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Fenbendazole improves pathological and functional recovery following traumatic spinal cord injury

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Abstract

During a study of spinal cord injury (SCI), mice in our colony were treated with the anthelmintic fenbendazole to treat pinworms detected in other mice not involved in the study. As this was not part of the original experimental design, we subsequently compared pathological and functional outcomes of SCI in female C57BL/6 mice who received fenbendazole (150 ppm, 8 mg/kg body weight/day) for four weeks prior to moderate contusive SCI (50 kdyn force) as compared to mice on the same diet without added fenbendazole. The fenbendazole-treated mice exhibited improved locomotor function, determined using the Basso mouse scale, as well as improved tissue sparing following contusive SCI. Fenbendazole may exert protective effects through multiple possible mechanisms, one of which is inhibition of the proliferation of B lymphocytes, thereby reducing antibody responses. Autoantibodies produced following SCI contribute to the axon damage and locomotor deficits. Fenbendazole pretreatment reduced the injury-induced CD45R-positive B cell signal intensity and IgG immunoreactivity at the lesion epicenter six weeks after contusive SCI in mice, consistent with a possible effect on the immune response to the injury. Fenbendazole and

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related benzimidazole antihelmintics are FDA approved, exhibit minimal toxicity, and represent a novel group of potential therapeutics targeting secondary mechanisms following SCI.

Keywords

pathogenic autoantibody; locomotor function; traumatic injury

Introduction

Mice used by another investigator in our facility were shown to test positive for pinworm. All animals in the facility were thereafter treated with a diet containing fenbendazole, a broad spectrum anthelmintic often used to prevent or treat pinworm infections in several animal species including rodents (Campbell, 1990). Fenbendazole binds β -tubulin, inhibits microtubule formations, and thereby blocks mitosis in nematodes (Friedman and Platzer, 1978). Fenbendazole has greater sensitivity for nematode as compared to mammalian tubulin, and is safely administered to mammals at therapeutic doses. However, it may also influence mammalian cells.

Although earlier studies indicated that fenbendazole had minimal effects on the murine immune response (Reiss et al., 1987, Cray et al., 2008), more recent findings demonstrate that fenbendazole suppresses B cell activation (Landin et al., 2009) and alters the onset and disease severity of murine experimental autoimmune encephalomyelitis (Ramp et al., 2010). Autoantibodies produced following CNS injury contribute to axonal degeneration and neurological dysfunction, such as locomotor disability (Ankeny et al., 2009, Ankeny and Popovich, 2010, Lucin et al., 2007, Lucin et al., 2009, Zhang and Popovich, 2011, Zhang et al., 2013). To determine if fenbendazole might influence CNS B cells, autoantibodies and neurological dysfunction, we evaluated the effects of fenbendazole treatment on spinal CD45R-positive B cells and IgG immunoreactivity, tissue damage, and locomotor deficits in a mouse model of spinal cord injury (SCI).

Experimental Procedures

Animals

Female; 12-wk-old, specific pathogen-free C57BL/6 mice (Charles River, Indianapolis, IN), weighing 20–25 g, were kept under standard housing conditions for at least 1 week following arrival. All animal care and surgeries were performed in accordance with the “Guide for the Care and Use of Laboratory Animals” of the US Department of Health and Humans Services and were approved by the IACUC at the University of Kentucky. During the course of these experiments, all animals were helminth-free.

Experimental groups and FBZ administration

Age- and weight-matched C57BL/6 female mice were randomly assigned into three groups: 1) control diet prior to sham operation (n=6 per group), 2) control diet prior to SCI (n=7 per group), and 3) FBZ-medicated diet prior to SCI (n=7 per group). The mice in the FBZ group received FBZ-medicated feed (Harlan Taklad 2018S, 18% protein rodent diet with 150 ppm

FBZ that ensures a minimum dose of 8 mg/kg/day) for 4 weeks prior to SCI. The mice in the control group or sham group received regular diet (Harlan Teklad 2018S, 18% protein rodent diet without FBZ) for 4 weeks prior to SCI or prior to sham operation. All mice received the regular diet following SCI or sham surgery. For one mouse in each of the SCI-control and SCI-fenbendazole groups, the amount of IgG and CD45R immunoreactivity was several-fold out of range as compared to values from other animals. These results were excluded from further statistical analysis.

Spinal Cord Injury

A thoracic level injury was chosen because more rostral injuries, such as cervical SCI, disrupt sympathetic control and result in immunosuppression (Lucin et al., 2007, Lucin et al., 2009, Ankeny and Popovich, 2010, Zhang et al., 2013). Following a T9 laminectomy, SCI was produced with a force of 50 kdyn using an Infinite Horizon SCI device (Precision Systems & Instrumentation) as previously described (Scheff et al., 2003, Yu et al., 2010). Briefly, mice were anesthetized with ketamine (80 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). A laminectomy was made to expose spinal segment T9. Contusion injury was then applied which produced moderate SCI. Injury parameters, including actual force applied to the spinal cord, displacement of spinal cord, and velocity were automatically recorded. For the contusive SCI, no significant differences in impact force, displacement, and velocity were found between treatment and control groups, indicating similar injuries to all animals (Table 1). Sham animals underwent laminectomy only. After surgery, mice were given prophylactic antibiotics (Baytril, SC mg/kg, subcutaneous, twice daily for four days post injury), saline (2 ml, subcutaneous, immediately post injury), and buprenorphine (0.02 mg/kg, subcutaneous, twice daily for two days post injury). Manual bladder expression was performed twice daily until bladder function returned. The surgical procedure and post-operative care were as described previously (Scheff et al., 2003, Yu et al., 2010).

Locomotor function (Basso Mouse Scale)

Locomotor deficit was assessed using the Basso Mouse Scale (BMS) including BMS scores and BMS sub scores (Basso et al., 2006). The BMS test was performed preinjury and 0, 1, 3, 7 14, 21, 28, 35, and 42 days postinjury in a blinded manner by two independent investigators who were trained and certified by the SCI Research Training Program at Ohio State University. BMS scores or sub scores for left and right hindlimbs were averaged to obtain a single value per mouse.

Histology

At six weeks post-SCI, the injured or sham-operated mice were euthanized and transcardially perfused with ice-cold saline followed by phosphate-buffered 4% paraformaldehyde. The fixed spinal cord (1.0 cm in length) centered at the lesion epicenter was removed and processed for sectioning and histological analysis, as previously described (Rabchevsky et al., 2001, Yu and Geddes, 2007). A modified eriochrome cyanine (EC) protocol was used to stain myelin and differentiate between white and gray matter, and the sections were also stained using cresyl violet. Images of stained sections and assessment of total tissue sparing, white matter sparing, and gray matter sparing in spinal cord sections

extending 5 mm rostral and caudal from the lesion epicenter were performed as described previously (Rabchevsky et al., 2001, Yu and Geddes, 2007).

Immunohistochemistry

The avidin-biotin-horseradish peroxidase method was used to perform immunohistochemical staining on spinal cord sections using rat adsorbed biotinylated anti-IgG antibody (Vector Laboratories, Inc., Burlingame, CA) as previously described (Hoane et al., 2006, Readnower et al., 2010). After washing in TBS/0.2% Triton X-100, the sections were treated with 0.3% H₂O₂ for ten minutes to eliminate endogenous peroxidase activity. To reduce non-specific background, the sections were further incubated with 10% normal horse serum in TBS/0.2% Triton X-100 for 1 hr at room temperature. The sections were incubated with the rat adsorbed biotinylated anti-IgG antibody overnight at 4°C (1:1,000). After the incubation with peroxidase conjugated streptavidin, immune complexes were visualized by incubation of sections in the DAB system (0.05% diaminobenzidine, 0.01% H₂O₂, and 0.3% imidazole, Vector Laboratories, Inc., Burlingame, CA) according to the manufacturer's instruction¹¹. IgG immunoreactivity was imaged using an AX80 microscope (Olympus, Center Valley, PA, USA) and quantitated by a gray-level-index (GLI) that corresponded to the area of IgG immunoreactivity using Image J software (Schneider et al., 2012) and Adobe Photoshop CS2 software (Adobe Systems Incorporated, New York, NY, USA) as previously described (Bisler et al., 2002, Readnower et al., 2010) (5 sections separated 100 µm apart/ per animal, n=6 animals per group). For each animal, the GLI for each section was calculated and averaged to obtain the percentage of total area with IgG immunoreactivity. Next, the average area per section of all animals in each group was averaged to obtain a mean value per section.

Immunofluorescence

Immunofluorescence staining was performed as previously described (Yu et al., 2010). Briefly, spinal cord cross-sections at the lesion site were incubated with a rat-anti mouse monoclonal antibody against CD45R (clone RA3-6B2, 1:50; B cell marker, Santa Cruz Biotechnology, Inc.) followed by incubation with FITC-conjugated goat-anti rat secondary antibody (Santa Cruz Biotechnology). Using a laser scanning confocal microscopy system (Nikon C2+, Melville, NY, USA), the fluorescent CD45R signals within the spinal cord section (512 × 512 µm) in the lesion site were captured (magnification 10×). Mean intensity was quantified within these regions of interest (ROI) in a blinded manner. Images were acquired at the laser setting HV 119 and 2.0 percent laser. Using a threshold setting of 1750, signal intensity was measured. Staining for CD45R was visualized within Z-stacks of 10 images with 2-µm steps corresponding to a 20-µm depth for each ROI. Four independent sections were evaluated for each ROI per animal (4 sections separated 100 µm apart per animal, n= 6 animals per group). For each animal, the calculated fluorescent mean intensity for expression of CD45R of four sections was averaged. Subsequently, the average intensity per section of all animals in each group was averaged to obtain a mean intensity value. Confocal microscopic images with double staining of CD45R (green) and DAPI (blue) to label cell nuclei were also captured at 100 × magnification.

Statistics

All data are presented as mean $\pm$ S.E.M. Group differences were evaluated by repeated measures ANOVA followed by Bonferroni post hoc test or t-test. Null hypotheses were rejected at the $P < 0.05$ level.

Results

Oral administration of fenbendazole (feed supplemented with 150 ppm ensuring 8 mg/kg body weight/day) for four weeks prior to contusive SCI (50 kdyn impact force, moderate severity) resulted in improved locomotor function, as measured by BMS scores and sub scores, six weeks post injury in C57BL/6 mice as compared to mice which received the same diet without added fenbendazole (Figure 1). Fenbendazole treatment was well-tolerated and did not result in alterations in body weight, as compared to animals on the control diet, following SCI (Table 2).

Analysis of EC-stained spinal cord sections demonstrated that fenbendazole pretreatment for four weeks prior to SCI resulted in significant improvements in total tissue sparing and white matter sparing at the lesion site six weeks after contusion SCI in mice (Figure 2). The gray matter sparing obtained with fenbendazole pretreatment was increased by 136%, but the differences between fenbendazole-treated and control groups were not statistically significant.

Immunohistochemistry analysis of spinal cord sections showed that contusive SCI resulted in a significant increase in IgG levels at the lesion site of spinal cord six weeks postinjury compared with sham animals (Figure 3). At six weeks following SCI, mice which had been treated with fenbendazole exhibited significant reduction in IgG immunoreactivity at the spinal cord lesion site, as compared to mice with SCI that did not receive fenbendazole (Figure 3).

Immunofluorescence staining of spinal cord sections showed that contusive SCI significantly increased CD45R-positive B cell signal intensity at the lesion site of spinal cord six weeks postinjury compared with sham animals (Figure 4). Fenbendazole pretreatment significantly reduced CD45R-positive B cell signal intensity at the spinal cord lesion site, as compared to the SCI control group which did not receive fenbendazole pretreatment (Figure 4).

Discussion

The purpose of this study was to investigate possible effects of fenbendazole treatment on experimental results obtained in a mouse model of spinal cord injury. Fenbendazole is widely used in veterinary medicine to treat animals for nematode infestation, including pinworm in rodents (Campbell, 1990). Although safe and well-tolerated at therapeutic doses, fenbendazole treatment may alter results of experimental studies. Effects of fenbendazole on behavioral tests of motor coordination and balance skills (Gadad et al., 2010) and the immune system and immune response to allergens have been reported in mice (Cray et al., 2008, Cai et al., 2009, Ramp et al., 2010).

Fenbendazole pretreatment reduced the extent of tissue damage and attenuated the impairment of locomotor function, measured by BMS, following contusion SCI in mice. The BMS scale is widely used to measure locomotor function after contusive SCI only for mouse including joint movement, weight support, stepping, trunk instability, and coordination (Basso et al., 2006). Fenbendazole cause modest impairment of select motor functions, such as the rotarod test, in the short term (Gadad et al., 2010), but these effects do not extend to the six week postinjury time points examined in the present study.

In the thoracic SCI model, although locomotor deficits are largely due to loss of axons and white matter damage (Magnuson et al., 2005), gray matter damage is also an important contributor to locomotor impairment after thoracic SCI, including postural muscle control and body position. Thus, the improved white matter and gray matter sparing observed following fenbendazole treatment is consistent with, and likely contributes to, the improved motor function in fenbendazole-treated mice, as compared to mice without fenbendazole treatment. While having relatively minor consequences in the thoracic injury model, gray matter sparing with the fenbendazole pretreatment could attenuate functional deficits following cervical or lumbar injuries where loss of motor neurons in gray matter contributes to the functional deficit (Magnuson et al., 2005, Onifer et al., 2005).

Benzimidazoles, including fenbendazole, inhibit microtubule polymerization by binding tubulin (Lacey, 1990). Benzimidazoles bind to a site on tubulin distinct from the Vinca alkaloids, and do not induce peripheral neuropathy, which is a dose-limiting toxicity of Vinca alkaloid microtubule inhibitors (Spagnuolo et al., 2010). While they have a greater binding to nematode as compared to mammalian tubulin at 37°C (Lacey, 1990), benzimidazoles can also disrupt mammalian microtubules, interfere with the division of rapidly dividing cells such as B lymphocytes and thereby suppress the immune system.

Following SCI, activation of B cells, production of autoantibodies and activation of downstream inflammatory cascades at sites of axon pathology and demyelination are important events in the secondary injury cascade (Ankeny et al., 2009, Ankeny and Popovich, 2010, Zhang and Popovich, 2011). This was originally described in mice, with supportive evidence of increased B cell activation and autoantibody production also evident in human SCI subjects (Hayes et al., 2002, Saltzman et al., 2013). Although normal IgG isolated from pooled human serum has been shown to attenuate neuroinflammation following SCI (Fehlings and Nguyen, 2010), IgG obtained following SCI activates intraspinal complement and cells bearing Fc receptors (Ankeny et al., 2009). In animal models of SCI, IgG mediates downstream inflammatory cascades causing axonal demyelination and degeneration and impairing locomotor recovery after injury (Ankeny et al., 2009, Ankeny and Popovich, 2010, Zhang and Popovich, 2011).

Fenbendazole, by inhibiting microtubule polymerization, reduces antibody responses by inhibiting proliferation of activated pre-B and B cells from the bone marrow and the spleen (Cabaj et al., 1994, Landin et al., 2009). The intact blood-spinal cord barrier limits the entry of cells and proteins into the spinal cord, but this barrier is damaged following SCI (Sharma, 2005). Similar disruptions to the blood-spinal cord or blood-brain barrier may also contribute to autoimmunity and neurodegeneration in other disorders such as Parkinson's

disease (Rite et al., 2007, Carvey et al., 2009, Zhang and Popovich, 2011). The reduction in CD45R⁺-B cell signal intensity and IgG immunostaining in the spinal cord could result from reduced B cell activity and autoantibody production and/or reduced penetration of IgG into the injury site due to the a reduction in damage to the blood-spinal cord barrier.

By disrupting microtubules and inhibiting cell division, FBZ may also have additional and off-target effects including potential effects on other proliferative cells including astrocytes, macrophages and microglia, endothelial cells, and neural progenitor cells. By potentially interfering with axonal transport, FBZ could also impair axonal regeneration and sprouting. Another microtubule disrupting agent, colchicine, can cause activation of astrocytes and microglia (Colburn and DeLeo, 1999), and also affect macrophages, neutrophils and endothelial cells (Crittenden et al., 2012). Following transient spinal cord ischemia in rabbits, the combination of chloroquine and colchicine decreased the numbers of mononuclear phagocytes within the damaged spinal cord, increased survival of motor neurons, and improved functional outcomes (Giulian and Robertson, 1990). However, colchicine alone was not investigated. Additional evidence that altered microtubule stability may influence recovery from SCI is provided by a recent study demonstrating that the microtubule inhibitor Taxol reduced glial scarring, promoted axonal regeneration, and enhanced functional improvements following experimental SCI (Hellal et al., 2011). While taxol stabilizes microtubules in contrast to the disruption by FBZ, both actions interfere with mitosis and cell division.

Although the anti-helminthic effects of fenbendazole and related compounds are largely ascribed to their effects on tubulin, additional mechanisms may be involved. In helminthes, benzimidazoles inhibit aminopeptidase activity, decrease glutamate catabolism, reduce glutamate uptake, increase cytosolic free calcium, and decrease glycogen levels (Jasra et al., 1990, Cumino et al., 2009). Whether the drugs have similar effects on mammalian cells remains to be determined.

The results of the present study demonstrate that FBZ pretreatment improves outcomes following SCI, and that this is accompanied by a reduction in IgG and CD45R⁺ B cell immunoreactivity at the lesion site. We hypothesize that the effects of FBZ on B cells contributed to the functional improvements, however the results are correlative and additional studies to examine the mechanisms and cellular effects of FBZ and related compounds are warranted. Benzimidazole antihelminthics are widely used in animals and humans, and therapeutic levels can be administered long term without causing toxicity or side effects (Fuchs, 1993). The results demonstrate that FBZ pretreatment alters outcomes following SCI and suggest that fenbendazole, related benzimidazoles, or possibly other compounds which disrupt microtubules may have potential as therapeutics for SCI.

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Highlights

- Fenbendazole improved pathological and functional outcomes following SCI in mice
- Fenbendazole disrupts microtubules and can impair B cell proliferation
- Fenbendazole reduced B cells and IgG at the spinal lesion site
- Fenbendazole and related compounds represent novel potential therapeutics for SCI

Figure 1.

Effects of fenbendazole on locomotor deficits 6 weeks following SCI. Fenbendazole treatment (triangles or white bar, $n=7$) for 4 weeks prior to SCI (50 kdyn) resulted in improved locomotor function measured by BMS scores (left) and BMS sub scores (right) 6 weeks after contusion SCI in C57BL/6 mice, as compared to mice treated in an identical manner but without fenbendazole supplementation in the diet (circles or black bar, $n=7$). Data are presented as the mean $\pm$ S.E.M. and analyzed with repeated measures ANOVA followed by Bonferroni post-hoc analysis, * $p<0.05$ and ** $p<0.01$.

Figure 2.

Effects of fenbendazole on tissue sparing 6 weeks following SCI. Fenbendazole treatment (white bar, n=7) for 4 weeks prior to SCI (50 kdyn) resulted in a significant increase in total tissue sparing (A) and white matter sparing (B) at lesion site 6 weeks following contusion SCI compared to control animals (Black bar, n=7), while the changes in Gray matter sparing (C) were not statically significant. Data are presented as the mean $\pm$ S.E.M. and analyzed with t-test, *p<0.05. D and E: Photomicrographs of representative transverse spinal cord sections 42 days following contusive SCI at the lesion epicenter. The sections were stained with eriochrome cyanine for myelin. The images shown were in D and E are tiled images blended into a montage. The calibrated Stage-Pro module of Image Pro 3D version 6 (MediaCybernetics, MD) was used to grab and reassemble images obtained from a 20 $\times$ objective on an Olympus BX51 microscope with an ASI XY automated stage. Scale bar: 100 μ m.

Figure 3.

Effects of fenbendazole treatment on IgG levels at the lesion epicenter 6 weeks following SCI. Photomicrographs are of representative transverse spinal sections taken from sham-injured (A) and SCI with control diet (B) or following fenbendazole pretreatment (C). The sections were immunostained using an antibody against mouse IgG. D: fenbendazole treatment (white bar, n=6) for 4 weeks prior to SCI (50 kdyn) resulted in a significant decrease in spinal levels of IgG at lesion sites 6 weeks following contusion SCI compared to animals on the control diet (black bar, n=6). Data are presented as mean $\pm$ S.E.M. and analyzed with repeated measures ANOVA followed by Bonferroni post-hoc analysis, ###p<0.001, compared to sham-animals, ***p<0.001, compared to SCI animals without fenbendazole pretreatment. Scale bar: 100 μ m.

Figure 4.

Effects of fenbendazole treatment on CD45R-positive B cell fluorescent signal intensity at the lesion site, 6 weeks following SCI. Photomicrographs of representative transverse spinal sections taken from spinal cord injured mice without fenbendazole pretreatment (A); with fenbendazole pretreatment (B), and a sham-injured mouse (C). The sections were immunostained with a primary antibody against CD45R (B cell marker, green) and counterstained with DAPI to identify cell nuclei. Quantitative results of the green fluorescent signal intensity are shown in (D). Fenbendazole treatment (white bar, n=6) for 4 weeks prior to SCI (50 kdyn) resulted in a significant decrease in CD45R intensity 6 weeks following contusion SCI, as compared to animals on the control diet (black bar, n=6). Data are presented as the mean $\pm$ SEM and analyzed with repeated measures ANOVA followed by Bonferroni post-hoc analysis, # $p < 0.05$, compared to sham-animals, * $p < 0.05$, compared to SCI animals without fenbendazole pretreatment. Scale bar: 10 μ m.

Injury Parameters

Table 1

Values are the mean $\pm$ SEM. No significant differences in impact force, displacement, and velocity were found between SCI-fenbendazole and SCI-control groups, indicating similar injuries to all animals.

Groups	Actual force (kdyn)	Displacement (microns)	Velocity (mm/sec)
SCI-FBZ	53.8 $\pm$ 0.24	386.8 $\pm$ 23.9	122.9 $\pm$ 0.29
SCI-Control	54.2 $\pm$ 0.52	364.7 $\pm$ 23.7	123.3 $\pm$ 0.84

Table 2**Weight (g)**

Values are mean $\pm$ SEM. Fenbendazole treatment did not alter body weight.

Groups	Pre	14	21	42 (DPI)
SCI-FBZ	24.5 $\pm$ 0.31	23.6 $\pm$ 0.29	23.9 $\pm$ 0.35	25.14 $\pm$ 0.37
SCI-Control	25.0 $\pm$ 0.71	22.2 $\pm$ 0.37	23.1 $\pm$ 0.67	23.4 $\pm$ 0.53

DPI: Days Post-Injury