A Toll-Like Receptor 9 Antagonist Improves Bladder Function and White Matter Sparing in Spinal Cord Injury

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Abstract

Spinal cord injury (SCI) affects motor, sensory, and autonomic functions. As current therapies do not adequately alleviate functional deficits, the development of new and more effective approaches is of critical importance. Our earlier investigations indicated that intrathecal administration of a toll-like receptor 9 (TLR9) antagonist, cytidine-phosphate-guanosine oligodeoxynucleotide 2088 (CpG ODN 2088), to mice sustaining a severe, mid-thoracic contusion injury diminished neuropathic pain but did not alter locomotor deficits. These changes were paralleled by a decrease in the pro-inflammatory response at the injury epicenter. Using the same SCI paradigm and treatment regimen, the current studies investigated the effects of the TLR9 antagonist on bladder function. We report that the TLR9 antagonist decreases SCI-elicited urinary retention and ameliorates bladder morphopathology without affecting kidney function. A significant improvement in white matter sparing was also observed, most likely due to alterations in the inflammatory milieu. These findings indicate that the TLR9 antagonist has beneficial effects not only in reducing sensory deficits, but also on bladder dysfunction and tissue preservation. Thus, modulation of innate immune receptor signaling in the spinal cord can impact the effects of SCI.

Key words: CpG ODN; inflammation; pattern recognition receptors; regeneration; traumatic spinal cord injury

Introduction

SPINAL CORD INJURY (SCI) causes disruption of the ascending and descending tracts, or the local circuitry, that subserve motor, sensory, and autonomic function. One such autonomic function that is frequently affected after SCI is control of the urinary bladder. An inability to fully empty the bladder produces pathologically elevated intrabladder pressures that can incur damage to the urinary tract. Consequently, urinary tract infections may develop in persons with SCI, which, if untreated, can be life-threatening.¹ This is one of the reasons why persons with SCI regard the recovery of bladder function as one of their highest priorities.²

SCI induces an inflammatory response at the injury epicenter, which has been described as a double-edged sword, having both beneficial and detrimental properties.³ Modulation of inflammation after SCI can alter the microenvironment at the injury epicenter, which, in turn, can impact the function of the residual cells in the spared tissue and their communication with other cells, including those that convey information to and from the bladder. Inflammation can also determine the extent of spared tissue,⁴ because an

inverse correlation between the intensity of the inflammatory response and the amount of spared tissue after SCI has been demonstrated.^{5–10}

Inflammation can be modulated by innate immune receptors including toll-like receptors (TLRs),^{11–15} which are pattern recognition receptors that mediate host-defense during infections. TLRs are expressed in cells of both the immune and central nervous systems (CNS). In addition to pathogen-derived ligands, endogenous ligands released by damaged cells can induce TLR signaling and, thereby, modulate sterile inflammation associated with CNS injury and disease.^{16,17}

Of the 13 TLRs that have been identified in the mouse,^{18,19} TLR2 and TLR4 have been the most extensively investigated with respect to their role in SCI.^{4,20} Although information about the contribution of TLR9 to SCI is limited, our previous investigations demonstrated that repeated intrathecal administration of a synthetic TLR9 antagonist, cytidine-phosphate-guanosine oligodeoxynucleotide 2088 (CpG ODN 2088), reduces the inflammatory response at the injury epicenter after SCI and ameliorates injury-induced thermal sensitivity, while having no effect on hindlimb locomotor function.²¹

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The current investigations were undertaken to complement these initial findings and determine whether intrathecal CpG ODN 2088 treatment ameliorates recovery of bladder function and improves tissue sparing in mice sustaining a severe contusion injury.

Methods

Animals

Ten-week-old C57Bl/6 female mice (Charles River, Wilmington, MA) were housed in a pathogen-free barrier facility and maintained on a 12-h dark/light cycle with food and water provided *ad libitum*. Sentinels were housed in the same room and periodically checked for pathogens. All animal protocols were approved by the New Jersey Medical School Institutional Animal Care and Use Committee.

Spinal cord contusion injury

Both injured and uninjured mice were used for the current study. Mice were handled for 1 week for acclimatization before surgery and behavioral testing. Mice were anesthetized with a mixture of ketamine (80 mg/kg; Vedco, St. Joseph, MO) and xylazine (10 mg/kg; Akorn Inc., Decatur, IL), administered intraperitoneally. Using aseptic approaches, a midline posterior incision was used to expose the dorsal elements of the T7–T9 vertebrae. A laminectomy was performed at the eighth thoracic vertebral level (T8), and a severe contusion injury (70 kdyn force) was induced using the Infinite Horizon Impactor device (Precision Systems & Instrumentation, Lexington, KY). A layered closure with sutures and wound clips completed the surgical procedure.

After surgery, the mice were group-housed in cages with Aspen shavings, and the cages were placed on a water-jacketed heating pad at 37°C. For postoperative care, mice received subcutaneous injections of lactated Ringer solution (Baxter, Deerfield, IL), physiologic saline (Baxter, Deerfield, IL), Baytril (0.05 mL; Bayer, Kansas City, KS), and buprenorphine (0.02 mL; Hospira, Lake Forest, IL). Antibiotics, analgesics, and 2 mL lactated Ringer solution were administered immediately postoperatively. Additional antibiotics, analgesics, and 1 mL saline were administered twice a day for 7 days. Mice were observed daily for signs of infection and to ensure appropriate wound healing.

Intrathecal delivery of TLR9 ligands by lumbar puncture

The antagonist CpG ODN 2088 was purchased from InvivoGen (San Diego, CA) and suspended in vehicle (endotoxin-free distilled water), aliquoted, and conserved at –20°C until use. Mice were assessed for locomotor recovery using the Basso Mouse Scale (BMS)²² on day 1 post-injury (PI) to confirm equivalent and proper injury severity, and were randomly divided into treatment groups. Intrathecal delivery of ligands and vehicle began 24 after injury, and was repeated every 48 h thereafter for the duration of the study.

The mice were anesthetized with isoflurane (1.0 L/min at a concentration of 3.0% in oxygen). A 27-gauge needle attached to a Hamilton syringe was inserted percutaneously into the interlaminar space between L5 and L6. On successful entry into the intrathecal space, CpG ODN 2088 (150 ng/g body weight; InvivoGen, San Diego, CA) or vehicle was injected in a total volume of 3 μ L. The needle was maintained in place for 30 sec before it was slowly removed to prevent egress of the injectate. Animals were allowed to recover in their home cages.

Evaluation of bladder function

The assessment of bladder function by measuring the weight of manually voided urine during daily bladder expression has been previously documented in the literature.^{23–25} Because this approach

was used primarily in injured rats, it was modified for mice because it is best for mice that have sustained a SCI to have their bladders expressed when prone, and also because the volume of urine that is expressed in uninjured mice is often too small to capture without significant error.

Bladders of all mice were expressed every 12 h after the surgical procedure for the duration of the study. Mice were anesthetized with isoflurane (1.0 L/min at a concentration of 3.0% in oxygen) and weighed on an analytic balance before and after the bladder was expressed. The weight of urine voided was determined by calculating the difference in the two weights. After bladder assessment, the mice were returned to their home cages.

To determine fluid intake, a cohort of uninjured and SCI mice were singly housed and treated as described above. The volume of water in the bottles was measured at 48-h intervals.

The urinary retention data are pooled from several iterations of this experiment, in which all three experimental groups were represented.

Urinalysis

Expressed urine underwent urinalysis once every 7 days. Urine was applied to Multistix® 10SG urinalysis strips (Siemens, Tarrytown, NY) and, following the prescribed amount of time (30–120 sec), strips were scored according to the manufacturer's instructions. Levels of ketones, bilirubin, protein, nitrites, leukocytes, and pH were determined by matching the color on the test strip with the reference material. Each biomarker had an ordinal scale assigned to each of the possible colors, with 2 to 6 possible levels, depending on the biomarker. A score of 1 corresponds with a negative reading for all biomarkers, with increasing numbers corresponding to increasing signal strength.

Immunohistochemistry and assessment of lesion volume and tissue sparing

Mice were sacrificed by sodium pentobarbital (1.95 mg/g) anesthesia followed by transcardial perfusion with saline and 4% paraformaldehyde in phosphate buffered saline (pH 7.4). Spinal cords, bladders, and kidneys were dissected, post-fixed overnight, cryoprotected in 27% sucrose, embedded in optimal cutting temperature (OCT) compound, frozen in a dry ice-ethanol slurry, and stored at –80°C. In the case of bladders, the inside of the bladder was also filled with OCT before embedding.

Two methods were used to determine the spinal cord lesion volume. In both cases, 30 μ m thick transverse sections, obtained at 150 μ m intervals and spanning the rostro-caudal length of the lesion, were used. In the first, sections were labeled with rabbit anti-glial fibrillary acidic protein (GFAP) antibody (1:1000; Dako). The signal was visualized using goat anti-rabbit Alexa Fluor 594 antibody (1:400; Invitrogen). The lesion was defined as the GFAP-negative area of the section that was bordered by the GFAP immunopositive astroglial scar.²⁶ Images were captured on either a Leica DM5500B microscope using Leica Application Suite 4.2 software or a Zeiss Axio Imager M2 using Axio Vision 4.8.2 software.

The lesion area was outlined and measured using NIH ImageJ analysis software.²⁷ The lesion area for each section was then multiplied by the distance between sections (150 μ m) to determine the lesion volume for that section. The overall lesion volume was calculated by summing each of these section volumes. The individual section with the greatest amount of GFAP-negative area was defined as the lesion epicenter.

For the second estimate of lesion volume, sections adjacent to those stained for GFAP were stained for Luxol fast blue and counterstained with hematoxylin and eosin (LFB-H&E).^{28–30} In these sections, the lesion is defined as any area of aberrant histopathology, notably recognized by degenerating white matter or

eosinophilic (degenerating) neurons.³¹ The images were captured and volume calculated as above.

For the quantification of white and gray matter sparing in the spinal cord, sections adjacent to those used for the measurement of lesion volume, and spanning the rostro-caudal length of the lesion, were stained for myelin with LFB.³² The amount of spared white matter and the total circumferential spinal cord area were delineated and measured using NIH ImageJ analysis software. Spared gray matter was calculated by subtracting the spared white matter and the GFAP-negative lesion area from the total spinal cord area for each section.

For kidney histology, the tissue was dissected, cryoprotected, and frozen as described above. Thirty micron thick sections were thaw-mounted onto slides and stained with H&E as described previously.²⁴ Three sections/mouse and six mice/group were used for the analyses.

Bladder weight and volume

After sacrifice, bladders were dissected out, blot-dried, and weighed. The length and width of each bladder was then measured. The bladder volume (V) was calculated by the equation for a pro-

late ellipsoid: $V = 4/3 * p * L/2 * W/2 * W/2$, where L is the bladder length and W is the bladder width. This method does not account for the elastic nature of the fully distended bladder and may therefore be an underestimation of the actual bladder volume.²⁵

Bladder wall thickness

Bladder sections (6 μ m thick) were prepared transverse to the longest axis of the bladder, beginning at the bladder dome, and thaw-mounted onto slides. Every fifth section was stained with H&E. The thickness of the bladder wall was determined using NIH ImageJ analysis software. Bladder sections between 900 μ m and 1200 μ m from the bladder dome were selected for measurement. On each section that was measured, four bladder regions were chosen at random and averaged to determine the thickness of that section.

Statistical analysis

IBM SPSS Statistics 20.0 (Armonk, NY) and GraphPad Prism 6.0 software (San Diego, CA) were used to determine the statistical differences between experimental conditions. Repeated measures

FIG. 1. Effects of intrathecal cytidine-phosphate-guanosine oligodeoxynucleotide (CpG ODN) 2088 treatment on bladder function and morphopathology after spinal cord injury (SCI). **(A)** Schematic diagram showing the treatment regimen with SCI on day 0 and lumbar punctures performed on alternate days (arrows). **(B)** Effects of CpG ODN 2088 on urinary retention. A significant difference between CpG ODN 2088- and vehicle-treated, injured mice was first observed on day 5 post-injury (PI) (** $p < 0.01$), again on days 7–10 PI (** $p < 0.01$), and this significant difference was maintained from day 13 PI until day 28 PI (** $p < 0.01$), the latest day examined; $n = 18$; Bonferroni post-hoc, following repeated measures analysis of variance (ANOVA). **(C)** Effects of CpG ODN 2088 on water intake. Graph shows the average daily water consumption per mouse ($n = 6$) with no significant difference being detected. **(D)** Effects of CpG ODN 2088 on bladder weight, bladder volume, and bladder wall thickness. Morphometric studies were performed at 28 days PI. Significantly different by ANOVA, Tukey post-hoc test * $p < 0.05$, $n = 8$.

analysis of variance (ANOVA) were used for comparison of daily urinary retention and urinalysis. Where appropriate, repeated measures ANOVAs were followed by multiple group comparisons using the Bonferroni correction. One-way ANOVAs were used to compare the results of daily water consumption, bladder weight, bladder volume, and bladder wall thickness. Two-way ANOVAs were used to compare white and gray matter sparing. Where appropriate, one-way and two-way ANOVAs were followed by the Tukey post-hoc test. The Student *t* test was used to compare the results for lesion volume. A *p* value <0.05 was considered to be statistically significant. Data are reported as mean $\pm$ standard error of the mean (SEM) throughout.

Results

CpG ODN 2088 treatment ameliorates bladder dysfunction

We assessed whether the TLR9 antagonist, CpG ODN 2088, modulates urinary retention after SCI. The treatment regimen is summarized in Figure 1A. Vehicle- or CpG ODN 2088-treated, injured mice retained significantly greater amounts of urine com-

pared with vehicle-treated, uninjured mice (Fig. 1B). Urinary retention, however, was significantly reduced in CpG ODN 2088-treated, injured mice compared with vehicle-treated, injured mice by repeated measures ANOVA ($F[1,54] = 2.380$, $p < 0.001$) (Fig. 1B), indicating improvement of bladder function.

This difference between the two SCI groups first manifests on day 5 PI, is present from day 7 through 10 PI, and is consistently different from day 13 PI onward. To ensure that the changes in retained urine were not because of different levels of water consumption in each group, water intake was also measured. Mice from all groups consumed equivalent amounts of water (Fig. 1C). Consistent with the decrease in urinary retention after treatment with CpG ODN 2088, SCI-induced increases in bladder weight, bladder volume, and bladder wall thickness were also reduced after administration of the antagonist to injured mice (Fig. 1D).

CpG ODN 2088 treatment increases white matter sparing

We measured lesion volume (Fig. 2A–D) as well as spared white and gray matter (Fig. 2A, 2E) as described in Methods. While

FIG. 2. Effects of intrathecal cytidine-phosphate-guanosine oligodeoxynucleotide (CpG ODN) 2088 treatment on lesion volume and tissue sparing after spinal cord injury (SCI). (A) Photomicrograph of representative transverse spinal cord sections near the injury epicenter. Adjacent sections are immunolabeled with an anti-glial fibrillary acidic protein (GFAP) antibody (left panel) or stained with Luxol fast blue (LFB) (right panel). The white line delineates the lesion (L). Arrows point at the astrocytic scar. The black line (right panel) delineates the spared white matter. Scale bar represents 200 μm. (B) Lesion volume (anti-GFAP) on day 28 post-injury (PI) in injured mice treated with CpG ODN 2088 or vehicle; $n = 6$; $p > 0.05$ by Student *t* test. (C) Photomicrograph of representative transverse spinal cord sections near the injury epicenter (adjacent to sections in panel A) and distal to epicenter (at caudal end of spinal cord). Sections are stained with LFB and hematoxylin and eosin (H&E). The white line delineates the area of aberrant tissue (L). Scale bar represents 200 μm. (D) Lesion volume (LFB-H&E) on day 28 PI in injured mice treated with CpG ODN 2088 or vehicle; $n = 6$; $p > 0.05$ by Student *t* test. (E) Spared white matter on day 28 PI, presented as a function of distance from the epicenter (left panel). Spared gray matter on day 28 PI, presented as a function of distance from the epicenter (right panel); $n = 6$; $*p < 0.05$ by Tukey *post hoc* test, following two-way ANOVA. Ascending numbers correspond to more rostral © distances from the epicenter and descending numbers correspond to more caudal (C) distances.

neither method of assessing total lesion volume demonstrated any differences between treatment groups, there was significantly more spared white matter in CpG ODN 2088-treated injured mice compared with vehicle-treated injured mice (Fig. 2E). A two-way ANOVA did not demonstrate a main effect for treatment group (consistent with the lesion volume results), but did for spinal location ($F[10,110]=15.09$, $p<0.001$). Further, there was a significant interaction of treatment group and spinal location ($F[10,110]=6.283$, $p<0.05$). Subsequent *post hoc* analyses showed there to be significantly more sparing of white matter both at the injury epicenter and 150 mm rostral to the epicenter. The amount of spared gray matter was not significantly different across groups.

CpG ODN 2088 treatment does not alter kidney function

Because our results indicated an improvement in bladder function, we determined whether this could be from effects of CpG ODN 2088 on the kidney. Weekly urinalysis and renal histology on day 28 PI were used as indices of kidney function. We specifically assayed levels of ketones, bilirubin, protein, nitrites, leukocytes, and pH. The levels were comparable across both injury groups and the uninjured mice for all markers tested (Fig. 3A–F). In addition, no overall qualitative differences were observed in kidney histology of injured mice treated with vehicle or CpG ODN 2088 (Fig. 3G–J). Glomerular integrity was similar across all groups, and no

FIG. 3. Effects of intrathecal cytidine-phosphate-guanosine oligodeoxynucleotide (CpG ODN) 2088 treatment on kidney function and histology after spinal cord injury (SCI). (A–F) Kidney function was assessed weekly using urinalysis test strips on expressed urine. Levels of biological markers were determined by matching the color on the strip with the reference material and plotted using an ordinal scale assigned to the number of possible colors for each of the biomarkers. For A–E, a score of 1 corresponds with a negative finding, with higher numbers corresponding to greater signal strength. Maximum signal detection is indicated by a score of 2 (nitrites), 4 (bilirubin), 5 (ketones and leukocytes), and 6 (protein). The pH of the urine (F) was plotted directly. There is no significant difference in levels of ketones, bilirubin, protein, nitrites, leukocytes, or pH for all groups at all time points. Data are shown as mean $\pm$ standard error of the mean. (G) A low magnification picture of a representative kidney section obtained from a vehicle treated, uninjured mouse and stained with hematoxylin and eosin. The cortex (CX), outer medulla (OM), and inner medulla (IM) are indicated. Higher magnification pictures of hematoxylin and eosin stained kidney sections from (H) a vehicle-treated, uninjured mouse, (I) a vehicle-treated, injured mouse on day 28 PI, and (J) a CpG ODN 2088-treated, injured mouse on day 28 PI. $n=5$ mice/group. Bar represents 700 μ m in (G) and 350 μ m in (H–J).

interstitial inflammation or fibrosis was detected in any of the groups. These results are consistent with the notion that repeated intrathecal delivery of TLR9 ligands does not induce systemic effects, as indicated in our previous report.²¹

Discussion

The studies reported here show, for the first time, that intrathecal administration of a TLR9 antagonist improves recovery of bladder function and white matter sparing after a severe, acute SCI in mice. This is an important finding because recovery of urinary control is ranked as one of the highest priorities within the SCI community.^{2,33} Despite current advances in bladder management, urinary tract infections remain the most cited reasons for hospital readmissions among those living with SCI.³⁴ If translated into the clinical setting, pharmacological approaches, such as the one presented here, could potentially ameliorate bladder function and, in so doing, improve the quality of life in persons living with SCI.

Our previous investigations demonstrated that treatment with CpG ODN 2088 significantly decreases the number of CD11b-, CD45-, and CD3-positive inflammatory cells and attenuates the proinflammatory cytokine response at the epicenter after SCI.²¹ In addition, we found that spinal cord neurons express TLR9. Thus, CpG ODN 2088 could modulate the function or survival of spared neurons in the spinal cord, including those that receive information from the pontine micturition center and the bladder. This modulation could be mediated via a reduction in the inflammatory reaction and the effectors released by these cells, which can act on neurons or through direct effects of the antagonist on neurons. In addition, even a small improvement in white matter sparing, most likely from attenuation of inflammation, could result in improved functional outcomes.

In our studies, we did not find any significant effects of the treatment on total lesion volume or spared gray matter. In contrast, white matter sparing was significantly better in CpG ODN 2088 treated mice. Because recent studies indicated a strong link between increased tissue sparing and suppression of the inflammatory response, it is possible that increased spared white matter at the epicenter is because of a reduction in the inflammatory reaction.^{5–10,35}

In the context of SCI, the best-studied TLRs are TLR2 and TLR4. Investigations have led to opposing conclusions with respect to their beneficial versus detrimental roles in SCI and SCI-associated complications, however.^{4,36–38} In contrast, our earlier and current studies consistently support the notion that TLR9 antagonism after SCI is beneficial and promotes recovery of sensory and autonomic functions, as reflected by improved thermal hypersensitivity and bladder voiding, respectively.

These beneficial outcomes may reflect the most dominant effect of CpG ODN 2088, *in vivo*, after SCI. It is possible that blockade of TLR9 negatively impacts select cell populations, which cannot be ruled out without further investigations. It is likely that the role of TLR9 in SCI is complex because of the multitude of cells that express this receptor, including those that infiltrate the site of injury and cells intrinsic to the spinal cord.

Conclusion

The current study highlights the therapeutic potential of a TLR9 antagonist, CpG ODN 2088, in recovery of bladder function after SCI. This finding, taken together with our earlier report showing a reduction in injury-induced pain hypersensitivity in mice treated with CpG ODN 2088,²¹ supports the notion that central administration of TLR9 antagonists could improve some secondary complications of SCI.

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Author Disclosure Statement

No competing financial interests exist.

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