Blood Glutamate Scavenger as a Novel Neuroprotective Treatment in Spinal Cord Injury

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Abstract

Neurotrauma causes immediate elevation of extracellular glutamate (Glu) levels, which creates excitotoxicity and facilitates inflammation, glial scar formation, and consequently neuronal death. Finding factors that reduce the inflammatory response and glial scar formation, and increase neuronal survival and neurite outgrowth, are of major importance for improving the outcome after spinal cord injury (SCI). In the present study, we evaluated a new treatment aiming to remove central nervous system (CNS) Glu into the systemic blood circulation by intravenous (IV) administration of blood Glu scavengers (BGS) such as the enzyme recombinant glutamate-oxaloacetate transaminase 1 (rGOT1) and its co-substrate. In this study we induced in mice an SCI (hemisection), and 1 h post-injury started administering BGS treatment for 5 consecutive days. The treatment reduced the expression levels of p-p38, which regulates apoptosis and increased the expression of p-Akt, which mediates cell survival. Moreover, this treatment decreased pro-inflammatory cytokine expression and microglia activation, reduced astrocytes’ reactivity, and facilitated expression of radial glia markers such as Pax6 and nestin. BGS treatment increased the survival of neurons at lesion site and enabled axonal regeneration into the injury site. These effects were correlated with improved functional recovery of the left paretic hindlimb. Thus, early pharmacological intervention with BGS following SCI may be neuroprotective and create a pro-regenerative environment by modulating glia cell response. In light of our results, the availability of the method to remove excess Glu from CNS without the need to deliver drugs across the blood–brain barrier (BBB) and with minimal or no adverse effects may provide a major therapeutic asset.

Keywords: blood glutamate scavenger; cytotoxicity; glial scar; neuroprotection; spinal cord injury

Introduction

Spinal cord injury (SCI) usually results in devastating lifelong functional impairment. The mechanical trauma causes ischemic damage that leads to a cascade of events including cell death and inflammatory cell infiltration. The ongoing cellular necrosis increases extracellular glutamate (Glu) levels, which creates a cytotoxic post-injury environment and facilitates inflammation, glial scar formation, and neuronal cell death. Large increase in extracellular Glu occurs shortly after SCI occurs; this elevated level may persist for days, depending on the severity of the injury.1–3 Therefore, reducing central nervous system (CNS) extracellular Glu levels may inhibit the anti-regenerative events and may significantly improve recovery processes after neurotrauma.

On top of the primary response to the injury, the secondary response includes astrocyte reactivity, very early following SCI, which remains chronic in a reactive state.4 Close to the injury center, a large portion of the astrocyte population also proliferates.5–7 Glu transporters play a central role in regulating extracellular Glu levels; they are almost exclusively expressed by astrocytes and account for ~90% of functional uptake of extracellular Glu.8 Glu transporters are also located on endothelial cells of brain capillaries.9,10 However, the presence of Glu transporters on brain capillaries has been largely ignored even though the existence of a brain-to-blood efflux of Glu was discovered over 50 years ago.11 When Glu accumulates in the endothelial cells to a concentration that exceeds plasma levels, it is moved via facilitated diffusion through the luminal side into the bloodstream. It has been reported that in pathological conditions associated with a large increase of Glu in brain tissue (such as ischemia), Glu diffuses from the extracellular space to the blood following a gradient of concentration, which could explain the increase of systemic Glu levels observed in these diseases.12 To date, strategies to reduce excitotoxicity have focused primarily on blocking Glu receptors or...
reducing the release of Glu from pre-synaptic terminals. These approaches have led to acute success in the laboratory, but have failed to translate into viable therapeutic applications in human clinical trials.\textsuperscript{13,14} This failure to translate laboratory findings to clinical success is due in part to unacceptable adverse side effects related to disruption of Glu signaling necessary for normal cellular communication. Therefore, we have developed an alternative strategy in which excess Glu is removed from the CNS into the systemic blood circulation following administration of blood Glu scavenger (BGS) such as the blood resident enzyme glutamate-oxaloacetate transaminase 1 (GOT1) and its co-substrate oxaloacetic acid (OxAc).\textsuperscript{15-18} BGS causes the transamination of Glu into 2-ketoglutarate. As a result of which a decrease in blood Glu levels takes place that increases the driving force for the efflux of the excess, deleterious Glu from the CNS into the blood. This concept has been proven using a rat model, where only animals whose blood Glu levels were reduced as a result of treatment with OxAc showed an efflux of radiolabeled Glu from the brain into the blood.\textsuperscript{17} Moreover, previous studies, including our own, have confirmed the efficiency of this novel neuroprotective treatment in paraxon intoxication, stroke, and traumatic brain injury (TBI) models by real-time monitoring of the reduction of the excess brain Glu levels following intravenous (IV) administration of BGS.\textsuperscript{15,16,19-21} For example, in the MCAO rat model single IV administration of rGOT1 0.13mg/kg in combination with 1.5 mM OxAc 80 min or 2 h after reperfusion induced a reduction in serum and brain Glu levels, resulting in a reduction in infarct volume and sensorimotor deficit.\textsuperscript{16} Similarly, in paraxon intoxication, rat model IV infusion of rGOT1 0.15 mg/kg in combination with 1.7 mM OxAc, 30 min post paraxon exposure, was able to rescue neurons in the piriform cortex of the treated rats. These data could provide proof of concept of the effectiveness of rGOT1/OxAc as a neuroprotective treatment for several neurotrauma models, including SCI. In this study, the therapeutic dose and therapeutic window were selected based on the studies described above.

In the present study, we examined the neuroprotective effect of BGS, using SCI on inflammatory signaling, secretion of pro-inflammatory cytokines, glial scar formation, neuronal survival, long-term axonal regeneration, and functional recovery.

Methods

Mice

Adult (2 months) male and female C57BL/6 mice were used. All experiments were conducted according to the Guidelines for the Use of Experimental Animals of the European Community approved by the Animal Care Committees of Tel Aviv University.

Spinal cord hemisections

As previously described,\textsuperscript{22} mice (20–30 g) were anaesthetized with isoflurane (5% for induction, 1–2% for maintenance) mixed with oxygen (1 L/min) and delivered through a nasal mask. The spinal cord was exposed at the low-thoracic to high-lumbar area. After laminectomy a complete left hemisection was made at T12 and the overlying muscle and skin were sutured. Mice were randomly assigned to the control phosphate buffered saline (PBS) or rGOT1/OxAc injection groups and allowed to survive for 2 h to 8 weeks post-injury.

Blood glutamate scavenger (BGS) treatment

The rat rGOT1 complementary DNA (cDNA) was cloned into the pET28 vector, transformed into Eschericia coli BL21(DE3) bacteria, and protein expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG; 0.2 mM). Next, the recombinant His-tagged rGOT1 protein was purified by Ni-agarose chromatography, and concentrated using a VivaSpin\textsuperscript{®} 20 centri-fuge. The quality and purity of the purified rGOT1 protein was assessed on SDS-PAGE, and quantified using a spectrophotometer (NanoVue, GE Healthcare).

One-hour post-injury, the treated group was injected via IV with a priming dose of 0.2 mg/kg of rGOT1 in combination with 0.03M OxAc (Sigma) followed by a daily subcutaneous (SC) injection of the same dose in 200 μL PBS. The pH of OxAc was adjusted to 7.0–7.4 using 0.5 M NaOH solution. The control group was injected via IV followed by a daily SC injection with 200 μL PBS (vehicle). Both groups were injected for the 5 consecutive days in the long-term experiments and for 1 or 2 days in the short-term ones. To examine pro-inflammatory cytokine increase by quantitative polymerase chain reaction (qPCR), we performed time course for the first injection of rGOT1/OxAc starting at h, 4 h, and 24 h post-SCI. In addition, only for this experiment we also performed dose response, using half-dose (0.5 dose; as indicated in Fig. 1A) of rGOT1/OxAc at 1 h post-SCI.

Blood glutamate determination

Two hundred microliters of mouse blood were collected 60 min after BGS IV injection. The blood was added to an ice-cold Eppendorf tube containing 200μl 1 mol/L perchloric acid and centrifuged at 10,000g for 10 min. The supernatant was collected and pH was adjusted to 7.2 with 2 mol/L carbonic acid and centrifuged again at 10,000g for 10 min. The supernatant was stored at –80°C until analysis. Glu concentrations were measured using the fluorometric method of Graham and Aprison.\textsuperscript{23} Briefly, 60 μL duplicated blood samples were added to a 90-μL solution containing 0.3 mol/L glycine, 0.25 mol/L hydrazine hydrate buffer adjusted to pH 8.6 with 0.5M H2SO4, and 7.5 U of Glu dehydrogenase in 0.2 mmol/L nicotinamide adenine dinucleotide. After incubation for 30–45 min at room temperature, the fluorescence was measured at 460 nm with excitation at 350 nm, and its concentration was determined using a Glu standard curve in the 0–60 mmol/L range.

Immunohistochemistry

Cryostat longitudinal sections (20 μm) of fixed frozen tissue were stained using standard immunohistochemistry. Primary antibodies: rabbit anti-glial fibrillary acidic protein (GFAP; 1:1000, Dako); mouse anti-GFAP (1:1000; Invitrogen), mouse anti-chondroitin sulfate proteoglycan (CSPG; 1:200; Sigma); rabbit anti-Ib1 (1:400; Abcam); rabbit anti-Ki67 (1:400; Thermo); rabbit anti Pax6 (1:400; Covance); and mouse anti-NeuN (1:1000; Millipore). Secondary antibodies: Alexa Fluor 488 or 568; 1:1000 (Invitrogen). Nuclei were visualized with DAPI (Sigma).

For GFAP and Ib1 density, CSPG intensity and lesion size, a series of 20-μm-thick longitudinal sections were cut. For each measurement, sections were taken at 200-μm intervals. Tissue included white and gray matter (15 sections per animal for GFAP and Ib1 staining, and 10 sections for CSPG staining; n = 6/group). DAPI immunofluorescence staining was used to define the edge of the lesion size and was calculated as comparable to control-vehicle treated SCI. All measurements were performed using Image J.

Lysate preparation and immunoblot

For protein analysis, 2 h after SCI spinal cords (3 mm from each side of the lesion) were homogenized using a manual homogenizer (Fisher Scientific Med 11-850-56) in lysis buffer. For immunoblot analysis, equal amounts of protein from each sample were loaded and resolved by SDS-polyacrylamide gel electrophoresis through 7.5–10% gels. The gels were electrophoretically transferred to a
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Blood glutamate (Glu) levels 1 h after BGS injection is significantly reduced compared with vehicle-control (n = 5 control, n = 6 rGOT1), results are mean ± standard error of the mean (SEM) (**p = 2.85402E-05, two-tailed t test, 95% confidence). (B) Two hours after SCI, p38 phosphorylation is significantly downregulated and Akt phosphorylation is upregulated in BGS-treated compared with vehicle-treated mice. Graphs show quantification of the results presented in western blot. Results are mean ± standard deviation (*p = 0.027483, **p = 0.003429; one way analysis of variance [ANOVA] followed by the Bonferroni’s multiple comparison test; n = 4 Intact group, n = 6 animals in each SCI group, untreated and treated).

Anterograde axonal tracing

Axonal regeneration was examined using anterograde tracing (vehicle control, n = 6; BGS, n = 7). Seven weeks after SCI, tetramethylrhodamine dextran (TMRD; “Fluoro-Ruby,” MW 10 000 kD; Molecular Probes) was injected into the spinal cord at the level of the lesion. To visualize the regenerating axons, membranes were blocked, and blotted with the corresponding primary antibodies followed by secondary antibody linked to horseradish peroxidase. Immunoreactive bands were detected by chemiluminescence reaction. The protein levels were quantified by a densitometry analysis of protein bands using the ImageJ software. p-p38 and p-Akt were compared with β-actin and Akt levels on stripped membrane/sec. Primary antibodies were rabbit anti-p-Akt (1:1000; Cell signaling), rabbit anti-p-p38 (1:1000; Cell signaling), mouse anti-β-actin (1:10000; MP Biomedical), and rabbit anti-Akt (1:1000; Cell signaling).

qPCR of spinal cord tissue

Mice were transcardially perfused with ice-cold PBS. Spinal cord tissues were dissected (wildtype control, n = 3, SCI, n = 6; SCI + BGS at 1 h, n = 5; SCI + half dose BGS at 1 h, n = 4; SCI + BGS at 4 h, n = 4; SCI + BGS at 12 h, n = 4) 3 mm on either side of the lesion and homogenized using a manual homogenizer (Fisher Scientific Med 11-850-56) in 1 mL TRIzol Reagent® according to manufacturer’s instructions (Invitrogen). Extracted RNA from the spinal cords was reverse transcribed into cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qPCR was performed using PerfeCTa SYBR® Green FastMix PCR Reagent according to manufacturer’s instructions (QuantaBio) in the 7300 Real-Time PCR System (Applied Biosystems), and analyzed the data using the 7300 System SDS RQ Study Software (Applied Biosystems). The following primers were used in the qPCR reactions: TNF-α forward, 5′-CTGTAGCCACCTGCTTCTAGCAA-3′ and reverse, 5′-CTG GCACCACTAGTGGTTGGTC-3′; IL-1β forward, 5′-ACCACCA AAGATGAAGGCT-3′ and reverse, 5′-GATACTGCTGCTGTA AAGCTTCT-3′; IL-6 forward, 5′-ATACCACTCCCCAAGACCT GTCT-3′ and reverse, 5′-CAGATTTGCACTGACACATCT-3′; iNOS forward, 5′-ACTCTGGATGAGCTCATCTTGGC-3′ and reverse, 5′-GTTCCCGAGCTCAGTACAGCCT-3′; and GAPDH forward, 5′-TCTTGTCACTGTAACGAGGCAGCT-3′ and reverse, 5′-CCATAGCGCCAAAACCGT-3′. GAPDH was used as a reference gene for normalization of relative mRNA expression.

Microscopy

Sections were imaged by fluorescence microscopy using an Axioplan Z1 (Zeiss) epifluorescence microscope. Photomicrographs (1300 × 1030 dpi) were obtained with 2.5 × and 5 × Plan-Neofluar (Zeiss, Germany) objectives, and acquired using an AxiosCam (Zeiss, Germany) digital camera using AxioVision software (v. 4.4; Zeiss, Germany), and a Nikon Eclipse Ni laser scanning confocal microscope. Images were sized using Adobe Photoshop 11 and Illustrator 14.

Behavioral analyses

Two examiners tested the mice weekly at 24 h and up to 5 weeks after SCI. The testing was done double-blinded.

Horizontal grid walking. After 2 min of free walking, missteps (normalized to total number of steps taken by the left hindlimb) were quantified. 22,24,25

Open field locomotion score. Evaluated for 3 min using the modified Basso-Beattie-Bresnahan (mBBB) scoring system of 20 points (PBS, n = 8; rGOT1/OxAc, n = 7). 26

Statistical analysis

All statistical analyses were conducted using the GraphPad Prism Program, Version 5.03 for Windows. Significance was evaluated...
using two-tailed \( t \) test with 95% confidence when comparing two parameters in data presented in Figures 2, 3, 4, and 5, and using one-way analysis of variance (ANOVA) followed by the Bonferroni’s multiple comparison test. One week after SCI. Results are mean ± standard deviation (one-way ANOVA followed by the Bonferroni’s multiple comparison test).

**FIG. 2.** Decreased inflammatory response in blood glutamate scavenging (BGS)-treated mice after spinal cord injury (SCI). (A) BGS treatment decreased cytokine messenger RNA (mRNA) 2 days after SCI as shown by quantitative polymerase chain reaction (qPCR). Decreased TNF-\( \alpha \), interleukin IL1-\( \beta \), IL6, and iNOS messenger RNA (mRNA) levels in BGS-treated mice compared with control mice (intact spinal cord \( n = 3 \), SCI \( n = 6 \), SCI + BGS at 1 h \( n = 5 \), SCI + half dose BGS at 1 h \( n = 4 \), SCI + BGS at 4 h \( n = 4 \), SCI + BGS at 24 h \( n = 4 \)). Results are mean ± standard deviation (*\( p < 0.05 \), **\( p < 0.01 \), one-way analysis of variance [ANOVA] followed by the Bonferroni’s multiple comparison test). One week after SCI. Results are mean ± standard deviation (one-way ANOVA followed by the Bonferroni’s multiple comparison test). (B) Iba1 immunostaining in control (vehicle-treated) mice compared with reduced levels in BGS-treated mice. Quantitative analysis of the reactive microglia marker Iba1 from 0.25 mm\(^2\) areas adjacent to the lesion site. Results are mean ± standard error of the mean (SEM) (**\( p = 1.03E-09 \), one way ANOVA followed by the Bonferroni’s multiple comparison test; \( n = 6 \) animals in each group). Scale bar at B = 200 \( \mu \)m.

**Results**

*Effect of glutamate scavenging on pro- and anti-apoptotic signaling following spinal cord injury*

To evaluate the peripheral blood effectiveness of the treatment with rGOT1 in combination with OxAc, to reduce blood Glu levels, we measured the Glu levels 30 min after their IV administration versus vehicle in SCI mice. Based on our previous experiments, we expected blood Glu levels to drop after the administration of rGOT1/OxAc.\(^{15}\) Indeed, blood GLU levels were significantly reduced in the BGS-treated animals (about 40%) compared with vehicle-control treated animals (Fig. 1). This result confirms that administration of BGS, in contrast to vehicle, significantly reduces blood Glu levels after SCI.

To further investigate whether the reduction in blood Glu affected the tissue pro-survival and anti-apoptotic signaling, we examined the levels of p-Akt and p-p38 expression a short time (2 h) after the treatment. p38 participates in inflammatory responses and cell fate decision. It regulates apoptosis by affecting pro-inflammatory cytokines such as TNF-\( \alpha \), interleukin (IL)-1\( \beta \), and IL-6, and contributes to the pathogenesis of SCI.\(^{27-30}\) Activation of Glu receptor has been shown to result in the suppression of PI3K/Akt activation and subsequently in the phosphorylation of p38 that induces apoptosis in neurons.\(^{31}\) To examine the protective effect of BGS treatment we next examined p38 and pAkt levels 2 h following the spinal hemisection, and 1.5 h after vehicle/BGS treatment. When compared with the vehicle-treated groups, a significant decrease in p-p38 protein expression was observed in mice following SCI and rGOT1/OxAc treatment (Fig. 1). In addition, this treatment induced significant increase in p-Akt protein expression compared with the vehicle-treated group.

Taken together these results suggest that the BGS treatment may have attenuated the primary excitotoxic effect that is seen upon induction of spinal injury and that may also decrease the secondary damage. To test this possibility we therefore aimed at examining the consequences on inflammation, gliosis, and neuronal death.

*Glutamate scavenger decreases inflammation at the lesion site*

SCI facilitates the release of pro-inflammatory mediators and cytotoxic factors by astrocytes and immune cells, which may activate nearby microglia and thus propagate the production of pro-inflammatory factors in a vicious cycle. Therefore, we examined whether treatment with BGS, at different times after SCI induction, would decrease pro-inflammatory cytokine production and
one week after SCI was significantly reduced in BGS-treated animals compared with vehicle-control (n = 6 in each group; ***p = 6.83097E-05; two-tailed t test with 95% confidence). (B) Glial fibrillary acidic protein (GFAP) density is significantly reduced in BGS-treated mice compared with control group (n = 6 in each group; *p = 0.025249 two-tailed t test with 95% confidence; on the right panel representative image of GFAP immunostaining. Quantitative analysis of the GFAP from 0.2-mm² areas adjacent to the lesion site. Results are mean ± standard error of the mean (SEM; two-tailed t test, 95% confidence; n = 6 animals in each group). Scale bar at B = 20 µm. (C) Quantitation of Ki67-positive cells at the lesion site show a significant decrease in BGS-treated compared with vehicle-treated mice (n = 5 in each group; ***p = 0.0009953; two-tailed t test with 95% confidence). (D) Representative images of lesion site of GFAP (red) and Ki67 (green) immunostaining. Quantitation of Ki67 positive that express GFAP cells at the lesion site show a significant decrease in BGS-treated compared with vehicle-treated mice (n = 6 in each group; ***p = 1.28938E-05 for GFAP positive cells and ***p = 4.21085E-05 for GFAP negative cells; one way analysis of variance [ANOVA] followed by the Bonferroni’s multiple comparison test). Scale bar in D = 50 µm. (E) Double immunostaining of chondroitin sulphate proteoglycan (CSPG; Red) and GFAP (Green) was performed on sections one week after SCI. Quantitation of CSPG density was measured from 0.2-mm² areas adjacent to the lesion site. Results are mean ± SEM (n = 6 in each group; ***p = 1.56593E-07, two-tailed t test, 95% confidence). Scale bars in E = 20 µm.

FIG. 3. Decreased glial scar formation in blood glutamate scavenging (BGS)-treated mice after spinal cord injury (SCI). One week after injury (A) lesion size was significantly reduced in BGS-treated animals compared with vehicle-control (n = 6 in each group; ***p = 6.83097E-05; two-tailed t test with 95% confidence). (B) Glial fibrillary acidic protein (GFAP) density is significantly reduced in BGS-treated mice compared with control group (n = 6 in each group; *p = 0.025249 two-tailed t test with 95% confidence; on the right panel representative image of GFAP immunostaining. Quantitative analysis of the GFAP from 0.2-mm² areas adjacent to the lesion site. Results are mean ± standard error of the mean (SEM; two-tailed t test, 95% confidence; n = 6 animals in each group). Scale bar at B = 20 µm. (C) Quantitation of Ki67-positive cells at the lesion site show a significant decrease in BGS-treated compared with vehicle-treated mice (n = 5 in each group; ***p = 0.0009953; two-tailed t test with 95% confidence). (D) Representative images of lesion site of GFAP (red) and Ki67 (green) immunostaining. Quantitation of Ki67 positive that express GFAP cells at the lesion site show a significant decrease in BGS-treated compared with vehicle-treated mice (n = 6 in each group; ***p = 1.28938E-05 for GFAP positive cells and ***p = 4.21085E-05 for GFAP negative cells; one way analysis of variance [ANOVA] followed by the Bonferroni’s multiple comparison test). Scale bar in D = 50 µm. (E) Double immunostaining of chondroitin sulphate proteoglycan (CSPG; Red) and GFAP (Green) was performed on sections one week after SCI. Quantitation of CSPG density was measured from 0.2-mm² areas adjacent to the lesion site. Results are mean ± SEM (n = 6 in each group; ***p = 1.56593E-07, two-tailed t test, 95% confidence). Scale bars in E = 20 µm.

microglia activation at the lesion site. For this aim 2 days after SCI that was followed by treatment with BGS, RNA was extracted from the various mice, and the messenger RNA (mRNA) levels of the pro-inflammatory factors were analyzed. As shown in Figure 2, the mRNA levels of TNFα, IL1-b, and IL6 were significantly decreased, whereas that of iNOS was reduced but not statistically significant in a time course and dose-dependent manner following BGS treatment. One week after SCI quantitation of Iba1 immunostaining, microglia/macrophage marker indicated that microglia density was reduced around the lesion site after BGS treatment (Fig. 3). Thus, it appears that BGS treatment mediated attenuation of inflammatory response at the lesion site. The results presented above demonstrate that early BGS treatment (1 and 4 h after SCI) with 0.2 mg/kg of rGOT1 in combination with 0.03 M OxAc decreased significantly the levels of TNFα, IL1β, and IL6 at the lesion site. Therefore, for the remainder of the experiments BGS treatment 1 h after SCI was chosen, although the window of treatment may be wider, at up to 4 h after injury.

**BGS reduces astrocyte reactivity at the lesion site**

To determine whether BGS can reduce astrocyte reactivity and scarring, we examined the levels of expression of GFAP and CSPG around the lesion site and glial cell proliferation at the lesion site. Our results demonstrate that in animals that were treated with BGS, the lesion site was significantly smaller compared with vehicle control (45.8% of the untreated control) (Fig. 3). Astrocyte reactivity was reduced at the lesion site in the BGS-treated animals, as demonstrated by the significant decreased density of GFAP expression at the borders of the lesion (Fig. 3). Quantitation of glia cell proliferation at the lesion site was performed by co-staining using the astrocytic marker GFAP, and the proliferation marker Ki67. As shown, a significant reduction in Ki67 labeled cells was observed at the lesion site in BGS-treated animals (Fig. 3; control 47.5 ± 19.9, BGS 34.1 ± 12.7). Moreover, we observed a significant lower number of cells co-expressing Ki67 and GFAP in BGS-treated compared with vehicle-control treated animals (Fig. 3; BGS 72.39% ± 15.63, con 88.7% ± 8.01). These results suggest that BGS treatment affected the extracellular environmental conditions that prevent reinforcement of proliferating glia cells from differentiating to reactive astrocytes. In addition, the CSPG secretion by reactive astrocytes at the lesion site was reduced in the BGS-treated group (Fig. 3). Taken together these results suggest that scar formation is strongly attenuated by BGS treatment.

**BGS mediates proliferation of glial progenitor cells at the lesion site**

The astrocytes response to SCI shows a spectrum of phenotypes, depending on the type, the proximity to the injury center, and to the
FIG. 4. Blood glutamate scavenging (BGS) treatment increased radial/progenitor-like cell presence at the lesion site. One week after spinal cord injury (SCI). (A) Number of Pax6 expressing cells at the lesion site in vehicle-control is significantly lower (n=6) compared with BGS-treated mice (n=6). Note the bipolar structure of the Pax6 positive cell processes (arrows) co-labeled with glial fibrillary acidic protein (GFAP). The total number of Pax6 positive cells at the lesion site in BGS-treated mice is significantly higher compared with vehicle-control (**p = 2.89E-5; one-way analysis of variance [ANOVA] followed by the Bonferroni’s multiple comparison test). The number of Pax6 positive/GFAP positive cells are significantly increased in BGS-treated mice (**p = 0.025099576; one-way ANOVA followed by the Bonferroni’s multiple comparison test). The number of Pax6 positive/GFAP negative cells are also significantly increased in BGS-treated mice (**p = 0.002734551; one-way ANOVA followed by the Bonferroni’s multiple comparison test). (B) Nestin expression is higher at the lesion of BGS-treated mice, not the bipolar structure of the cell processes (arrows). Results are mean ± SEM (n=6 in each group; ***p = 4.8416E-15, two-tailed t test, 95% confidence). Scale bars in A and B = 50 μm.

severity of the injury.32 Reactive proliferating astrocytes can de-differentiate to increase the number of radial glia at the lesion site.33,34 For example, as we showed in our previous study, the expression of Pax6, which is an important functional indicator of neurogenic radial glia,35 may be increased within the proliferating population of astrocytes after supporting pro-regenerative treatment33 compared with vehicle-control, whereas the level of Pax6 remains lower within glia cells at the lesion site. As shown in Figure 2D, we observed a higher percentage of Ki67+/GFAP- cells following BGS treatment; we thus examined Pax6 expression at the lesion site. One week after SCI, Pax6 expression was significantly higher around the lesion site in BGS-treated compared with vehicle-control mice (Fig. 4; 39.9 ± 9.7 cell/field; 11 ± 5.1, respectively), and Pax6+/GFAP- cells in BGS treatment were around 41% of the total Pax6 positive cells compared with control, where only 27% of the Pax6 positive cells were GFAP negative. This suggests that reduced Glu levels may contribute to better pro-regenerative conditions at the lesion site, and allow proliferating astrocytes to regain characteristics of neurogenic radial/progenitor glia. To confirm that the Pax6 positive cells are glia progenitor cells we examined nestin expression at the lesion site. All Pax6 positive cells were nestin positive in both, BGS and vehicle treatments, but nestin expression was significantly higher at the lesion site in BGS-treated animals (Fig. 4). These cells, expressing Pax6 may be the cells that can give rise to new neurons or bipolar glia that could support axonal regeneration, as we demonstrated in our previous studies using other treatment approaches.33

BGS is neuroprotective and decreases neuronal apoptosis at the lesion site

Animals subjected to BGS treatment following SCI, showed a dramatic increase in the number of neuronal cells at the lesion site (50% increase: from 24.17 ± 6.52 in vehicle-control to 36.38 ± 9.21 in BGS treatment), as observed when counting the number of NeuN positive cells per field, suggesting an increase neuronal survival (Fig. 5). Also, a larger number of neurons was observed in a close proximity to the lesion site (up to 500 μm) compared with vehicle-control, suggesting that BGS had a protective effect on neurons by reducing the excitotoxicity at the lesion site. This observation is further supported by co-localization of caspase-3 with NeuN positive cells (Fig. 5). As shown, less cells expressing caspase-3 at the lesion site were observed upon BGS treatment (Fig. 5). Thus, our data demonstrate that BGS treatment elicits a strong neuroprotective effect in SCI.

BGS improves axonal regeneration toward the lesion site and improves functional recovery

Because BGS treatment promoted neuronal cell survival we next examined axonal regeneration, using the anterograde tracer tetramethylrhodamine dextran (TMRD). We injected TMRD 8 weeks post-injury, at the cervical level, upstream to the lesion. Opposed to vehicle-control, we observed that in BGS-treated animals there are axons entering the lesion site (Fig. 6A–C; enlargements in i, ii, and iii). Also the treatment with BGS resulted in a significant increase in the number of axons upstream to the lesion site (Fig. 6D; 400 μm upstream to the lesion), although there were not significantly more traced axons labeled up to 1000 μm rostral to the lesion site between BGS-treated and control (Fig. 6D).

Examination of functional recovery, assessed up to 5 weeks after SCI, showed that BGS treatment improved locomotor abilities. The mBBB scores in the SCI with vehicle treatment group were significantly lower compared with those of animals that were treated with the combination of rGOT1 and OxAc for 5 weeks after the injury (Fig. 6F). Although not statistically significant, the grid walking test showed a better performance by mice treated with BGS compared with those that were treated with vehicle (Fig. 6G).

Discussion

Damage to the spinal cord has a lifelong impact on the injured individual. CI caused by external force most commonly occurs in
young adults, thus most victims live with the consequences of their injury for decades. This fact highlights the importance of finding therapeutic approaches that will improve the environmental condition of the injury site and increase the capacity for neuronal regeneration. Numerous studies over the past 40 years have consistently shown that high levels of extracellular Glu in the CNS can damage and kill neurons.1,36,37 In this study we demonstrate that decreasing Glu levels by BGS after SCI in mice results in a reduced inflammatory response and decreased astrocyte reactivity and glial scar formation. The glial scar was reduced not only by decreasing the number of astrocytes and their processes, but also by reducing pro-inflammatory cytokine and CSPG secretion at the lesion site. Moreover, this treatment promoted the formation and propagation of radial/progenitor bipolar glia cells, which can at later stages mediate better axonal regeneration toward the lesion site.

Glu transporters, especially sodium-dependent transporters like EAATs that are expressed by astrocytes, play a central role in regulating extracellular Glu levels.8 Despite this robust response that can increase the number of astrocytes, in these reactive cells Glu transporter expression is reduced following SCI, suggesting an inability to properly regulate extracellular Glu homeostasis.38 It has been shown that Glu transporters are also located on endothelial cells of brain capillaries.9,10 In the last 2 decades, accumulating data suggest the existence of an influx of excess Glu from the brain extracellular fluids into the bloodstream, whereas Glu accumulates in the endothelial cells of the BBB to a concentration that exceeds those that are measured in the blood.12,18 Blood Glu levels are mainly regulated by two enzymes, glutamate-oxaloacetate transaminase (GOT1 or AST) and glutamate-pyruvate transaminase (GPT or ALT), both enzymes are able to metabolize blood Glu and facilitate the lowering of extracellular levels of brain Glu.39 Low levels of AST and ALT enzymes may be a significant limiting factor for the brain-to-blood Glu efflux. Studies in human patients have shown that low levels of AST in the blood accompanied with high levels of blood Glu after cerebral ischemia correlates with poor neurological outcome.40,41 In contrast, higher AST and ALT levels on admission were independently associated with good functional outcome at 3 months after stroke.12 This association was stronger for AST than ALT levels. This favorable effect was also supported by positive effects on the reduction of lesion volume. In

FIG. 5. Blood glutamate scavenging (BGS) treatment is neuroprotective by reducing apoptotic neuronal death at the lesion site following spinal cord injury (SCI). One week after SCI (A,B) significantly more NeuN positive cells are observed at the lesion site compared with vehicle-control. Results are mean of NeuN positive cells in the field ± standard error of the mean (SEM; n = 6 in each group; **p = 0.002958, two-tailed t test, 95% confidence). (C) Immunostaining for active caspase-3 co-localized with NeuN (D) and increased in control compared with BGS treatment. Scale bars in A and D = 100 μm, in C = 50 μm.
FIG. 6. Blood glutamate scavenging (BGS) treatment result in axonal regeneration into the lesion site and improves motor function after spinal cord injury (SCI). Two months post-injury (A) anterograde tracing demonstrates that axons in vehicle-treated mice reach but do not enter the lesion; however, (B,C) in BGS-treated mice axons enter the lesion site. Enlarged panels (i, ii, iii) are shown of the boxed areas in A, B, and C, respectively. (D) Quantitation of traced axons in 0.2-mm² frames, 400 μm upstream to the lesion shows significantly more axons in BGS treatment. No difference in axonal number at 1-mm upstream of the lesion site between the two groups (**p<0.001, ***p<0.001; n.s. = non-significant one-way analysis of variance [ANOVA] followed by Bonferroni’s multiple comparison test, vehicle n=8; BGS n=7). (E) Confocal image of double-labeled tetramethylrhodamine dextran (TMRD; red) with GFAP (green) shows that in vehicle-control (upper panel) astrocyte processes are going in random directions and do not support traced axons, whereas in BGS (lower panel) axonal processes are located along glial fibrillary acidic protein (GFAP) processes. (F) The modified Basso-Beattie-Bresnahan (mBBB) score was measured up to 5 weeks after SCI in control (n=8) and BGS (n=7) treated mice and significantly improved in BGS treatment on week 5. (G) Walking on a grid was improved following BGS treatment. Results are mean ± standard deviation (*p<0.05; non-parametric Mann-Whitney test, α set to 5%). Scale bars in A, B, and C = 500 μm; in i, ii, iii, and E = 100 μm.
addition, previous studies have shown that a decrease in blood Glu concentration, achieved by administration of rGOT1 and co-substrate OxAc, leads to a larger natural Glu gradient between the brain and blood, facilitating the efflux of excess extracellular CNS Glu into the blood in various animal models.17–20 The concept of blood-to-brain Glu scavenging is well recognized as a novel and attractive protective strategy to reduce the excitotoxic effect of excess extracellular Glu that accumulates in the brain following various pathological conditions. Our published data on BGS technology demonstrated that this approach turned out to be extremely effective in providing neuroprotection in animal models of acute excitotoxic conditions such as TBI,21 stroke,19 and paraxoxin intoxication,32 and chronic conditions such as glioblastoma34 and amyotrophic lateral sclerosis (ALS).35 The neuroprotection was detected in terms of neurological and behavioral tests, as well as in histological analysis.

In the present study we demonstrated that a short time after SCI (2 h), treatment with rGOT1/OxAc increased pro-survival signaling, p-Akt, and decreased pro-apoptotic mediator, p-P38. This may suggest that the decreased levels of Glu at the lesion site, as a result of Glu scavenging, reduced the excitotoxicity and therefore improved the extracellular conditions at the injured area followed by reduced inflammatory response, neuronal cell death, and astrogliosis. Animals that were treated with BGS demonstrated a significantly reduced lesion size, which partially formed by decreased astrogliosis, characterized by reduced cell proliferation and GFAP expression levels. Moreover, this treatment enables the proliferating astrocytes at the lesion site to gain their progenitor cell characteristics, as we have demonstrated by increased expression of nestin and Pax6. These astrocytes that express progenitor glia markers can support axonal regeneration, as we have demonstrated also in our previous studies, using pro-regenerative treatments.5,33

Although BGS did not lead to extensive axonal regeneration through the lesion site, it resulted in some axonal regeneration through the glial scar area and significant neuronal survival close to the lesion site compared with vehicle-control treatment. Astrocytes at the lesion site in animals treated with BGS are more of a bipolar morphology rather than multi-polar morphology. This structure is more supportive for axonal regeneration as the axons are not blocked by the astrocytes processes as is evident in vehicle-control animals (Fig. 6A,E). These results may be the reason for the functional improvement in BGS-treated mice in locomotor tests we described.

The clear advantage of BGS technology is that there seems to be no direct intervention with any of the CNS Glu receptors or Glu transport systems. The administration of rGOT1/OxAc only temporarily affects the blood Glu levels and this creates a gradient between the levels of Glu in the blood and in the CNS, causing a rapid influx of excess Glu from the CNS to the blood without affecting any other CNS functions. As plasma Glu levels normally fluctuate by 50% during the circadian cycle,34 we speculate that in the transition of the BGS technology into clinical trials, no severe side effects are expected. Moreover, preliminary toxicity studies in rodents injected with BGS revealed no toxic effects at a dose up to 100-fold higher than a therapeutic dose (data not shown). Therefore, we suggest that BGS treatment can be administered to patients shortly after an accident by paramedics, which makes it the fastest available treatment for all types of neurotrauma, to decrease the initial damage at the injury site followed by lesser functional impairment in the future. Because our preliminary results at 4 h after the injury show some positive effect, further research is needed to evaluate the efficacy of a wider and more attractive clinical treatment window.

In light of our results, the availability of the method to remove excess Glu from the CNS without the need to deliver drugs across the BBB and with minimal or no adverse effects may provide a major therapeutic asset. Clearly, a treatment of SCI ought to be a complex therapy, and our technology may increase the effectiveness of the standard of care or of other emerging treatments.

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