Spinal Cord (2008) 46, 39–44

www.nature.com/sc

ORIGINAL ARTICLE

Immunomodulatory effect of the purine nucleoside inosine following spinal cord contusion injury in rat

AC Conta[1,3] and DJ Stelzner[1,2]

[1]Neuroscience Program, College of Graduate Studies, SUNY Upstate Medical University, Syracuse, NY, USA and [2]Department of Cell and Developmental Biology, College of Graduate Studies, SUNY Upstate Medical University, Syracuse NY, USA

Study design: *In vivo* study using a moderate spinal cord contusion injury (SCI) model in adult rat.
Objective: To assess the immunomodulatory effects of the purine nucleoside inosine on macrophage/microglia activation at and near the lesion site and in white matter areas remote from the injury epicenter.
Setting: Department of Cell and Developmental Biology, SUNY Upstate Medical University, Syracuse, NY, USA.
Methods: Animals ($N=56$) were injured using a moderate SCI at T9–T10 spinal level and were divided into three groups, depending on treatment paradigm. Rats received either intraperitoneal or subcutaneous injections of inosine ($N=28$) or vehicle ($N=28$). Spinal cord tissue was processed for ED-1 immunoreactivity and the volume fraction of ED-1$^+$ profiles was calculated using the Cavalieri method and unbiased stereology.
Results: The volume fraction of ED-1$^+$ profiles within gray and lateral white matter regions at and around the lesion site was significantly reduced only following a twice daily-6 week treatment course, compared with vehicle controls, and white matter areas remote from the lesion were unaffected by all inosine treatment paradigms.
Conclusions: Continued subcutaneous delivery of inosine, beginning 15-min post-SCI and persisting throughout the survival period of 6 weeks exerted immunomodulatory effects at and around the lesion site.
Spinal Cord (2008) **46**, 39–44; doi:10.1038/sj.sc.3102057; published online 3 April 2007

Keywords: inosine; contusion injury; macrophage/microglia; immunomodulatory; secondary damage

Introduction

Secondary tissue damage following traumatic spinal cord contusion injury (SCI) is the consequence of a series of cellular, biochemical and molecular events, which take place along a temporal continuum, beginning as early as a few seconds and extending for as long as weeks and months following the primary insult.[1] Resident microglia that become activated after SCI as well as infiltrating immune cells like macrophages and peripheral monocytes that are recruited to the site of injury, take part in the localized secretion of inflammatory cytokines and other cytotoxic molecules.[2,3] It has been suggested that the formation of a cystic cavity that replaces the initial central necrotic region within gray matter areas, is in part fueled by this microglial/macrophage response, which peaks between 3 and 7 days post-SCI.[4] Although their numbers decrease between 7 and 14 days post-SCI, activated macrophages/microglia can still be found around the lesion site and in cord areas rostral and caudal to it, in numbers higher than those seen in non-injured controls.[3,4] White matter (WM) regions remote from the injury epicenter show a fairly consistent expression of ED-1$^+$ profiles weeks and months post-SCI, suggesting the chronic presence of phagocytes within previously reported degenerating long tracts[4–6] (present study). The molecules released by activated microglia/macrophages following SCI are capable of inducing paracrine and autocrine mechanisms that lead to increased vascular permeability and leukocyte infiltration as well as continued expression and production of additional proinflammatory cytokine mRNAs.[7,8] Free radical molecules, in particular, are believed to mediate neurodegeneration via lipid peroxidation, thus participating in bystander damage processes.[9,10]

Inosine is a naturally occurring purine nucleoside that results from the deamination of adenosine, by adenosine

Correspondence: Dr DJ Stelzner, Department of Cell and Developmental Biology, College of Graduate Studies, SUNY Upstate Medical University, Syracuse, NY 13210, USA.
E-mail: stelzned@upstate.edu
[3]Current address: Department Neurobiology and Anatomy, Drexel University College of Medicine, 2900 W. Queen Lane, Philadelphia, PA 19129, USA.
Received 1 December 2006; revised 3 February 2007; accepted 11 February 2007; published online 3 April 2007

deaminase, and is released from cells in response to metabolic stress. The immunomodulating effects of inosine have been confirmed in a variety of *in vitro* injury paradigms.[11,12] *In vivo*, the immunomodulating properties of inosine were studied in a number of injury paradigms outside the central nervous system (CNS), including animal models for endotoxin-induced shock, acute lung injury, septic shock, colitis and arthritis,[11,12] and within the CNS, in animal models of multiple sclerosis, optic nerve axotomy, SCI and stroke.[13–16]

In this study, we investigated the effects of intraperitoneal and/or subcutaneous inosine administration following moderate (25-mm weight drop) SCI in rats. In light of inosine's reported anti-inflammatory effects,[17] we specifically analyzed the effects of this molecule on the macrophage/microglial response.

Materials and methods

Adult, Long–Evans female rats (Simonsen Laboratories, Gilroy, CA, USA, $n=56$) were used for this study. All procedures were conducted in compliance with protocols for animal care approved by the Department of Laboratory Animal Resources and the Committee for the Humane Use of Animals at SUNY Upstate Medical University and Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) guidelines. Animals were housed with exposure to a 12:12-h light/dark cycle. Food and water were provided *ad libitum*.

Surgical procedures

Spinal cord contusion injuries. After anesthesia induction with sodium pentobarbital (Nembutal, intraperitoneally 0.1 ml/100 g body weight), a laminectomy was made at the T9–T10 vertebral level, exposing dura, followed by a 25-mm contusion injury using the NYU IMPACTOR device and MASCIS protocols.[18] Rats were given Cefazolin (0.02 cc subcutaneously) twice daily for the first postoperative week as a prophylactic measure to prevent bladder infections. Bladders were expressed twice daily until rats recovered the urinary reflex. Animals were kept alive for 6 weeks following SCI.

Animal groups and treatment

Animals were randomly assigned to inosine (Sigma, St Louis, MO, USA; 100 mg/kg in 0.9% sterile saline; $N=28$) or vehicle (0.9% sterile saline; $N=28$)-treated groups and received inosine/saline either intraperitoneally (twice daily for 1 week, during the third week ($N=10$/group) or the first week, beginning 15-min post-SCI; $N=10$/group) or subcutaneously (twice daily, every day for 6 weeks, beginning 15-min post-SCI; $N=8$/group). We chose the subcutaneous route for chronic drug delivery to minimize peritoneal irritation caused by repeated intraperitoneal injections. Such irritation led to increased hindlimb spasticity in pilot studies beginning after the first full week of injections. Subcutaneous injections are unlikely to affect the kinetics of drug delivery; inosine should be present at the SCI site for a similar overall time course as for intraperitoneal injections (A Conta and O Brown, personal communication).

Histology and immunohistochemistry

At the end of the survival period, animals were deeply anesthetized and transcardially perfused with phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4). Spinal cords were dissected, post-fixed, cryoprotected and embedded in OCT embedding medium (Tissue-Tek, Sakura Finetek USA Inc., Torrance, CA, USA). Tissue blocks were cut on a cryostat at 20 μm in the transverse plane, except for the 1-cm cord segments centered at the lesion site, which were cut in either the transverse or the horizontal plane. Tissue sections were serially mounted on Superfrost Plus slides (Fisher Scientific, www.fishersci.com). In all, 10 series were collected, and any given series contained sections separated by 200 μm.

ED-1 immunofluorescence. A series of slides was processed for immunofluorescent detection of ED-1/CD68.[3] Following pretreatment with 0.5% cupric sulfate ($CuSO_4$; Sigma) in ammonium acetate buffer (50 mM, pH 5.0; Sigma) to attenuate endogenous lesion-induced autofluorescence, sections were incubated in block (4% normal goat serum and 0.5% bovine serum albumin in 0.5 M Tris-buffered saline) for 2 h at room temperature, followed by primary antibody (1:800 mouse anti-ED-1/CD68; Serotec, Raleigh, NC, USA) diluted in blocking solution, for 24 h at 4°C. Mouse anti-ED-1 was detected with a goat anti-mouse fluorescein isothiocyanate secondary antibody (1:50; Zymed, South San Francisco, CA, USA). Sections were coverslipped with Vectashield (Vector Laboratories, Burlingame, CA, USA).

Data acquisition and image analysis

The volume fraction of ED-1-positive profiles was determined on transverse and horizontal cord sections using the Cavalieri method and unbiased stereology. Briefly, transverse sections throughout high cervical, cervical enlargement, high-mid thoracic, as well as transverse and horizontal sections through the lesion-containing segment were all analyzed for ED-1 immunoreactivity. Three to five sections per animal were randomly and systematically chosen for volume fraction analysis. This procedure is detailed in Figure 1.

All data were analyzed in Microsoft Excel 2004 for Mac Version 11.2 and Microsoft Excel 2000 for Microsoft Windows 2000 Professional, using one-way analysis of variance (ANOVA) and the Student–Newman–Keuls *post hoc* test. Significance was set at the 95% confidence interval. In all graphs, error bars are standard error of means.

'We certify that all applicable institutional and governmental regulations concerning the ethical use of animals were followed during the course of this research.'

Results

Chronic inosine treatment reduces macrophage/microglia infiltration at the SCI lesion site

Intraperitoneal administration of inosine for 1 week, either during the third week or the first week post-SCI, did not

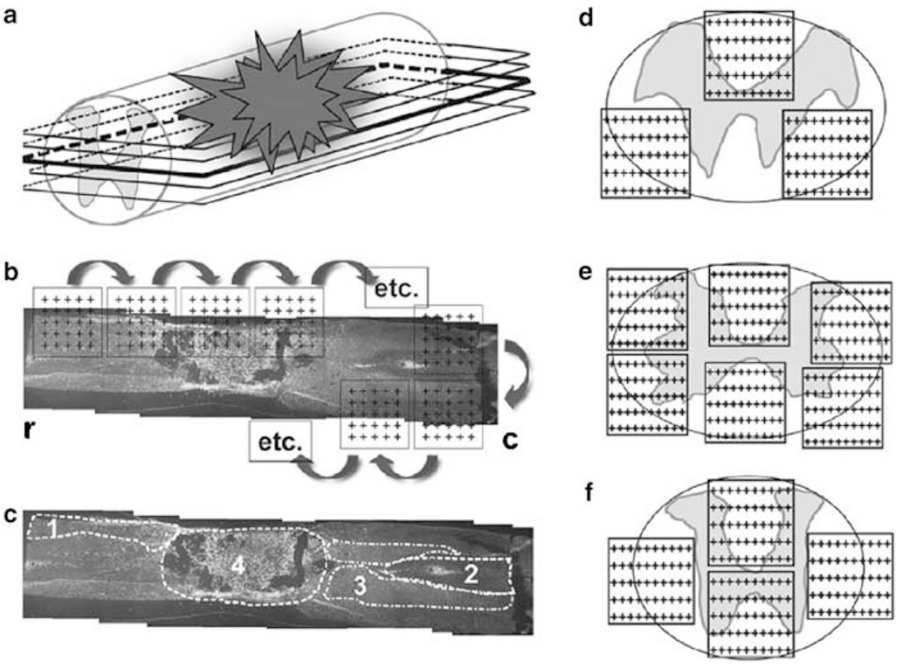

Figure 1 Schematic of stereological sampling in different cord regions at and rostral to the lesion site. (**a**) Cartoon illustrating the serial collection of sections through the lesion site segment that were cut in the horizontal plane at 20 μm. For stereological analysis of ED-1$^+$ profiles, only sections taken from the center of the segment (bolded lines) were used, to ensure sampling from similar cord regions across all cases. (**b**) Volume of ED-1$^+$ profiles at and around the lesion epicenter was determined using unbiased stereology, by randomly placing a 1-mm^2 point grid over horizontally sectioned 1-cm long cord segments centered at the lesion epicenter. Each region where the grid was placed constituted a field. To ensure sampling from the entire section, the grid was moved from field to field; in all, anywhere from 11 to 20 fields were analyzed per section (between three and five sections per animal). Within each field, the total number of points (reference points) and the number of points falling on ED-1$^+$ profiles (points of interest) were recorded, thus obtaining reference area and area of interest. Reference volumes and volumes of interest were calculated using the Cavalieri method and the following equation: $V = T * a/p * \sum P_i$, where T is the average thickness of the sections (20 μm), a/p is the area associated with each point on the grid (100 μm^2) and $\sum P_i$ is the sum of the number of points that fall on the object within a field on the ith section. (**c**) Sampling on horizontal cord sections containing the lesion site was carried out within discrete cord compartments, delineated with dotted lines, including lateral white matter (1; LWM), dorsal funiculus (2; DF), gray matter (3; GM) and lesion site proper (4; LS). (**d**–**f**) Cartoons showing grid placement for stereological assessment of ED-1$^+$ profiles in long tracts within WM regions remote from the lesion site. We calculated the volume fraction of ED-1$^+$ profiles in high cervical (**d**), cervical enlargement (**e**) and mid-thoracic (**f**) cord levels, by placing the grid on defined WM regions (gracile fasciculus, right and left dorsolateral WM, and right and left ventrolateral WM, LWM – for thoracic levels – and ventral WM).

significantly reduce the volume fraction of ED-1$^+$ profiles, compared with saline controls. This finding was the same across all cord compartments analyzed (Figure 2a and b; $P>0.05$). Conversely, chronic repeated subcutaneous administration of inosine over the course of the entire survival period significantly reduced the volume fraction of ED-1$^+$ profiles within lateral WM and gray matter cord compartments around the lesion epicenter, compared with saline controls (Figure 2c; $P<0.05$, one-way ANOVA and Student–Neumann–Keuls *post hoc* test).

Inosine treatment does not reduce macrophage/microglia reactivity in remote WM regions rostral to the lesion site

We assessed the location and volume fraction of ED-1$^+$ profiles in WM areas remote from the lesion site (i.e. high cervical, cervical enlargement and mid-thoracic spinal cord; see Materials and methods and Figure 1 for details). Profiles positive for ED-1 were generally rounded in shape and had a foamy appearance that was indicative of phagocytic and reactive macrophages/microglia (Figure 3a). These ED-1$^+$ profiles could be seen in clearly defined WM regions spanning the length of the spinal cord rostral to the lesion epicenter. These specific WM areas contain descending and ascending axonal tracts that are differentially damaged following SCI. In mid-thoracic cord, we found wedge-shaped foci of ED-1$^+$ profiles in the gracile fasciculus as well as in dorsolateral, lateral and ventrolateral, ventral and ventromedial WM regions (Figure 3c–f). At levels of the cervical enlargement, we found a similar distribution (Figure 3b). High cervical cord levels had less ED-1 immunoreactivity, overall. The only obvious foci were circumscribed to the gracile fasciculus wedge and ventrolateral portions of the WM.

We found no significant decrease in the volume fraction of ED-1$^+$ profiles within these discrete WM areas following inosine treatment, compared with vehicle controls ($P\geqslant0.05$ at all spinal levels and WM regions analyzed), regardless of treatment paradigm and cord level analyzed (data not shown). We found that macrophage/microglia above baseline levels of reactivity were evident at longer time points than those documented previously,[4] up to 42 days post-SCI.

Discussion

Treatment paradigm rationale

In this study, we assessed the effects of inosine following moderate SCI in three different treatment paradigms. In the first paradigm, animals received inosine or saline intraperitoneally twice daily for 1 week, during the third week post-SCI ($N=10$/group). The rationale for this approach addresses the possibility that inosine may be capable of exerting immunomodulatory effects at subacute/chronic times following injury, particularly in light of evidence showing the presence of ongoing inflammatory and cytotoxic processes, not only at the lesion site, but also in cord regions far from the lesion epicenter, even weeks after the initial trauma.[2,4–6] In the second paradigm, animals received inosine or saline intraperitoneally for 1 week during the first week, beginning 15-min post-SCI and twice daily thereafter, for 7 days ($N=10$/group). In the third paradigm, animals received inosine or saline subcutaneously for the duration of the survival period, 6 weeks, beginning 15-min after surgery and continuing twice daily thereafter, for 42 days ($N=8$/group). The rationale for treatment paradigms two and three takes into consideration previously reported anti-inflammatory and antioxidant properties of inosine and its potential involvement in 'early' secondary damage mechanisms known to occur within hours following traumatic injury to the CNS.[4] In such situations, inosine would be most beneficial if given during a time that includes this acute period. As the purpose of this study was to assess the effects of inosine following SCI in a minimally invasive therapeutic context, we elected not to deliver inosine directly into the spinal cord via intrathecal or intraspinal methods, at this juncture. Such an invasive approach would warrant additional controls, which are beyond the scope of this study.

Twice daily delivery of inosine over the entire 6-week period post-SCI was the only treatment paradigm capable of significantly reducing the volume fraction of tissue occupied by activated macrophage/microglia around the lesion epicenter. This finding indicates inosine is able to exert immunomodulatory functions following contusion lesions in adult rat. The fact that only immediate/acute and continual delivery of inosine led to a significant reduction in volume fraction of ED-1^+ profiles suggests that this molecule's immunomodulatory effects are on one or more 'early' secondary damage mechanisms following SCI. It is also possible that the immunomodulatory effect of inosine is only temporary, and a delay between treatment and analysis masked any positive effects of inosine in the short-term treatment groups. Additional studies are needed to address this possibility.

Mechanisms of action of inosine

Although inosine acts mainly in a receptor-mediated fashion, via the A3 adenosine receptor[12] it has also been shown to enter the cell via facilitated diffusion, and carry out its functions intracellularly.[12] It is likely, however, that a large

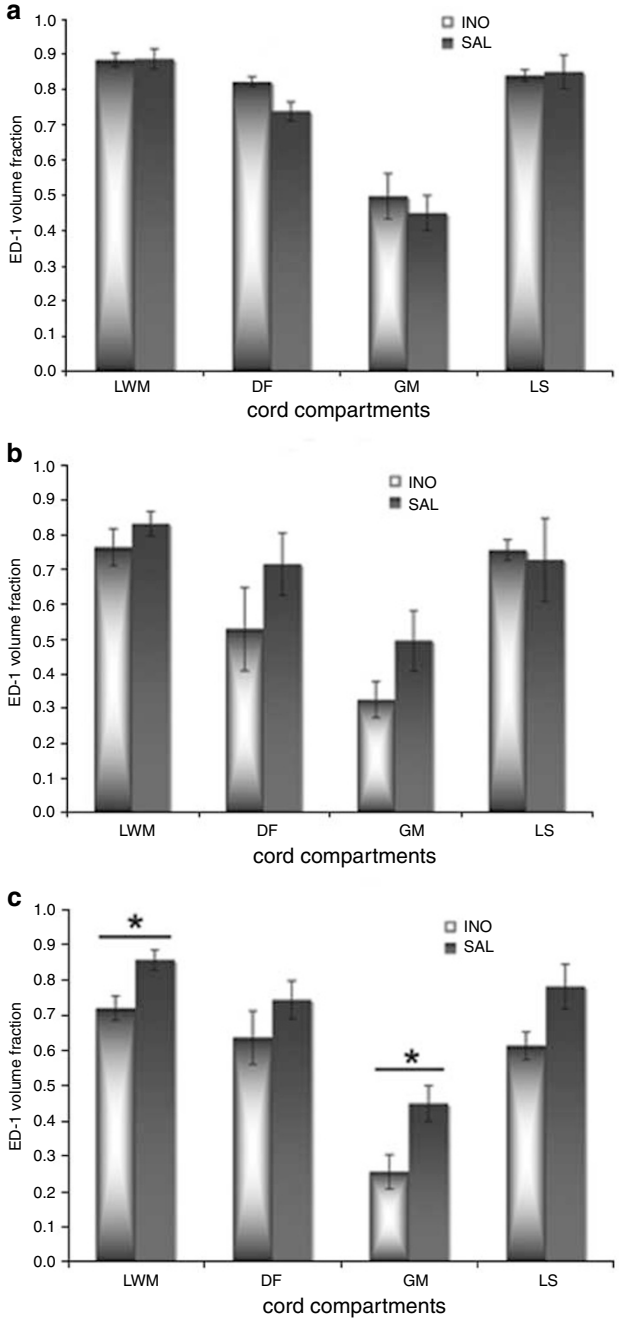

Figure 2 Volume fraction of ED-1^+ profiles within specific cord compartments at and around the lesion epicenter. The volume fraction of phagocytic macrophages/microglia is decreased in gray and WM areas at and adjacent to the lesion site, in animals that received inosine (100 mg/kg subcutaneously, $2\times$/day for 6 weeks) compared with vehicle controls and to the third week and first week treatment paradigms. (**a**) Mean volume fractions of ED-1^+ profiles in discrete cord compartments at and around the lesion epicenter following intraperitoneal inosine or saline administration during the third week post-SCI. (**b**) Mean volume fractions of ED-1^+ profiles in inosine vs saline groups treated during the first week post-SCI. (**c**) Mean volume fractions of ED-1^+ profiles in individual cord compartments at and around the lesion epicenter following inosine treatment for 6 weeks. When compared with vehicle controls, there was a statistically significant decrease in phagocytic macrophages/microglia (*$P=0.05$, one-way ANOVA) in gray and WM areas at and adjacent to the lesion. Error bars are s.e.m. LWM, lateral white matter; DF, dorsal funiculus; GM, gray matter; LS, lesion site.

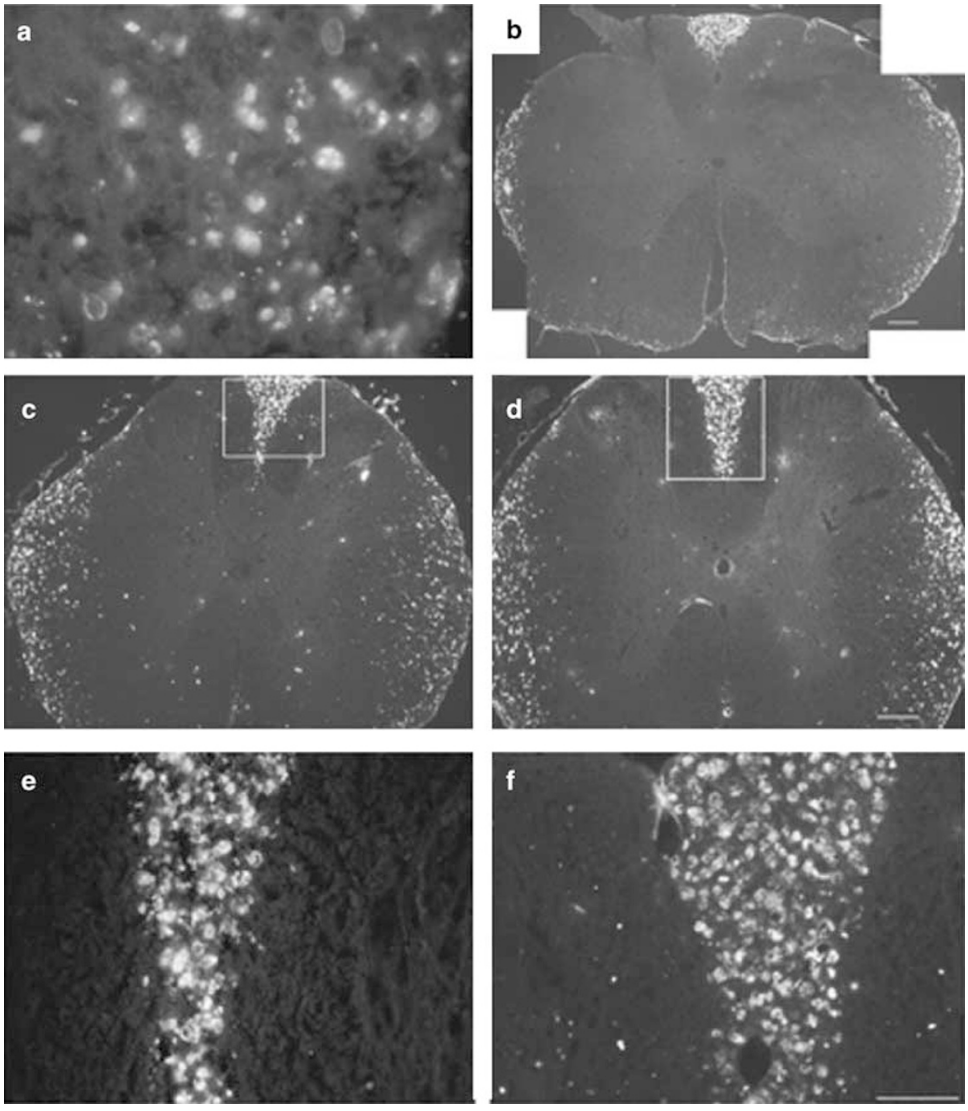

Figure 3 Pattern of labeling of ED-1$^+$ profiles within WM regions remote from the lesion epicenter. (**a**) High-power ($\times$400) representative image showing the rounded and foamy morphology of ED-1$^+$ profiles, characteristic of reactive microglia and/or phagocytic macrophages found within WM regions in areas remote from the lesion epicenter, following moderate SCI. (**b**) Representative fluorescent photomicrograph of a cross-section through the cervical enlargement that was processed for ED-1 immunofluorescence. ED-1$^+$ profiles can be appreciated within the gracile fasciculus as well as in lateral and ventral WM tracts. Image taken at $\times$100. (**c** and **d**) Representative images in cross-section taken from mid-thoracic cord levels from an inosine-treated (**c**) and a saline-treated (**d**) animal showing foci of ED-1 immunoreactivity within discrete WM regions. Images were taken at $\times$50. (**e** and **f**) Higher magnification images of boxed areas in **c** and **d**, respectively, showing ED-1$^+$ profiles with a rounded, foamy morphology within the gracile fasciculus in thoracic cord. Images were taken at $\times$200. Scale bars in (**b**, **d** and **f**) = 100 μm.

part of the neuroprotective effects of inosine in CNS disease and/or traumatic brain and SCI models are due to inosine's antioxidant properties, by virtue of inosine being metabolized to uric acid (UA).[19] UA is considered a potent peroxinitrite scavenger shown to be neuroprotective during oxidative stress conditions both *in vitro* and *in vivo*.[16,19]

Inosine has been used extensively in humans, having been marketed for a number of years as an energy supplement, despite little evidence to support this claim.[12,19] Inosinic acid, the 5′-monophosphate of inosine, is used as a flavor enhancer, and is consumed extensively by humans.[19] The only known side effect of inosine administration in humans is a significant increase in UA levels, resulting from the normal metabolism of inosine to ribose 1 phosphate and hypoxanthine; hypoxanthine in turn is further metabolized to UA and allantonin (UA being the end product in this metabolic chain). High UA levels appear to be beneficial in multiple sclerosis cases, where UA concentrations are abnormally low.[10,19] Further, it has been hypothesized that abnormally low levels of UA could be considered a risk factor for other neurodegenerative conditions like Alzheimer's and Parkinson's disease.[16]

Inosine and the microglial/macrophage response to SCI

The chronic presence of phagocytic microglia/macrophages within the spinal cord lesion site and within WM tracts far

from the lesion has been well documented.[4,20] Such activated immune cell foci provide a continual source of inflammatory and cytotoxic molecules that are believed to partly account for the apoptotic cell death of neurons and oligodendrocites following SCI.[5,6,20] Enhanced ED-1 immunoreactivity is usually directly correlated with the increased production of reactive microglia and infiltration of phagocytic macrophages. Our finding that inosine is capable of significantly reducing the volume fraction of ED-1$^+$ profiles within gray and lateral WM at and around the lesion, following moderate SCI, suggests that one potential mechanism of action of inosine, or its metabolic breakdown product UA, is to curb the infiltration and accumulation of phagocytic macrophages and reactive microglia at the site of injury, supporting reports by others.[10,19] Our observation that the volume fraction of ED-1$^+$ profiles is reduced in gray matter regions at the lesion site is important in light of the fact that reactive microglia and phagocytic macrophages have been implicated in the process of cavitation that eventually replaces the necrotic regions observed within gray matter after SCI.[4] Because inosine is broken down to UA and allantonin relatively quickly following peripheral administration, by 15 min in rodents (AC Conta and DC Hooper, personal communication), it is very likely that the immunomodulatory effects of inosine we report in this study are the result of inosine's breakdown product, UA and its potent-free radical scavenger properties. Additional studies are needed to specifically assess this possibility.

The present findings are important because they are the first, to our knowledge, to assess the effects of inosine treatment beginning after experimental SCI induction and over a time course longer than 2 weeks. In fact, inosine treatment following moderate SCI appeared to have a beneficial effect after moderate contusion lesion only when administered beginning 15-min post-injury and twice daily thereafter over the entire 6-week time course of this experiment. These findings emphasize the chronic nature of secondary injury processes after SCI and the need for more effective and long-lasting interventions.

Acknowledgements

This work was supported by The New York State Spinal Cord Injury Research Trust Fund, Contract Number C020931, and a Biomedical Sciences Grant from SUNY Upstate Medical University.

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