gradient directions, resolution at $0.5 \times 0.5 \times 0.5$ mm³, sampling bandwidth = 250 kHz, and NEX = 16. B_0 maps were also computed from two gradient echo images ($TE = 2$ ms) to enable distortion correction of DTI data.

Image analysis and quantification

All images were analyzed in MATLAB 2011a (The Mathworks). We registered all volumes used for quantification using a rigid body algorithm based on mutual information (49). EPI distortion correction based on the B_0 map and non-linear transformations was also implemented. The MTR was defined as follows:

$$MTR = (S_0 - S_{sat}) / S_0 \quad (1)$$

where S_0 and S_{sat} are the signals without and with MT saturation, respectively.

For CEST images, the Z-spectra were normalized to the control scan ($Z = S/S_0$) and MTR_{asym} was calculated using the formula

$$MTR_{asym} = Z(\Delta\omega) - Z(+\Delta\omega) \quad (2)$$

where $Z(+\omega)$ is the normalized signal at the positive offset $+\omega$ and $Z(-\omega)$ its counterpart at the negative offset $-\omega$.

The *in vivo* Z-spectra and MTR_{asym} may be contaminated by MT and nuclear Overhauser effects (NOE). A 3-point method was used to calculate MTR_{asym}^* from a labeled image with RF offset δ where maximum MTR_{asym} observed and two boundary images with minimum MTR_{asym} and an offset separation $\pm \Delta$.

$$MTR_{asym}^* = \{Z(\delta - \Delta) + Z(\delta + \Delta)\} / 2 - Z(\delta) \quad (3)$$

CEST ratio (CESTR) was measured as the percent peak height of each resolved peak from the fitting of the regional averaged spectrum. We took a fitting approach because the peaks in the Z-spectra of the cyst region (Supplementary Fig. S1B) overlapped and cannot be accurately measured with the 3-point method. Thus, we applied iterative least-squares peak fitting (Supplementary Fig. S1D) with 3 Lorentzian bands to resolve the peaks around 1.2, 2.2 and 3.5 ppm following a correction for non-specific macromolecular MT and direct saturation on water with a single Lorentzian band fitting (Supplementary Figure S1C) (50).

An NOE* map was calculated from three points at -2.0, -3.5 and -5.0 ppm

$$\text{NOE}^* = \{Z(2.0 \text{ ppm}) + Z(5.0 \text{ ppm})\} / 2Z(3.5 \text{ ppm}) \quad (4)$$

Mean ADC and FA maps were calculated from the principal eigenvalues (λ_1 , λ_2 , and λ_3) of the diffusion tensor (51).

$$FA = \sqrt{\frac{1}{2} \frac{\sqrt{(\lambda_1 - \lambda_2)^2 + (\lambda_2 - \lambda_3)^2 + (\lambda_3 - \lambda_1)^2}}{\lambda_1^2 + \lambda_2^2 + \lambda_3^2}} \quad (5)$$

Mean ADC is a scalar measure of overall diffusion in a voxel.

$$ADC = \frac{\lambda_1 + \lambda_2 + \lambda_3}{3} \quad (6)$$

High-resolution MTC images were used to identify regions of interest (ROIs) (e.g. WM, GM, and cyst) manually for quantification. To minimize partial volume effects, we excluded voxels along the spinal cord - CSF (cerebrospinal fluid) boundary. Building upon prior detailed descriptions of the lesion cavity in animal models of SCI (52), we further divided the observed lesion into rim and core sub-regions, which were distinguishable as having a high-MTR and a low-MTR value, respectively (MTR cutoff, 0.2). The two regions with high and low MTR_{asym} were also defined by a cutoff value set to half of the maximum MTR_{asym} value of the lesion. For longitudinal comparisons, the averaged regional values and standard deviations were then calculated across voxels within each ROI and compared using Student's t-tests.

Histology

The postmortem spinal cord from each animal was examined histologically. Series of coronal tissue sections were stained for HE (hematoxylin and eosin), CTB (cholera toxin B) (53), GFAP (glial fibrillary acidic protein) (54), and myelin (55) to reveal the lesion level and extent (CTB), amount of afferent disruption (CTB terminals in the brainstem), reactivated glia (GFAP), axon demyelination (myelin), and other pathological changes (HE). Histological information was used to confirm the pathological changes at and surrounding the lesioned spinal cord. Because only a limited subset of slices was processed for each stain, we did not perform a quantitative histological study.

RESULTS

SCI lesions and behavioral deficits

Despite the great effort devoted to creating a reproducible lesion, the resultant injuries demonstrated by MRI and the behavioral deficits varied across animals. Monkey SM-P showed impairment of his food reach and retrieval function during the first 6 weeks after SCI. After week 10, his general behavior appeared to be normal. Monkey SM-G showed a moderate behavioral impairment within two weeks after SCI. Monkey SM-T showed a relatively mild behavioral impairment after the first lesion, and a second lesion was applied

3 weeks later. This apparent inter-subject variability in behavioral deficits mimics the often-observed different functional/behavioral impairments in patients with SCI. To simplify the presentation, we report the detailed observations for SM-P, and then follow with brief descriptions of the findings from the other monkeys.

Multi-parametric MRI findings

Enhanced visualization of spinal cord lesions and cysts with MTC imaging—

Compared to conventional T1W images, MTC provided enhanced visualization of GM and WM contrast, as well as improved detection of tissue changes around the injury site. Figure 1 shows data obtained before and after SCI in subject SM-P. In a normal spinal cord (Fig. 1B, c-d), the GM and WM contrast was about 29% greater in MTC images (intensity ratio of ~ 1.54) than in T1W images (intensity ratio of ~ 1.19). This increase in contrast level is consistent with increased MT effects (and hence lesser signal) in WM compared to GM. Note that in spine, the contrast between GM and WM is different to brain, with WM showing lower intensity on our T₁-weighted acquisitions because the higher equilibrium magnetization and water content in GM contribute strongly. A very thin layer of hyperintense cerebrospinal fluid (CSF) (Fig. 1B, a-c) was visible around the spinal cord. The comparable rostral-caudal symmetry and minimal variations in cord size and shape at different time points illustrate the reproducibility of our imaging protocol.

At the targeted lesion level of C4 (Fig. 1C), a signal void ($\sim 1.5 \times 3.0 \text{ mm}^2$ shown in Fig. 1C) was clearly visible in both MTC and T1W images obtained at 10 weeks after SCI. This signal void region likely corresponds to the lesion and associated blood products.

Interestingly, a high-intensity abnormal volume (AV), which was very likely a cyst, was also detected rostral to the lesion. AV appeared as an area of high-MT contrast (Fig. 1C, a-c), but was barely distinguishable from GM or WM in T1W images (Fig. 1C, d). The cyst-WM contrast in MTC images was about 100% stronger than that in T1W images (Fig. 1C, c-d). The AV in this particular animal was about 14.4 mm^3 ($4 \times 2 \times 1.8 \text{ mm}$) at this time point (see supplementary Figure S2).

MTC images and DTI (ADC and FA) maps revealed dynamic progression of spinal cord lesions and cyst formation—

Figure 2 shows a series of MTC images and ADC and FA maps obtained at multiple time points within a 24-week window after SCI, which demonstrate the lesion progression and the development of the cyst. As early as 2 weeks after SCI, when the first MRI scan was taken, MTC images revealed a clear lesion as a black hole in the spinal cord tissue (Fig. 2A, b) with a small hyperintense cyst region at a more rostral location (Fig. 2A, b). By 8 weeks after SCI, the lesion region appeared to be smaller, but the hyperintense cyst region appeared to be larger than it had been at week 2, with lateral and rostral expansion (Fig. 2A, c). The cyst was located mostly in the WM region, but pushed a GM strip toward the ipsilateral side.

ADC and FA maps, obtained in parallel with MTC images at 8, 12 and 24 weeks after SCI (Fig. 2B & C), also showed dynamic changes. ADC values can be indicative of the cell density of tissues whereas FA values are associated with various microstructural factors such as coherence of fibers, axonal size and density, and myelination. At week 8 after SCI,

the mean ADC value of the cyst was larger, and the mean FA value was smaller than those of GM and WM. Both the ADC and FA values approximated those of dilute solutions. Over the following 16 weeks, the cyst volume decreased markedly (Fig. 2B) and the ADC value for the cyst decreased concomitantly by about 60% (from $\sim 2.4 \mu\text{m}^2/\text{ms}$ to $1.9 \sim \mu\text{m}^2/\text{ms}$). The FA value of the cyst core was maintained while that of the surrounding WM tissue increased (Fig. 2C).

In summary, the features of a high ADC value, a low FA value, and a lack of MT effects supports interpretation of the lesion as a cyst (17,56-59). The cyst was initially filled with fluids or semi-solids with low cellular density and few axons. Over time, the cellular density of the cyst increased.

Longitudinal characterization of the macromolecular signatures of the cyst

with MTR—We measured MTR to understand how the macromolecular content within the cyst changed over time following injury (Fig. 3). MTR values of spinal cord WM (0.645 ± 0.063) were greater than those of GM (0.530 ± 0.031 ; $p < 0.05$), which were also significantly greater than those of core and rim regions of the cyst (Fig. 3B). The rim has higher MTR values than the core ($p < 0.05$), and both of these values persisted over time. The size of the rim, however, changed drastically, peaking at post-lesion week 10, but returning to its size at post-lesion week 2 by post-lesion week 24 (Fig. 3C). Together, these results indicate that the rim region underwent a shrinking process, but contained constant levels of proteins during the 6-month recovery period.

Longitudinal characterization of the micromolecular composition of the cyst

with CEST—The Z-spectra and MTR_{asym} curves of the cyst showed dynamic concentration changes in metabolites after SCI (Fig. 4A). For example, at week 10 post-lesion, the Z-spectrum of the cyst was asymmetric with three peaks significantly resolved on the positive frequency offsets around 3.5 ppm, 2.2 ppm, and 1.2 ppm, which are related to the levels of amides, amines and hydroxyls, respectively (Fig. 4A). As controls, positive peaks for GM and WM were barely detectable at corresponding offsets both before and after SCI (Fig. 4A). More importantly, from week 10 to week 12, we observed differential changes in these three peaks in the cyst region (Fig. 4A). The peaks at 2.2 and 1.2 ppm (corresponding to amines and hydroxyls) diminished considerably, while the peak at 3.5 ppm (corresponding to amides) remained constant. At 24 weeks, all three peaks had diminished to baseline level (Fig. 4A). As controls for potential partial volume and motion artifacts, CSF did show much smaller peaks at 1.0, 1.5-1.8, 2.2 and 4.0 ppm offsets (Supplementary Figure S3), likely related to constituents such as glucose and protein (60).

Similar to MTR, MTR_{asym} maps also revealed heterogeneous low- and high- intensity sub-structures within the cyst (orange-yellow patches in Fig. 4B b-d) and lesion cavity (blue patches). Lower levels of amines and hydroxyls characterized the low-intensity region, which corresponded to the rim in the MTR map. The low NOE* value (Fig. 4C) indicated that the cyst contained lower levels of mobile macromolecule than the rest of spinal cord tissue. Figure 4D shows the dynamic changes of CESTR values during the recovery period of 24 weeks. From post-lesion week 10 to week 12, the CESTR values of the high- (left graph) and low-intensity (right graph) region at ~ 3.5 ppm (black columns) did not vary. In

contrast, values at ~2.2 ppm and ~1.2 ppm dropped significantly. By post-lesion week 24, all of the CESTR values had returned close to the zero line. These data demonstrate that the levels of amides, amines, and hydroxyls changed at different rates during the recovery period following SCI.

Table 1 summarizes all the measures in subject SM-P longitudinally. In this subject, the AV onset is 2 weeks and peak is 10 weeks. The AV did not fully disappear in 6 months after SCI. In addition, smaller MTR and FA, and bigger ADC values were observed for the abnormal tissues below 1 level around AV and along the lesion side. No evident changes were observed beyond 1 level.

Observations in the other two monkeys—We observed different progression patterns for the lesion and cyst in the second monkey, SM-G. Compared to SM-P, the cyst in SM-G differed in two ways. First, the cyst developed around the lesion site (Fig. 5A), rather than toward the rostral side of the lesion as had been observed in SM-P. Two weeks after SCI, the hyperintense cyst showed a more irregular shape while the lesion appeared as a hypointense hole within the cyst. The size of the cyst was very small at about 9 mm³ (1.5 × 2 × 3 mm³). Second, the molecular composition of the cyst changed much more quickly over time in SM-G, than was observed in SM-P. As shown in Figure 5C, at week 2 post-SCI, compared to control GM data, the MTR values for the cyst in SM-G were significantly reduced, and those values had decreased slightly further by 14 weeks post-SCI (-0.079 ± 0.043 in the core and 0.340 ± 0.110 in the rim, respectively). The most significant decrease in the MTR values of the cyst occurred at the early stage of recovery, with the size of the cyst stabilizing around week 7 (Fig. 5C) after SCI.

The CEST measures for the cyst in SM-G showed corresponding dynamic changes (Fig. 6). While the MTR_{asym} at ~3.5, ~2.2, and ~1.2 ppm were elevated at week 2 after SCI, by week 14 they had dropped to normal levels (Fig. 6A). Similar to SM-P, SM-G also exhibited the largest CESTR values at ~3.5 ppm when the cyst progressed to its peak (Fig. 6D). However, the speed and trend for each specific metabolite in the cyst of SM-G differed from those of SM-P. At week 7, the CESTR peak at ~1.2 ppm, corresponding to the mobile level of hydroxyls, was even higher than that at 2 weeks (Fig. 6A); in contrast, the peaks at 2.2 and 3.5 ppm had decreased. By 14 weeks after SCI, all CESTR values (Fig. 6D) had dropped back to normal levels. In summary, the levels of proteins and metabolites in the cyst changed in a manner similar to the patterns observed for subject SM-P, but recovered more quickly. In the third subject, SM-T, the cyst showed similar features to those observed for subject SM-G at 16 weeks post-SCI (see supplementary Figure S4). Table 2 summarizes these findings.

Histological evaluation of the spinal cord lesion and cyst formation

Gross and histological examination of the postmortem spinal cords confirmed several MRI observations in all three monkeys. First, we confirmed that the MTC images depicted the location of each lesion accurately, particularly in terms of spinal level. In SM-P, the lesion was confirmed to be at level C4, where the digit 1 (D1) afferents bundle entered the spinal cord. In SM-G and SM-T, the lesions were confirmed to be at between C4 – C5 levels.

Examination of coronal CTB- and GFAP-stained sections confirmed the rostro-caudal status of the lesions (red arrows in Fig. 7A, SM-P shown) and the cyst region revealed in MTC images (red asterisks in Fig. 7A-C). The lesion site was filled with soft connective tissue while the cyst region had a tissue cavity; this phenomenon has been described previously (61,62).

The histological signatures in HE, CTB, and GFAP stained sections confirmed that the cyst formed in all three animals (6,62). The tissue surrounding the high-MTC contrast cyst region contained heavy, dark GFAP-positive cells that extended along the middle-lateral and rostral-caudal directions (Fig. 7A-B, right). Since GFAP is a marker for reactivated glia (62,63), the presence of heavy GFAP-positive cells around the cyst cavity indicated that glial scars had formed in these locations. Examination of the HE-stained spinal cord tissue sections also revealed degenerated swollen axons, swollen axonal sheaths, and gliosis (Fig. 7D, SM-P shown). Spinal cord tissue sections from other two subjects showed similar histological features, supporting the presence of cysts (Fig. 7B&C).

DISCUSSION

In this longitudinal multi-parametric MRI study, we characterized the dynamic spontaneous recovery of damaged spinal cords in squirrel monkeys from structural, cellular and molecular composition perspectives. A combination of ADC, FA, MTR, NOE* and CESTR measures enabled us to monitor longitudinally subtle changes in cell density, fiber density, immobile/mobile macromolecular levels, metabolites and neurotransmitter byproducts within the AV that formed around the lesion sites, over recovery periods following SCI. The most novel finding of this study is observation of dynamic changes of macromolecules (e.g. proteins) and metabolites (e.g. amines, amides, and hydroxyls) within the cysts.

The main findings of this study are summarized in a modified schematic illustration (Fig. 8) of the neuropathological features of the SCI adapted from Figure 2 in the review paper by Thuret and colleagues (6). The signature changes of a cyst formation, tissue and cell properties of the scar, and the presence of reactivated glia and microglia are all well described previously. We now extend the framework by including the temporal progression (6,9) of cellular and molecular changes during the recovery period after SCI.

Advantages of multi-parametric high-field MR imaging

A combination of MRI measures allowed us to quantify longitudinally the changes in spinal tissue after traumatic injury. The high MRI field (9.4 T) was crucial for the success of this study. It is well known that the higher signal- and contrast-to-noise ratios (SNR and CNR) offered at high field permit higher resolution images to be obtained. The high-resolution anatomic images allowed us to make regional quantitative assessments. For example, the MT contrast image enabled clear segmentation of the GM and WM, as well as of the rim and core subregions of the cysts. Improving segmentation accuracy is important because spinal cord imaging is known to be sensitive to partial volume effects from neighboring tissues and CSF pulsation. Additionally, it was more straightforward to separate the CEST effects since the differences in resonant frequencies of exchanging species increase with the field strength.

Clinical implications of multi-parametric MRI observations

One important goal of MRI studies on animal models of SCI is to develop tools and optimize imaging parameters that can be referenced in human clinical studies. Traditional T1W, T2W and DWI are often used to differentiate edema, tumors and cysts in spinal cord (and brain), typically based on the location, geometry, shape, rim thickness, and homogeneity of tissue abnormalities (17,56-59). A cyst on MRI images is often described as showing a very thin rim surrounding the fluid. Edema, by contrast, often exhibits as a relatively uniform volume with a smooth edge. The observed T1, T2, MTC and DWI contrasts in MRI indicate the nature of the formation to be consistent with a fluid-type cyst of low cellularity. Additionally, CEST imaging suggests that the AV is cyst like but not edema, based on its high level of amide, amine and hydroxyl, and low NOE and solid MT effects. The unusual low MTR, high ADC and low FA were also indicative for a cyst formation.

Previous studies have shown that injured spinal cord tissue can form a cyst, which can be filled with fluid, semi solid substances or a mixture of blood, minerals, tissue and other fluid including CSF (17,56-59). In our cases, the MRI measures indicate that the fluid inside AV was neither blood nor CSF, but liquid containing low levels of aliphatic protons in mobile macromolecules and high compositions of metabolites in the early weeks after lesion. Later on, the liquid content was reduced with increased cellular density. We speculate that the high levels of freely exchangeable amine and amide inside the cyst could be an accumulation of the byproducts of amino acids from neurotransmitters (64). It is known that extracellular concentration of excitatory neurotransmitters such as glutamate increased drastically within days to weeks after injury, which in turn contribute to the secondary damage at the injury site (65), for a review see (66). Thus, the high levels of amine and amide we observed at the cyst region could be a reflection of such secondary injury. Nevertheless, the histological features of the spinal cord tissue at the lesion and AV locations are quite similar to the previous descriptions of the cyst in other models of spinal cord injury (67,68).

Longitudinal monitoring of SCI progression

We have demonstrated that multi-parametric MRI offers a novel way for early detection and longitudinal visualization of the lesion and of cyst formation, and for providing important information for future clinical applications. For example, cyst and scar tissue formation around the lesion are known to be the two main obstacles for axon regeneration and functional reinstallation. Many ongoing studies and clinical trials are focused on replacing the cyst or scar tissue with stem cells or peripheral nerves to promote or assist axons' reaching their targets. In this respect, besides the ability to monitor integrity of WM fiber tracks by DTI (13,22), and the early detection and visualization of cyst formation, multi-parametric MRI is likely valuable for *in vivo* monitoring of spontaneous recovery of spinal cord from injury and for evaluating the effects and outcomes of therapeutic interventions. Axonal loss and recovery are known to be major consequences of SCI, and our DTI findings captured such axonal changes. CEST in combination with MT and DTI enables greater sensitivity for detecting changes in the cellular and molecular composition of injured spinal cord tissue. With these methods, early cyst formation could be detected 2 weeks after SCI,

when the first measurement was taken. In principle, data acquisition could be moved even more toward the acute phase, such as days after lesion.

Our data showed that the progression of the lesion and of cyst formation varied between animals, even though each lesion was created in a reproducible manner. *In vivo* longitudinal MRI offers a unique opportunity to obtain systematic quantitative evaluation of inter-subject variability and its impact on behavioral and interventional outcomes at the group level. Importantly, the observed differential recovery patterns observed via MT and CEST of two animals highlights the facts that the cyst in each animal exhibited its own molecular signature. Quantification of inter-subject variability has additional clinical relevance given that highly variable situations are often observed in human SCI patients.

Finally, the pathological changes captured by multi-parametric MRI reflect underlying cellular and molecular changes, which were later confirmed histologically. The histological features were consistent with previous observations in rats up to 6 weeks after contusion injuries of the spinal cord (52,61,68) (for reviews see (6,62,66)). We also acknowledge that microdialysis could increase confidence in CEST observations of cyst metabolites. However, microdialysis sampling would affect longitudinal progress and recovery after SCI. Nevertheless, here, we showed that repeated ADC, FA, MTR, NOE* and CESTR quantification could detect subtle changes in cellular and fiber density, as well as concentration changes in the compositions of proteins and metabolites and possible changes in neurotransmitter byproducts in the cyst at different times after injury.

Primate model of SCI

The primate SCI model used here has proven to be valuable for understanding the role of cortical plasticity in mediating functional recovery. For example, using longitudinal functional MRI methods, we have shown that plastic topographic reorganization occurs in multiple cortical areas after a dorsal column lesion in the cervical spinal cord of monkeys (48). Here we demonstrated that parallel longitudinal imaging of the injured cord and brain can be achieved in individual animals. Finally, given the functional and structural differences between primate nervous systems and those of other commonly used animals, conducting preclinical trials with primates has the advantage of providing safety and efficacy data more relevant to humans (69,70).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. MTC (Magnetization Transfer Contrast) images reveal spinal cord injury and AV (cyst) at C4 location in subject SM-P

(A) Top: White box on sagittal brain image indicates the imaging field of view over cervical spinal cord. Bottom: schematic illustration of the targeted lesion of unilateral dorsal column (black patch). (B) Pre-lesion MTC images on sagittal (a), transverse (b), and coronal (c) views, and T₁-weighted image on coronal view (d). C2 indicates nerve bundle entering the 2nd cervical segment. GM: gray matter; WM: white matter. H: head; R: right. (C) MTC (a, b, c) and T₁-weighted (d) images obtained at 10 weeks after the lesion. Les: lesion. White dotted lines in a and b indicate the location of coronal imaging plan.

Figure 2. Longitudinal MTC and DWI (ADC and FA mapping) reveal the progression of lesion and the development of the AV (cyst) in subject SM-P

(A) Coronal MTC images obtained at pre-lesion (a), 2 (b), 8 (c), 10 (d), 12 (e), and 24 (f) weeks after lesion. H: head and L: left. (B) Mean ADC maps obtained at week 8 (a), 12 (b), and 24 (c) after lesion. (C) FA maps obtained at week 8 (a), 12 (b), and 24 (c) after lesion. White arrows indicate the formation of cyst. Red arrows indicate the lesion sites.

Figure 3. Longitudinal quantifications of MTR (magnetization transfer ratio) value and size of rim and core regions of the AV (cyst) in subject SM-P
 (A) Representative MTC image (a) and MTR map (b) at post-lesion 10 weeks show the region of interest (red outline) used for longitudinal quantification. Two sub-regions within cyst with high and low MTR values were classified as *rim* and *core* for quantification. MTR quantification threshold is set at 0.2. (B) The averaged MTR value of the GM and the time courses of MTR values of rim and core at 2, 8, 10, 12 and 24 weeks after injury. The standard deviations (error bar) were calculated from voxels within each ROI. * $p < 0.001$ vs. control, and # $p < 0.01$ vs. control. (C) Time courses of the area sizes of rim and core after lesion.

Figure 4. Longitudinal CEST imaging reveals dynamic changes of metabolites levels at the rim and core regions weeks after spinal cord lesion in subject SM-P

(A) Comparison of the averaged Z-spectra (solid markers) and MTR_{asy} curves (open markers) of GM, WM, and AV (cyst) derived from CEST imaging at post-lesion 10 weeks, 12 weeks, and 24 weeks. 2-head arrows indicate solid phase MT effects from macromolecules. Single big-head arrows indicate intramolecular and intermolecular nuclear Overhauser effects (NOEs) in mobile macromolecules such as proteins/peptides and lipids. Black asterisks indicate the peaks of metabolites (with hydroxyl, amine, and amide groups). (B) Representative MTC image (a) and MTR_{asy} maps (b-d) obtained at different RF offsets at post-lesion 10 weeks. Two sub-regions with high and low MTR_{asy} values within cyst are identified for quantification. The cutoff threshold is set at the half of the MTR_{asy} maximum value of the entire cyst region. (C) NOE* map. (D) Time courses of the regional CESTR values of high and low sub-regions at 3.5, 2.2, 1.2 ppm frequency offsets at 10, 12 and 24 weeks after lesion. The control CESTR value is from GM of normal spinal cord. The standard errors were calculated across voxels within each region of interest. * $p < 0.001$ vs. control, and # $p < 0.001$ vs. week 10 data.

Figure 5. Representative MTC and T₁-weighted images, MTR maps, and time courses of MTR value and size of AV (cyst) sub-regions weeks after lesion in the second subject SM-G
 (A) Images obtained at 2 weeks after SCI. MTC images of the sagittal (a), transverse (b) and coronal (c) views of the cervical spinal cord, T₁-weighted coronal image (d), and MTR map (e). Two sub-regions of rim and core with high and low MTR values are identified within cyst region. Cutoff MTR threshold is set at 0.2. (B) Corresponding images obtained at 14 weeks after lesion. (C) The averaged MTR values of control GM and the time courses of MTR values at rim and core regions within cyst obtained at 2, 7 and 14 weeks post-lesion. The standard deviations were calculated across voxels within each region of interest. * $p < 0.001$ vs. control, and # $p < 0.01$ vs. control. (D) Time course of the area sizes of rim and core regions after lesion. Control is normal GM.

Figure 6. Longitudinal CEST Imaging reveals dynamic changes of metabolites in the cyst sub-regions in the second subject SM-G

(A) Comparison of the averaged Z-spectra (solid markers) and MTR_{asy} curves (open markers) of GM, WM, and AV (cyst) derived from CEST imaging at post-lesion 2 weeks, 7 weeks, and 14 weeks. 2-head arrows indicate solid MT effect from macromolecules. Single big-head arrows indicate NOE. Black asterisks indicate the peaks of corresponding metabolites (hydroxyl, amine, and amide). (B) Representative MTC image (a) and MTR_{asy} maps (b-d) obtained at different RF offsets at 2 weeks post-lesion. Two regions of rim and core with high and low MTR_{asy} values were isolated for quantification. The cutoff threshold was set at the half of the MTR_{asy} maximum value of the entire cyst region. (C) NOE* map. (D) Time courses of the regional CESTR values of high and low sub-regions at 3.5, 2.2, 1.2 ppm frequency offsets at 2, 7 and 14 weeks after lesion. The control CESTR value is from GM of normal spinal cord. The standard errors were calculated across voxels within each region of interest. * $p < 0.001$ vs. control, and # $p < 0.001$ vs. week 2 data.

Figure 7. Histological evaluation of tissue properties with CTB, GFAP, and HE staining in all three animals

(A) MTC map, CTB and GFAP stains of spinal cord tissue obtained at week 26 after lesion for SM-P. Red arrows in A indicate scar type tissue formed at the lesion site. (B) CTB and GFAP stains of spinal cord tissue obtained at weeks 16 after lesion for SM-G, and (C) CTB stain of spinal cord tissue obtained at week 18 after lesion for SM-T. Red asterisks indicate the cyst formation. (D) HE stain of the tissue surrounding the cyst formation in SM-P shows swollen axons (black arrows), nuclear enlarged microglia, pallor and pleomorphism (yellow arrows). H: head and L: left.

Figure 8. Schematic illustration of the progressions of lesion and cyst after spinal cord lesion

Table 1

Longitudinal comparison of MRI parameters of subject SM-P

Parameter	ADC ($\mu\text{m}^2/\text{ms}$)	FA	MTR (%)	NOE* (%) (-3.5 ppm)	MTR _{asym} * (%) (3.5 ppm)	MTR _{asym} * (%) (3.5 ppm)	CESTR (%) (3.5 ppm)
WM	1.352	0.823	64.5	3.13	-13.55	2.02	2.78
GM	0.831	0.390	53.0	3.46	-8.45	3.46	4.76
AV	Week 2	0.275	25.7	1.54	-3.67	3.37	4.40
	Week 8	0.153	21.9	NA	NA	NA	NA
	Week 10	0.149	22.0	1.01	37.10	24.73	34.91
	Week 12	0.184	22.6	1.08	36.70	18.73	34.88
	Week 24	0.246	21.4	0.96	-2.32	1.29	3.02

Note: CEST results are not available (NA) at week 8 due to position change of the subject during CEST imaging

Table 2

Comparison of MRI parameters

Parameter	ADC ($\mu\text{m}^2/\text{ms}$)	FA	MTR (%)	NOE* (%) (-3.5 ppm)	MTR _{asym} (%) (3.5 ppm)	MTR _{asym} * (%) (3.5 ppm)	CESTR (%) (3.5 ppm)
WM	1.26 $\pm$ 0.10	0.778 $\pm$ 0.039	62.3 $\pm$ 2.9	3.38 $\pm$ 0.71	-11.0 $\pm$ 2.9	2.58 $\pm$ 0.50	3.51 $\pm$ 0.56
GM	0.89 $\pm$ 0.07	0.388 $\pm$ 0.031	54.2 $\pm$ 2.2	4.34 $\pm$ 0.53	-8.40 $\pm$ 1.58	3.09 $\pm$ 0.80	4.27 $\pm$ 0.51
AV (onset)	2.13 $\pm$ 0.52*	0.208 $\pm$ 0.061*	26.4 $\pm$ 5.0*	1.24 $\pm$ 0.74*	4.06 $\pm$ 14.76*	6.39 $\pm$ 5.48*	8.12 $\pm$ 6.88*
AV (peak)	2.56 $\pm$ 0.30*	0.146 $\pm$ 0.010*	24.8 $\pm$ 4.8*	0.65 $\pm$ 0.32*	29.59 $\pm$ 8.06*	20.87 $\pm$ 7.07*	28.95 $\pm$ 11.16*
AV (end)	1.87 $\pm$ 0.08*	0.228 $\pm$ 0.017*	25.3 $\pm$ 2.6*	0.80 $\pm$ 0.15*	-2.89 $\pm$ 0.71*	2.23 $\pm$ 1.05	3.49 $\pm$ 0.81

Note: n=9 for normal white matter (WM) and gray matter (GM), and n=3 for AV. Standard deviations are across subjects. The stage when AV occurs is defined as onset (2 weeks after SCI), the stage when the CESTR of AV is maximum during the progress is defined as peak, and the stage the CESTR of AV is minimum is defined as end since it is the end time point in the longitudinal study.

* P < 0.05 vs. normal tissue.