Safety and Efficacy of Rose Bengal Derivatives for Glial Scar Ablation in Chronic Spinal Cord Injury

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Abstract

There are no effective therapies available currently to ameliorate loss of function for patients with spinal cord injuries (SCIs). In addition, proposed treatments that demonstrated functional recovery in animal models of acute SCI have failed almost invariably when applied to chronic injury models. Glial scar formation in chronic injury is a likely contributor to limitation on regeneration. We have removed existing scar tissue in chronically contused rat spinal cord using a rose Bengal-based photo ablation approach. In this study, we compared two chemically modified rose bengal derivatives to unmodified rose bengal, both confirming and expanding on our previously published report. Rats were treated with unmodified rose bengal (RB1) or rose bengal modified with hydrocarbon (RB2) or polyethylene glycol (RB3), to determine the effects on scar components and spared tissue post-treatment. Our results showed that RB1 was more efficacious than RB2, while still maintaining minimal collateral effects on spared tissue. RB3 was not taken up by the cells, likely because of its size, and therefore had no effect. Treatment with RB1 also resulted in an increase in serotonin eight days post-treatment in chronically injured spinal cords. Thus, we suggest that unmodified rose Bengal is a potent candidate agent for the development of a therapeutic strategy for scar ablation in chronic SCI.

Keywords: chronic spinal cord injury; photochemical scar ablation; rose Bengal

Introduction

THERE ARE NO EFFECTIVE clinical therapies currently to ameliorate the loss of function that occurs after spinal cord injury (SCI). In addition, although numerous research studies have demonstrated functional recovery in animal models of acute or subacute SCI, the proposed treatments have failed when tested in models of chronic injury. One factor that has been suggested to contribute to this lack of response is the formation of the glial scar that is generated in response to the cord injury.^{1,2} Initial damage to surrounding blood vessels triggers blood clotting, and activation of complement and other cytokines in the primary injury area attracts macrophages and other immune cells. Hemorrhage in the gray matter after contusion injury also activates microglia and astrocytes in the surrounding region, contributing to formation of the glial scar.

In the period after the injury, immune cells clear away debris in the central region of the cord and the secondary injury cascade leads to the formation of a multi-component fibrotic and astrocytic barrier that surrounds the cavity at the location of the primary injury.^{3–5} Hypertrophic astrocytes form a tightly intertwined layer of entangled filamentous processes around the traumatic SCI site.

These reactive astrocytes usually exhibit high expression of the intermediate filament protein glial fibrillary acidic protein (GFAP) and members of the chondroitin sulfate proteoglycan (CSPG) family.⁶ Some astrocytes, however, especially those derived from endogenous neural stem cells, may not express GFAP.⁷

The fibrotic component of the glial scar results primarily from fibroblasts that invade the site of injury as a consequence of the disruption of the blood–brain barrier and the associated inflammatory processes after trauma. These cells remain in the lesion core and produce extracellular matrix (ECM) proteins including collagen, fibronectin, and laminin, which may inhibit regenerating axons.⁸ The invading fibroblasts along with reactive astrocytes also act to re-establish the glia-pial barrier referred to as glia limitans.⁹ After SCI the glial scar serves to seclude undamaged tissue from the lesion by limiting the propagation of inflammatory processes from increasing the lesion size.¹⁰ Thus, in an acute injury phase, the glial scar has an important role, whereas in the chronic phase, the glial scar may act as both a physical and chemical barrier to axonal regrowth.¹¹

Treatment of chronically injured spinal cord in rats with chondroitinase ABC (ChABC) has been shown to digest CSPGs and can

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also enhance regeneration of axons and increase collateral sprouting by spared axons and presents one promising therapeutic strategy.^{12,13} ChABC treatment has also been shown to have a synergistic effect when combined with other regenerative strategies including mesenchymal stromal cell transplantation and treadmill rehabilitation.^{14,15} Certain disadvantages, however, have been reported for the use of ChABC, including incomplete digestion of inhibitory glycosaminoglycan side chains from lesion core proteins, short enzymatic activity half-life at body temperature, and the inability of ChABC to cross the blood–brain barrier. Bacterial ChABC may also induce an immune reaction after repeated injections.¹⁶ A further potential disadvantage is that ChABC only addresses one specific component of the scar, whereas we propose that rose bengal treatment will affect multiple components.

We hypothesize that to treat chronic SCI optimally, all components of the inhibitory glial scar layer must be removed without further damaging the surrounding spared tissue. The most effective method remains to be elucidated, however. Although attempts have been made to remove the scar physically through surgical or laser methods, this may prove to be too traumatic. Photo ablation of scar tissue after infusion of photo reactive dye into the lesion is less invasive and provides an attractive clinical alternative. Rose bengal (4,5,6,7-tetrachloro-2',4',5',7'-tetraiodofluorescein) is a commonly used vital staining histological dye and is approved by the Food and Drug Administration as a diagnostic agent for conjunctival abrasions or corruptions in routine ocular examinations. Rose bengal is locally phototoxic to cells that have taken up the dye when exposed to light. It is also an effective generator of reactive oxygen, which initiates direct peroxidation reactions within endothelial membranes.¹⁷ This property has been used to develop the use of rose bengal infusion and focal light exposure in animal models of photochromic stroke induction.^{18,19}

The utility of rose bengal for noninvasive glial scar photo ablation in a rat model of chronic SCI has been demonstrated previously

by our group.²⁰ Here we report the development and characterization of lipophilic rose bengal derivatives that might represent promising novel therapeutic approaches. We compare two chemically modified rose bengal derivatives with unmodified rose bengal and expand on our previous work with further characterization of the effects in chronic SCI.

A number of properties for molecule design were considered including polarity, diffusion, ablative properties, and solubility. These derivatives were generated by the addition of hydrophobic molecules or large inert steric conjugates with the goal of increasing the specificity of their photo-ablative action and increasing the safety profile of the technique by reduction of diffusion into spared tissue. We have investigated the effects of these three versions of rose bengal for photo ablating the glial scar of chronically injured rat spinal cords with examination of potential therapeutic safety and efficacy through characterization of the spinal cord tissue after treatment.

Methods

Rose bengal derivatives

Figure 1 shows the molecular structures of unmodified rose bengal (RB1) and two modified derivatives. The first derivative (RB2) has the addition of a decyl carbon unit analog synthesized with the purpose of slowing diffusion into spared tissue, thereby reducing further damage to adjacent healthy tissue. The second derivative (RB3) has the addition of polyethylene glycol (PEG) as an alternate diffusion retardant.

Contusion injury

Female Long-Evans adult rats weighing 200–220g were used for this study, and all protocols were approved by the Institutional Animal Care and Use Committee at the University of Minnesota. Rats were anesthetized by inhalation of isoflurane, and a laminectomy

Chemical Structure of Rose Bengal and its Derivatives

FIG. 1. Chemical structure of rose bengal and its derivatives.

was performed at the T8–T9 vertebral level. A moderate contusion injury was made with a 200 Kdyn force using the Infinite Horizon Spinal Cord impactor (IH 0400) (Precision System and Instrumentation LLC).

Scar ablation technique

All rats underwent SCI as described above. All rats then received either scar ablation treatment or saline injection six weeks after injury. Rats were anesthetized by inhalation of isoflurane, and the injury site was re-exposed. The photo ablation procedure is described in Zhang and associates.²⁰ Briefly, 1 μ L of 2% (diluted in 0.9% saline) rose bengal or derivative was injected into the cavity at the injury site through a 26-gauge blunt Hamilton metal needle connected to a Hamilton syringe. This injection was made at a depth of 0.8–1.0 mm for 1 min. Eight minutes after injection, the spinal cord was illuminated for 5 min with the full-spectrum light of a halogen bulb (150W, 7 cm distance). To prevent damage by the heating source from the halogen light, the spinal cord was bathed with saline solution during the illuminating time. Immediately after illumination, the wound was sutured.

Diffusion analysis

Six weeks after contusion injury, six rats ($n=2/\text{RB}$ group) were utilized to localize the rose bengal injected into the cavity, with the same injection technique as described above. The rats were euthanized without perfusion immediately after injection, and the spinal cord was harvested, fixed overnight in 4% paraformaldehyde, and then immersed in 30% w/v sucrose at 4°C. Spinal cord of 1 cm length including the cavity epicenter was then sectioned and examined.

Rose bengal derivatives

There were four treatment groups: Group 1—unmodified rose Bengal; Group 2—decyl carbon rose Bengal; Group 3—PEG rose Bengal; Group 4—saline only. Within each group (six rats/group), rats were allocated randomly to be sacrificed at two time points; 24 h after scar ablation ($n=3$) and eight days after scar ablation ($n=3$). These time points were selected to examine both acute and subacute changes of our treatment.

Tissue harvesting

Rats were fully anesthetized with intraperitoneal injection of ketamine hydrochloride and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4. Spinal cords were removed and post-fixed overnight in the same fixative solution, then immersed in sucrose (30% w/v) and washed with PBS. A segment of the spinal cord 1.0 cm in length encompassing the injury site was removed and embedded in Tissue-Tek OCT embedding compound (VWR, Mississauga, ON, Canada). The tissue was sectioned in the transverse plane at 20- μ m intervals using a Leica CM3050 S cryostat.

Histology and cavitation analysis

To analyze cavitation, every eighth section from the spinal cords of rats sacrificed at 24 h and 8 days for all four treatment groups was processed for hematoxylin and eosin (H&E) and Luxol Fast Blue (LFB) staining. Ten sections per rat (rostrocaudally 1.6 mm from the epicenter of the cavity) were imaged on a Leica DMI8 inverted microscope. To compare the cavity area between groups, a modified protocol that combines H&E (background) and LFB (myelin) staining was developed. The area of maximum cavitation (epicenter) of each section was traced using Image J software from Fiji (v.1.45) (National Institutes of Health [NIH]; Bethesda, MD). The

measurements obtained were then used to generate values for the cavity area for each of the cords from each treatment group ($n=3$).

Immunohistochemistry (IHC): Glial scar analysis

After transcardial perfusion, spinal cords were processed for immunohistochemical analysis. To identify effects on the components of the glial scar, serial transverse sections surrounding the lesion epicenter (every eighth section) were utilized to identify astrocytes with both GFAP and aldehyde dehydrogenase 1 family member (ALDH1), which assists in the identification of GFAP negative astrocytes. The CSPGs were also characterized, and collagen type I alpha 1 (COL1A1) was used to describe the fibroblastic component of the glial scar.

Briefly, after blocking by 0.01 M PBS containing 1% horse serum for 1 h at room temperature, the sections were incubated with primary antibodies at 4°C overnight. The primary antibodies were anti-GFAP (1:500, Dako), anti-ALDH1 (1:200, NeuroMab), anti-CSPGs (1:200, Sigma), and anti-COL1A1 (1:250, Novus Biologicals). Sections were then washed three times for 30 min in PBS. Sections were incubated with their corresponding fluorescent secondary antibody conjugated with Alexa Fluor 488 (donkey anti-mouse, donkey anti-rabbit, 1:1000), or Alexa Fluor 555 (donkey anti-goat, donkey anti-mouse, and donkey anti-rabbit 1:500) (ThermoFisher Scientific) for 1 h at room temperature. After three washes for 10 min in PBS, sections were counterstained with DAPI and coverslipped with Immu-Mount mounting medium (ThermoFisher Scientific). Images were obtained with a Leica DMI8 inverted microscope.

IHC: Spared tissue analysis

Serial transverse sections surrounding the lesion epicenter (every eighth section) were utilized to identify effects on the remainder of the injured spinal cord. Serial sections were used to identify neurons (NeuN), oligodendrocytes (adenomatous polyposis col—APC), endogenous neural stem cells (nestin), serotonergic axons (5HT), and macrophages/microglia (ED1). Briefly, after blocking by 0.01 M PBS containing 1% horse serum for 1 h at room temperature, the sections were incubated with primary antibodies at 4°C overnight. The primary antibodies were anti-NeuN (1:200, Abcam), anti-APC (1:20, Calbiochem), anti-nestin (1:100, BD Pharmagen), anti-5HT (1:7500, Immunostar), and anti-ED1 (1:250, Novus Biologicals).

Quantification

For the quantification of the immunofluorescent signal, images of sections (at epicenter) per rat were acquired on a fluorescent microscope (Leica DMI8 inverted microscope) at 10x magnification. Six random fields of view ($0.6 \times 10^2 \text{ mm}^2$ per field) per section within an area of 0.35 mm^2 around the cavity were considered for quantification. Immunoreactivity was quantified with the Image J software from Fiji (v.1.45) (NIH; Bethesda, MD) by measuring the integrated optical density (intensity of fluorescence per unit of surface area). Biological variation in relation to any differences in stain absorption of any individual section was accounted for by defining the threshold.²¹ To define the threshold (T), the number ($n=10$) of known negative and positive regions were randomly selected from three different sections from both control and treated samples. Mean intensities (u_1 and u_2) and standard deviations (σ_1 and σ_2) were obtained for negative and positive regions. Later threshold was calculated as $T = u_1 * \sigma_2 + u_2 * \sigma_1 / \sigma_1 + \sigma_2$. A different threshold was calculated for each antibody.

The average integrated density of an antibody signal was recorded per section around the lesion cavity, averaged for each rat, and compared across treatment groups. The results were expressed in arbitrary units. Quantification of NeuN was performed

by counting the number of positive cells per section in the gray matter of the spinal cord from each rat.

The sections that were selected to illustrate the various scar components in the figures were obtained from the area surrounding the epicenter of the lesion. Some biological variation is seen because they are not all from the same animal but from one of three animals in each group. These are representative sections only and the quantitative data were calculated for each rat around the entire lesion cavity.

Functional testing

Rats were evaluated using Basso, Beattie, Bresnahan (BBB) open field locomotor testing.²² Evaluation time points included immediately before and after SCI, day 1 after injury, and then weekly at days 7, 14, 21, 28 post-injury, and after scar ablation. Observers were blinded as to the treatment group.

Statistical analysis

All data are presented as mean $\pm$ standard error. BBB scores of all rats were analyzed using repeated measures analysis of variance (ANOVA). Cavitation area and all quantitative IHC data were analyzed using one-way ANOVA. The Bonferroni *post hoc* test was used to identify differences among groups. Statistical significance was determined at $p < 0.05$. All IHC quantitation, cavitation analysis, and functional testing were performed in a blinded fashion.

Results

Diffusion analysis

Six weeks after contusion injury, unmodified rose bengal (RB1) or chemically modified derivatives (RB2 and RB3) (Fig. 1) were injected into the injury cavity without photo activation. The injected animals were euthanized without perfusion immediately after injection, and the injected tissue was collected, fixed, and prepared for cryosectioning to examine diffusion of the injected compounds at the periphery of the lesion cavity. We observed that RB1 and RB2 had diffused into the injured spinal cord tissue, whereas RB3 had only minimal diffusion across the interface between the cavity and the lesioned tissue (not shown).

Histology and cavitation analysis

The histologically apparent glial scar comprises activated astrocytes and other inhibitory cells and molecules. Six weeks after injury, rose bengal or one of its derivatives was injected into the lesion site. Transverse sections surrounding the epicenter of the cavity were subjected to H&E/LFB staining, and representative images of the cavity epicenter are depicted in Figure 2. As expected, this demonstrated that the chronic lesion contains a multi-component scar that lines the entire perimeter (arrows around the lesion epicenter) of the cavity in the control (Fig. 2A, 2E) and RB3 treated spinal cords (Fig. 2D, 2H). In contrast, the arrows in Figure 2F indicate that the scar tissue was almost completely ablated in RB1 treated cords. Treatment with RB2, however, demonstrated that the glial scar lining was not completely ablated (Fig. 2C, 2G).

Cavitation analysis was then undertaken to assess any change in size of the cavity after glial scar ablation eight days post-injury (Fig. 2I). The area of the lesion was measured at the injury epicenter. No statistically significant difference in lesion area was detected between the groups examined; however, the size of the cavity area trended toward an increase in RB1 and RB2, compared with RB3 and the control group.

IHC: Glial scar analysis

The IHC was performed to determine the effects of scar ablation on components of the glial scar at 24 h and eight days post-treatment (Fig. 3 and 4). Representative images (Fig. 3A, 4A) show detection of GFAP (GFAP positive astrocytes), ALDH1 (GFAP negative astrocytes), COL1A1 (fibroblastic/meningeal component), and CSPG for each treatment group at each time point. This analysis showed that both GFAP and ALDH1 were significantly reduced at 24 h (Fig. 3B) and eight days post-treatment (Fig. 4B) after RB1 treatment compared with control ($p < 0.05$). The expression of CSPG was decreased at 24 h and eight days post-treatment with RB1 compared with control, but this did not reach statistical significance (Fig. 3B, 4B). Similarly, RB1 treatment reduced COL1A1 antigen expression at 24 h and eight days post-treatment compared with control; however, this only reached statistical significance at the 24 h time point (Fig. 3B).

Expression for glial scar components measured showed a trend toward reduction at 24 h post-treatment with RB2 compared with control, but these differences were not statistically significant (Fig. 3B). At eight days post-RB2 treatment, however, expression of both GFAP and ALDH1 were significantly decreased when compared with control ($p < 0.05$) (Fig. 4B). Similar to RB1 treatment, CSPG expression was also reduced at eight days post-treatment with RB2, compared with control, but this did not reach statistical significance. Finally, in contrast to treatment with RB1, COL1A1 expression at eight days post-treatment was not reduced compared with control after injection of RB2.

The IHC analysis demonstrated that the scar components were unchanged at both time points when treated with RB3 compared with control.

IHC: Spared tissue analysis

The IHC was performed to confirm there was no damage to spared tissue after glial scar photoablation. The following cells were examined at 24 h and eight days: NeuN, APC, and nestin. There were no differences in IHC detection for each of these antibodies ($p > 0.05$) in treated samples when compared with control treated tissue, and no significant difference was observed between tissues treated with the three versions of rose bengal ($p > 0.05$). (data not shown).

The IHC was also performed to identify 5HT (Fig. 5) and to characterize the inflammatory response involving ED1 (Fig. 6) at 24 h and eight days after photoablation. The 5HT expression was elevated in the RB1 and RB2 treated groups at 24 h after treatment (Fig. 5B), but was only significantly increased in the RB1 treated group at eight days (Fig. 5C) when compared with treatment with RB2, RB3 or the saline control ($p < 0.05$).

Expression of ED1 was increased at 24 h (Fig. 6A) after treatment with RB1 and RB2, suggesting that there was an inflammatory reaction caused by our intervention. We observed that this was significantly higher at 24 h ($p < 0.05$) and remained elevated, but no longer statistically significant, at eight days post-treatment in RB1 and RB2 treated groups, when compared with control treatment (Fig. 6B). We did not observe this phenomenon in RB3 treated rats. No difference in ED1 expression was observed between RB1 and RB2 treated rats at either time point.

Functional testing

Rats were evaluated using BBB open field locomotor testing to determine whether there were deficits or improvements after glial

FIG. 2. Cavitation analysis after photo ablation with rose bengal (RB) derivatives in a rat model of chronic spinal cord injury (SCI). (A–D) Hematoxylin and eosin/Luxol Fast Blue staining of rat spinal cord eight days post-treatment with either saline, RB1, RB2, or RB3 administered six weeks after SCI. Scale bar 100 μ m. (E–H) Corresponding images at higher power. Scale bar, 50 μ m. The images demonstrate the glial scar tissue around the cavity (arrowheads) in both control (E) and RB3 (H) treated groups. This scar was almost completely ablated in RB1 (F) and was partially ablated in RB2 (G) treated groups. (I) Comparison of cavity sizes between the four treatment groups. While RB1 and RB2 have a slightly increased cavity size, this did not reach statistical significance.

scar ablation. We did not observe any differences in motor scores between the control group and any of the treatment groups at any time point (data not shown).

Discussion

Chronic glial scar is the result of a complex network of cellular and molecular interactions within the central nervous system after injury. Brain and/or spinal cord injury results in scar tissue formation at the lesion site and creates an inhospitable environment for axonal regeneration.^{23–27} This scar consists of both glia (mainly astrocytic) and fibrotic components.²⁸ GFAP is a conventionally used astrocytic marker but only labels a limited number of astrocytes.²⁹ Therefore, along with GFAP, we included ALDH1 as a second astroglial marker that is primarily and selectively expressed in spinal cord astrocyte,³⁰ together with collagen (COL1A1), which is an important component of the fibrotic scar.

Recent literature has suggested that contrary to the idea that formation of the glial scar prevents axonal regeneration, astrocytes actually aid central nervous system axonal regeneration.³¹ In response to this controversial suggestion, however, Silver³² points

out that the glial scar consists of more than just astrocytes, and Göritz and colleagues³³ suggest that other inhibitory cell types, such as fibroblasts and pericytes, also contribute to the glial scar and its negative effects on regeneration. We hypothesize, therefore, for a photoablative technique to be successful, several components of the glial scar need to be targeted by the treatment.

The goal of this study was to develop further an effective and innovative technique for removing glial scar associated with chronic SCI. The functionality of rose bengal as an ablative technique has been demonstrated previously by our group,²⁰ but that preliminary study was limited in its characterization and examined only one version of rose bengal (RB1). Because there is evidence that more than one component of the glial scar is inhibitory, it is important to determine the effects of our ablative method on these various components. To further optimize this technique, we have introduced two additional rose bengal derivatives and expanded our characterization in a rat model of contusive chronic SCI.

In the present study, RB1 (unmodified rose bengal) diffused effectively into the glial scar, as we had reported previously. It attenuated expression of all four of our measured components of the glial scar at both 24 h and eight days. This was significant for GFAP

FIG. 3. Effect of photo ablation with rose bengal (RB) on the composition of the glial scar in a rat model of chronic spinal cord injury (SCI) at 24 h post-treatment. Spinal cords were examined for components of the glial scar at 24 h post-treatment with either saline, RB1, RB2, or RB3 administered six weeks after SCI. **(A)** Astrocytes (glial fibrillary acidic protein [GFAP], aldehyde dehydrogenase 1 [ALDH1]), matrix (chondroitin sulfate proteoglycan [CSPG]) and fibrotic (collagen COL1A1) components were detected at 24 h post-treatment among the four groups. Scale bar, 100 μ m. Representative sections are shown. **(B)** Quantitative analysis of the glial scar at 24 h after treatment for each of the four treatment groups. The analysis showed that expression of both GFAP and ALDH1 were significantly reduced after RB1 treatment when compared with control. Significant reduction in the expression of COL1A1 was observed when treated with RB1 compared with control. Expression of CSPG was decreased, however, after the treatment with RB1 or RB2 but was not statistically significant compared with control. Statistical analysis utilized one-way analysis of variance with Bonferroni *post hoc* test. * $p < 0.05$. Data represent mean $\pm$ standard error of the mean.

FIG. 4. Effect of photoablation with rose bengal (RB) on the composition of the glial scar in a rat model of chronic spinal cord injury (SCI) at eight days post-treatment. Spinal cords were examined for components of the glial scar at eight days post-treatment with either saline, RB1, RB2, or RB3 administered six weeks after SCI. (A) Astrocytes (glial fibrillary acidic protein [GFAP], aldehyde dehydrogenase 1 [ALDH1]), matrix (chondroitin sulfate proteoglycan [CSPG]) and fibrotic (collagen COL1A1) components were detected at eight days post-treatment among the four groups. Scale bar, 100 μ m. Representative sections are shown. (B) Quantitative analysis of the glial scar at eight days after treatment for each of the four treatment groups. The analysis showed significant reduction in expression of GFAP and ALDH1 compared with control after RB1 and RB2 treatment. Treatment with RB1 showed decreasing trend in COL1A1 expression compared with control but did not reach statistical significance. Expression of CSPG, however, was decreased after the treatment with RB1 or RB2 but was not statistically significant when compared with control. Statistical analysis utilized one-way analysis of variance with Bonferroni *post hoc* test. * $p < 0.05$, ** $p < 0.01$. Data represent mean $\pm$ standard error of the mean.

FIG. 5. Effect of photoablation with rose bengal (RB) on serotonin in a rat model of chronic spinal cord injury (SCI) at 24 h and eight days post-treatment. Spinal cords were examined for serotonin at 24 h and eight days post-treatment with either saline, RB1, RB2, or RB3 administered six weeks after SCI. (A) Serotonin was detected at both 24 h and eight days post-treatment among the four groups. Scale bar, 100 μ m. Representative sections are shown. (B, C) Quantitative analysis revealed that expression of serotonin increased at 24 h after the treatment with RB1 and RB2 but was not statistically significant compared with control. Dramatic increase in the expression, however, was seen at eight days post-treatment with RB1 when compared with the other three groups. Statistical analysis of serotonin levels was performed using one-way analysis of variance with Bonferroni *post hoc* test. * $p < 0.05$, ** $p < 0.01$. Data represent mean $\pm$ standard error of the mean.

and ALDH1 at 24 h and eight days post-treatment, suggesting that RB1 was most effective in ablating the astrocytic component. The fibroblastic/collagen component was significantly reduced at 24 h only compared with control, and one possible explanation is the onset of reformation of the glial scar by eight days. The CSPG component was reduced but not significantly at both 24 h and eight days. This may indicate less of a treatment effect on CSPGs, but it is also important to note that the IHC signal was not as robust for the CSPGs, and therefore this may represent the difference in antigen detection.

In contrast, RB2 attenuated expression of all four of our measured components at 24 h and eight days except for collagen at eight days, but to a lesser extent than RB1. This is consistent with our hypothesis that while RB2 would diffuse into the glial scar, it would be slightly retarded, and thus have a smaller effect than RB1. This reached significance in the reduction of astrocytes (GFAP and ALDH1) at eight days only. In a recent study, collagen was identified as a prominent factor in the scar-forming phase of SCI,²⁵ and COL1A1 was chosen as a representative marker for the fibrotic scar

component. We found that collagen expression was the only scar component that was not reduced at eight days by RB2 treatment, and again this could indicate early recurrence of the fibrotic component of the glial scar. In contrast to RB1 and RB2, RB3 had no effect on any of the measured components of the glial scar.

These results suggest that RB1 more effectively reduces the expression of scar components compared with treatment with RB2, RB3, or control. RB2 as hypothesized had similar but lesser effects than RB1. This is likely attributed to the varied diffusion rate of RB1 and RB2 into the injured spinal cord, and these results suggest that RB3 did not cross the interface between the cavity and the lesioned tissue, likely attributed to its size.

A major concern when removing scar tissue from an injury site is to minimize the collateral effect of ablation on functioning spared tissue. Quantification indicated that the number of neurons (NeuN), endogenous stem cells (nestin), and oligodendrocytes (APC) did not differ significantly between the groups treated with saline, RB1, RB2, or RB3 at either 24 h or eight days post-treatment. We also

FIG. 6. Effect of photoablation with rose bengal (RB) on macrophages/microglia (ED1) in a rat model of chronic spinal cord injury (SCI) at 24 h and eight days post-treatment. Spinal cords were examined for macrophages/microglia at 24 h and eight days post-treatment with either saline, RB1, RB2, or RB3 administered six weeks after SCI. Quantification of immunoreactivity of ED1 at (A) 24 h and (B) eight days post-treatment. The analysis showed significant increase in expression of ED1 at 24 h post-treatment with RB1 and RB2 compared with control and remained elevated at eight days post-treatment but was not statistically significant when compared with control. Statistical analysis was performed using one-way analysis of variance with Bonferroni *post hoc* test. * $p < 0.05$, ** $p < 0.01$. Data represent mean $\pm$ standard error of the mean.

found that open field locomotion was unchanged immediately after scar ablation or up to eight days after treatment when compared with the control group. This further supports the idea that scar ablation did not damage the spared tissue, but also indicates that scar ablation alone is not sufficient to provide any meaningful functional recovery, and that it would be best utilized in a combinatorial therapy with neural progenitor cell transplantation. This is a subject for future study.

The inflammatory reaction is an important factor in SCI. Activated macrophages/microglia persist at the site of SCI³⁴ for at least one year post-injury.¹⁰ These activated macrophages/microglia respond rapidly to disturbances within the microenvironment and lead to a cascade of complex reactions. Our findings suggest that the number of macrophages/microglia were significantly increased at 24 h post-treatment in the RB1 and RB2 treated rats, indicating that there was likely an immediate inflammatory reaction induced by our intervention. The observed increase in ED1 expression remained elevated but was no longer significant at eight days post-treatment.

Although Anderson and associates³¹ found that ablating chronic astrocytic scar failed to result in spontaneous sprouting or regeneration of supraspinal axons, and further suggested a necessary role for the astrocytic scar in this process, our findings do not support this. Our method of glial scar ablation resulted in an increase in serotonin levels. Serotonin was found to be elevated in the RB1 group at 24 h after photoablation, and this reached significance at eight days after treatment when compared with control. This phenomenon was also observed to a lesser extent in the RB2 group at 24 h post-treatment. We attribute this observation to upregulation of serotonin in preparation for sprouting of existing axons in a more permissive environment. Additional studies will now be required to further explain this finding.

The expression of serotonin was initially apparent next to the lesion site at 24 h, and a highly significant increase in the amount of

serotonin was detected throughout the cord around the injury site when treated with RB1 at eight days (Fig. 5A). This increase may play an important role in the regenerative process. Similar findings were reported after administration of chondroitinase ABC.^{14,35–39} The authors suggested that treatment with ChABC into the injured spinal cord promoted serotonergic sprouting within the spinal cord and induced plasticity of spared tracts.

Conclusion

These results indicate that the use of rose bengal is a safe and effective technique to remove various components of the glial scar after chronic contusion injury. These results confirm our previous findings²⁰ but also characterize these effects in further detail. Further, we have investigated the use of two rose bengal derivatives and compared these results with our original rose bengal. We found that RB1 (unmodified rose Bengal) is more efficacious than our RB2 formulation (modified with hydrocarbon) without measurable damage to surrounding tissue. The use of either the RB1 or RB2 analog could be predicated on the level of ablation required for any combination treatment along with cellular transplantation, a strategy that we are investigating currently.

In this study, scar ablation did not lead to functional recovery, but our studies did demonstrate serotonergic sprouting up to eight days after scar ablation when treated with RB1. While we believe that the scar will likely recur before any meaningful functional recovery could occur in the absence of any additional treatments, our experiments here were only conducted for eight days. Therefore, it is also possible that our scar ablation treatment experiment was not long enough to appreciate any functional difference. In contrast, RB3 (modified with polyethylene glycol) was too large to be taken up into the cells and therefore had no effect. An increase in macrophages/microglia was observed after both RB1 and RB2 treatment, but this did not have any measurable detrimental effects.

We have demonstrated that rose bengal can be utilized as a safe and effective method of glial scar ablation. We do not believe that the use of this therapy alone will be an optimal treatment for chronic SCI, but rather that it is a promising strategy in combination with other therapies that have shown promise in acute or subacute SCI. In our future studies, we plan to utilize RB1 in combination with cellular transplantation for the development of a new treatment for chronic SCI.

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Author Disclosure Statement

No competing financial interests exist.

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