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Salmon fibrin treatment of spinal cord injury promotes functional recovery and density of serotonergic innervation

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Abstract

The neural degeneration caused by spinal cord injury leaves a cavity at the injury site that greatly inhibits repair. One approach to promoting repair is to fill the cavity with a scaffold to limit further damage and encourage regrowth. Injectable materials are advantageous scaffolds because they can be placed as a liquid in the lesion site then form a solid *in vivo* that precisely matches the contours of the lesion. Fibrin is one type of injectable scaffold, but risk of infection from blood borne pathogens has limited its use. We investigated the potential utility of salmon fibrin as an injectable scaffold to treat spinal cord injury since it lacks mammalian infectious agents and encourages greater neuronal extension *in vitro* than mammalian fibrin or Matrigel®, another injectable material. Female rats received a T9 dorsal hemisection injury and were treated with either salmon or human fibrin at the time of injury while a third group served as untreated controls. Locomotor function was assessed using the BBB scale, bladder function was analyzed by measuring residual urine, and sensory responses were tested by mechanical stimulation (von Frey hairs). Histological analyses quantified the glial scar, lesion volume, and serotonergic fiber density. Rats that received salmon fibrin exhibited significantly improved recovery of both locomotor and bladder function and a greater density of serotonergic innervation caudal to the lesion site without exacerbation of pain. Rats treated with salmon fibrin also exhibited less autophagia than those treated with human fibrin, potentially pointing to amelioration of sensory dysfunction. Glial scar formation and lesion size did not differ significantly among groups. The pattern and timing of salmon fibrin's effects suggest that it acts on neuronal populations but not by stimulating long tract regeneration. Salmon fibrin clearly has properties distinct from those of mammalian fibrin and is a beneficial injectable scaffold for treatment of spinal cord injury.

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Keywords

dorsal hemisection; three-dimensional; biomaterial; axon; BBB score; bladder; serotonin

Introduction

Spinal cord injury (SCI) severs descending motor and ascending sensory tracts, cutting off the lower regions of the spinal cord from the brain. Secondary stages of CNS injury such as hemorrhage and infiltration of immune cells lead to increasing neuronal losses and functional impairment (Tator and Fehlings, 1991). The injured region of the human spinal cord degenerates leaving a cystic cavity surrounded by a glial (astrocytic) scar that prevents regrowth of axons, thereby prohibiting recovery from the motor and sensory deficits accompanying the injury.

One approach to SCI is to implant a bridging, or scaffold, material in the cystic cavity with the goal of providing a substrate for axonal regrowth and limiting glial scar formation along the lesion edges. A variety of scaffold materials have been tested, including natural polymers, biodegradable and non-biodegradable synthetics, and composite conduits composed of guidance tubes with material fills (Novikova, et al., 2003, Straley, et al., 2010). Several are associated with improved functional recovery, making it likely that a repair-promoting scaffold will be an important component of a multi-pronged approach to SCI treatment. However, questions remain regarding optimal structure and composition of the scaffold.

Scaffolds with linear, aligned axonal guidance channels have been developed to mimic spinal cord architecture and encourage longitudinal axonal extension through the scaffold. These types of scaffolds must be fabricated prior to implantation in the lesion site in order to generate the linear channels and prevent exposure of spinal cord tissue to the toxic chemicals or processes necessary for their formation. Injectable scaffolds provide an alternative to preformed scaffolds. These materials are placed in the lesion site as a liquid and allowed to polymerize in situ. This approach is therefore restricted to scaffolds for which the polymerization process is biocompatible, preventing the use of some synthetic polymers since toxic byproducts are generated during polymerization. One advantage to injectable scaffolds is complete filling of the lesion site and tight association of the scaffold with the edges of the tissue since the material conforms to the shape of the lesion cavity. Scaffolds that polymerize in situ can also be used to embed transplanted stem cells and growth promoting molecules can be incorporated into the polymerized matrix; thus these scaffolds provide specific benefits in combinatorial therapeutic approaches (Straley, et al., 2010, Taylor, et al., 2006). Importantly, the mechanical properties, or compliance, of the scaffold should match that of the surrounding nervous system tissue to encourage the greatest degree of regrowth and reduce mechanical stresses on the surrounding tissue (Balgude, et al., 2001, Elkin, et al., 2007, Flanagan, et al., 2002, Oudega, et al., 2001, Prange and Margulies, 2002). Injectable, non-toxic materials that match the compliance of spinal cord tissue may provide the best scaffolds for repair.

A variety of biomaterials have been tested as injectable scaffolds, including the biopolymer fibrin. In fact, human fibrin is FDA-approved and commonly used as a surgical glue (Tisseel®). Fibrin formation is non-toxic and occurs during the coagulation cascade when fibrinogen is cleaved by thrombin to form fibrin monomers, which then spontaneously polymerize to form a three-dimensional matrix (Mosesson, 2005). Varying the concentration of thrombin used to induce polymerization controls the rate of fibrin gel formation, which is advantageous for maintaining a liquid state during injection while forming a solid scaffold *in*

vivo. Fibrin has been successfully utilized in repair strategies for a variety of *in vivo* neuronal injury models (e.g. (Patist, et al., 2004) (Iwaya, et al., 1999, Stokols, et al., 2006, Taylor, et al., 2006, Tsai, et al., 2006, Williams, 1987). In spite of these advantages, mammalian fibrin gels degrade rapidly (Bensaid, et al., 2003, Novikova, et al., 2003, Sieminski and Gooch, 2004) and may be contaminated with blood-borne pathogens such as HIV, hepatitis C, and prion proteins (Fischer, et al., 2000). In animal studies, autologous mammalian fibrinogen may contribute to CNS damage and lack of repair by inhibiting neurite outgrowth (Schachtrup, et al., 2007) and activating resident astrocytes and microglia (Adams, et al., 2007, Schachtrup, et al., 2010).

The limitations of mammalian fibrin have led to the use of fibrin from other species, most notably North Atlantic salmon (Uibo, et al., 2009). Salmon and mammalian fibrin polymerize similarly and salmon fibrinogen and thrombin are in ready supply from the aquaculture industry (Wang, et al., 2000). Salmon fibrin may be safer than mammalian fibrin for use in humans since known salmon viruses are not transmissible to mammals (Wolf, 1988); in part because fish are coldwater animals and most viruses that infect them are inactivated at human body temperatures. Fibrin prepared from salmon proteins encourages greater cell motility and neurite extension than mammalian fibrin (Ju, et al., 2007, Sieminski and Gooch, 2004), matches the compliance of CNS tissue (at ~3 mg/ml fibrin concentrations) (Ju, et al., 2007, Prange and Margulies, 2002), and degrades more slowly than human fibrin (Ju, et al., 2007, Laidmae, et al., 2006). Salmon fibrin treatment does not cause immune-mediated toxicity (guinea pigs, mice, rabbits) (Michaud, et al., 2002) but can induce antibodies to salmon fibrinogen and thrombin in host animals (rat, rabbit, and swine) (Laidmae, et al., 2006, Laidmae, et al., 2010, Rothwell, et al., 2009, Rothwell, et al., 2010, Wang, et al., 2000). However, these antibodies do not cross-react with the host's proteins, alter the levels of host fibrinogen or thrombin, or create coagulation problems (Laidmae, et al., 2006, Laidmae, et al., 2010, Rothwell, et al., 2009, Rothwell, et al., 2010, Uibo, et al., 2009).

Salmon fibrin's positive effects on neurite extension, slower degradation kinetics, and lack of potentially infectious agents led us to test whether it would be a beneficial injectable scaffold for the injured CNS. We used a dorsal hemisection model of spinal cord injury commonly utilized to test scaffolds and completed a thorough assessment of animals treated with salmon fibrin, human fibrin (Tisseel®), or left untreated (controls). We show that salmon fibrin-treated animals have greater recovery of locomotor and bladder function and more serotonergic innervation caudal to the lesion site as compared to animals treated with human fibrin or untreated controls. Surprisingly, there was no effect of salmon fibrin on glial scar formation or lesion volume.

Methods

Experimental animals

Experimental animals were adult female Sprague-Dawley rats (Harlan, San Diego, CA), each weighing 200–250 g. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of California Irvine.

Pre-injury locomotor training

Prior to surgery, all rats were assessed for locomotor behavior with the Basso, Beattie, and Bresnahan (BBB) open-field locomotor scale to acquaint the rats with the task and ensure they had no noticeable ambulatory impairments (Basso, et al., 1995).

SCI surgery, fibrin implantation, and post-operative procedures

Rats were randomly assigned to three separate groups (untreated controls, human fibrin-treated, salmon fibrin-treated) with 8 rats in each group. Rats were anesthetized with an intraperitoneal injection of ketamine and xylazine (100 and 10 mg/kg, respectively; Western Medical Supply, Arcadia, CA). The hair overlying the mid-thoracic area from T3-T11 was removed by shaving and the exposed skin sterilized with Betadine. A small midline incision was made through the skin at the T6-T10 vertebral level and underlying connective and muscle tissue were bluntly dissected to expose the lower thoracic portion of the spinal column. A laminectomy was performed at the T8-T10 vertebral level to remove vertebrae overlying the dorsal spinal cord. A dorsal hemisection at T9 was created using a Micro Feather Ophthalmic Scalpel with a 15 degree angle blade (Electron Microscopy Sciences, catalog number 72045-15, Hatfield, PA) by extending the cut ventrally to the central canal to ensure ablation of all dorsal pathways. The following steps were taken to standardize the lesions: 1) a 1 mm mark was placed on the blade to ensure the same depth was achieved for each lesion, 2) the blade was passed through the lesion three times to ensure that all dorsal fibers were ablated, and 3) all injuries were created by one surgeon to reduce variability.

Fibrin gels were implanted into the lesion site after dorsal hemisection by diluting fibrinogen (3.0 mg/ml) and thrombin (0.15 U/ml) into phenol-free EMEM and gently injecting ~5 µl of the liquid mixture into the lesion site using a Hamilton syringe, being careful not to touch the edge of the needle to the tissue. Low thrombin concentrations were used to delay fibrin polymerization until after filling of the injury site. Preliminary experiments verified sufficient polymerization within 5 minutes at this thrombin concentration. Rats were maintained in a stationary position for at least 5 minutes after fibrin treatment to allow polymerization to occur *in situ*. Fibrin solution from each injection was also placed on a surface and visually monitored to ensure complete polymerization had occurred. Human fibrinogen and thrombin stocks were Tisseel® and salmon fibrinogen and thrombin were from Sea Run Holdings, Inc. (Michaud, et al., 2002, Wang, et al., 2000).

After dorsal hemisection, and in some cases fibrin treatment, back muscles were sutured with 4-0 chromic gut (Ethicon, catalog number G181H, Cornelia, GA) and the skin was closed with wound clips (Fisher Scientific, Pittsburgh, PA). Post-operatively, rats received subcutaneous injections of 5 ml per 100 kg of 0.9% saline for hydration for 10 days, 2.5 mg/kg Baytril to reduce the risk of bladder infection for 10 days, and 0.01 mg/kg buprenorphine for pain control for 3 days. Rats were placed on water circulating jacketed warming pads at 37° C to maintain body temperature for three days post-injury. Rats were group housed (4–5 per cage) and were monitored twice daily for general health, coat quality (indicative of normal grooming activity) and mobility within the cage. Rats were also monitored for signs of skin lesions on the paralyzed limbs or autophagia of the toes. If a rat exhibited severe autophagia, it was euthanized.

Bladder functional analysis

Analysis of bladder function was carried out by team members blinded to treatment group. Bladders were manually expressed twice daily until each rat regained autonomic bladder function (approximately two weeks post-injury). Retained urine for each rat was collected from the morning expression prior to the administration of fluids and the volume (ml) of retained urine recorded.

Locomotor functional testing

Locomotor testing was carried out by team members blinded to treatment group. At least 2 trained evaluators scored each animal per session for several weeks postinjury and treatment. Hindlimb locomotor function was assessed with the 21-point Basso, Beattie, and

Bresnahan Locomotor Rating Scale (BBB) (Basso, et al., 1995). The scale measures a wide range of hindlimb function, from complete paralysis to normal walking behavior, by assessing hind limb joint movements, stepping, trunk position and stability, forelimb-hindlimb coordination, paw placement, toe clearance, and tail position. Rats were assessed in an open field (150 × 100 cm) for 4 minutes at a similar time of day for each testing. BBB subscores of rats scoring 11 or above on the BBB scale were assessed during the same testing period with a 13-point scale that allows for investigation of fine locomotor movements, including details of paw position, toe clearance, tail position, and trunk stability (Basso, 2004, Lankhorst, et al., 2001, Popovich, et al., 1999).

Pain testing

Pain testing was conducted on the plantar surface of the paws by the von Frey filament method as described in Chaplan et al. 1994 (Chaplan, et al., 1994). Mechanical threshold measurements for each paw were obtained using manual application of the von Frey monofilaments to determine the force stimulus threshold. Rats were measured near the end of experiments (68 days after treatment) to enable comparison of behavioral responses with underlying anatomical changes revealed by immunohistochemical analysis.

Salmon fibrinogen antibody production

New Zealand white rabbits were immunized twice (four weeks apart) i.p. with 11 mg/kg purified salmon fibrinogen in 0.9% NaCl. Blood was drawn 10 days after the second immunization and serum prepared by centrifugation. Serum samples were tested by Western blotting to determine specificity for the salmon fibrinogen chains.

Western blotting and fibrin gel cryosections

Purified salmon, human, or bovine fibrinogen (Calbiochem, catalog number 341573, San Diego, CA) (1 µg per lane) were loaded onto 8–16% gradient polyacrylamide gels (BioRad, catalog number 161–1226, Hercules, CA) for SDS-PAGE. Proteins were electrophoretically transferred onto nitrocellulose paper, and blots were stained with 0.1% wt/vol Ponceau S in 1% acetic acid to detect total protein and verify equal loading and transfer of each lane. Ponceau S was removed with sequential rinses of TBS-Tween (50 mM Tris buffer, pH 7.6, 150 mM NaCl, 0.05% Tween 20), and the blots were blocked in 5% nonfat dry milk in TBS-Tween. Anti-salmon fibrinogen antibody was diluted 1:50,000 in 5% nonfat dry milk in TBS (50 mM Tris base, pH 7.6, 150 mM NaCl) and blots were rinsed in TBS-Tween. Primary antibody was detected with an HRP-conjugated goat anti-rabbit IgG secondary antibody diluted 1:3000 (Promega, catalog number W4011, Madison, WI), and secondary antibodies were detected with an enhanced chemiluminescence system (Pierce Chemical Co., catalog number 32106, Rockford, IL).

Fibrin gels were prepared from fibrinogen (3 mg/ml) and thrombin (1.5 U/ml) proteins from salmon or from rat (Sigma-Aldrich, catalog numbers F6755 and T5772, St. Louis, MO). Fibrin was polymerized into 100 µl gels on tissue culture plates, fixed with 4% paraformaldehyde, carefully excised from the surface, and cryoprotected and sectioned as described below for spinal cords. Fibrin gel cryosections were immunostained with anti-salmon fibrinogen antibody diluted 1:6000 using methods as described below for spinal cords.

Histological procedures

At the end of each experiment, rats were euthanized by anesthetic overdose and perfused transcardially with 4% paraformaldehyde. Spinal cords from brainstem to cauda equina were carefully dissected and post fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH

7.4. Spinal cords were cryoprotected overnight in 27% sucrose and an approximately 16 mm long region of the spinal cord containing the lesion site was dissected and frozen in TissueTek (VWR, catalog number 25608-930, West Chester, PA). Spinal cords were cryosectioned sagittally into 5 serial sets of 20- μ m thick sections collected on slides. One set of sections was stained with hematoxylin and eosin and coverslipped with DPX mounting media (Sigma-Aldrich, catalog number 44581, St. Louis, MO).

Sections to be immunohistochemically processed for salmon fibrinogen, serotonin (5-HT), GFAP, and neurofilament heavy chain (NFH) were washed three times in PBS, blocked in 5% normal goat serum (NGS) in PBS and incubated in diluted antibodies overnight at room temperature. Conditions were as follows: rabbit anti-salmon fibrinogen (described above) 1:6000 in PBS with 5% NGS; rabbit anti-serotonin (Sigma-Aldrich, catalog number S-5545, St. Louis, MO) 1:5000 in PBS, 5% NGS and 0.5% Triton X-100; rabbit anti-GFAP (Dako, catalog number Z0334, Carpinteria, CA) 1:2000 in PBS with 5% NGS; mouse anti-NFH was a mixture of SMI-31 and SMI-32 (Sternberger Monoclonals, Baltimore, MD) 1:100 in PBS with 5% NGS. Following incubations in primary antibodies, slides were washed 3 times in PBS then incubated two hours in 1:250 dilution goat anti-rabbit Alexa Fluor 488 (Molecular Probes, catalog number A11034, Carlsbad, CA) in PBS with 5% NGS or 1:250 dilution goat anti-mouse Alexa Fluor 555 (Molecular Probes, catalog number A21424, Carlsbad, CA) in PBS with 5% NGS. In some cases, all cell nuclei were stained with Hoechst 33342, 2 μ g/ml in PBS (Molecular Probes/Invitrogen, catalog number H1399, Carlsbad, CA). Following secondary incubation, the slides were washed three times in PBS, dried, and coverslipped with Kaiser's mounting medium (Humason's Animal Tissue Techniques, 1972). Controls included sections stained with secondary antibody alone. All sections stained were from rats 77 days post-injury and treatment unless otherwise noted.

Image Acquisition and Data Analysis

Images were taken using a Nikon TE2000-U inverted fluorescent microscope or Nikon e600 fluorescent microscope in conjunction with a Spot RT camera and Spot Advanced software. Confocal images were taken using a laser scanning confocal microscope (Olympus FV1000, Center Valley, PA) at ~300 nm resolution and 2 μ m optical slice. Creating the composites for each region of interest was completed through manual photo stitching using Photoshop from Adobe's Creative Suite 3. Images were evaluated with quantitation methods listed below and all data analyzed using Kaleidagraph or GraphPad Prism.

Quantification of GFAP signal, serotonergic-positive fibers, and lesion volume was performed using Simple PCI (Hamamatsu Corporation, Bridgewater, NJ). GFAP-positive signal was measured in 2 mm regions adjacent to the lesion site. The 5-HT immunoreactivity was measured along both intermediolateral (IML) tracts since they were noted to be among the densest 5-HT-positive regions in the mouse spinal cord (Steward, et al., 2008). The area occupied by 5-HT-positive fibers was calculated for 2 mm regions rostral and caudal to the lesion site and the percentage of caudal signal calculated.

Lesion volume was calculated from H&E stained sections. The primary lesion edges were traced three times manually for each section. The primary lesion was defined as the actual cavity of the injury site excluding Wallerian degeneration and other areas of secondary injury. If necessary, adjacent GFAP-stained sections were analyzed as a means to more accurately define the lesion. The area of the three manual traces per section were averaged, the areas from 20–25 sections collected serially with defined spacing per cord were summed, and the known distance between each section was used to calculate the lesion volume of each cord in cubic millimeters.

Statistical analysis

BBB scores, BBB subscores, and bladder function for each group were analyzed using repeated measures two-way ANOVA. The Bonferroni test was used for post-hoc analysis to correct for multiple comparisons. Relationships between BBB scores and either bladder function, GFAP staining, lesion volume, or 5-HT fiber density was determined by the Pearson correlation coefficient. Data from von Frey hair mechanical stimulus testing was analyzed by Student's t-test. Comparisons of quantitative immunohistochemistry data implemented Student's t-test. Analyses were performed using Graph Pad Prism (La Jolla, CA) and Kaleidagraph (Reading, PA).

Results

Salmon fibrin forms a gel in the lesion site

We initially tested fibrin polymerization *in vitro* to establish appropriate conditions for *in vivo* experiments. We titrated the thrombin concentration to a level that would allow injection of a liquid mixture of salmon fibrinogen and thrombin and formation of a solid gel in the lesion site. In order to test whether polymerization occurred *in vivo* as expected, we developed an antibody specific for salmon fibrinogen. Western blots of salmon, human, and bovine fibrinogen show that the anti-salmon fibrinogen antibody has much greater affinity for salmon fibrinogen than mammalian fibrinogens (Fig. 1A). Fibrinogen is cleaved as part of the polymerization process to form fibrin, so to assess the ability of the antibody to detect polymerized fibrin we formed fibrin gels *in vitro* and prepared cryosections of the gels for immunostaining. The anti-salmon fibrinogen antibody robustly stained salmon fibrin but did not stain rat fibrin at levels above background, confirming the utility of this antibody for detecting salmon fibrin in rat tissue with minimal cross-reactivity for endogenous rat fibrin (Fig. 1B).

Formation of salmon fibrin gels *in vivo* was assessed by allowing salmon fibrin to polymerize in the lesion sites of rats subjected to a dorsal hemisection injury. We used a rat model system since injury to the rat spinal cord results in a cystic cavity and a glial scar that mimics those found in human injury. A dorsal hemisection was chosen in order to draw comparisons to a number of studies in the literature that have tested the efficacy of three-dimensional material implants for treating neural injury (Johnson, et al., 2009, Menezes, et al., 2010). Sections of spinal cords through the lesion sites of injured rats (untreated, human fibrin-treated, and salmon fibrin-treated) at time points ranging from 2 to 10 days after surgery and scaffold implantation were stained with the anti-salmon fibrinogen antibody. While no specific staining was observed in untreated controls or rats treated with human fibrin, significant signal was detected in the lesion sites of rats treated with salmon fibrin (Fig. 1C). Salmon fibrin was detected after 2 and 5, but not 10, days post-injury and fibrin treatment. These data confirm that salmon fibrin forms a gel in the lesion site that persists after the initial implantation but eventually degrades.

Rats treated with salmon, but not mammalian, fibrin exhibit improved locomotor recovery

We tested whether fibrin treatment would affect locomotor recovery after dorsal hemisection SCI by comparing untreated controls and animals treated with salmon or human fibrin at the time of injury. Rats (8 per group) were assessed by the BBB locomotor scale for ~10 weeks post-injury and treatment. Animals treated with salmon fibrin scored significantly higher on the BBB scale than animals treated with human fibrin or untreated controls (Fig. 2A). Analysis of individual time points indicated significant differences between the salmon fibrin-treated group and the other groups on days 1 and 35 after treatment (Fig. 2A, BBB scores at day 1 salmon fibrin-treated 7.4 $\pm$ 1.3, human fibrin-treated 2.7 $\pm$ 1.2, untreated 3.5 $\pm$ 1.3; day 35 salmon fibrin-treated 14.1 $\pm$ 0.9, human fibrin-treated 10.9 $\pm$ 0.3,

untreated 11.6 ± 0.3). Although in this experiment differences were evident between groups as early as 1 day after treatment, in other experiments the groups scored similarly in BBB testing until day 7 (data not shown). The improved locomotor behavior of salmon fibrin-treated animals was stable and maintained throughout the 10 week course of the experiment (Fig. 2A). The BBB scores indicate that rats in the salmon fibrin-treated group (BBB score ~ 14) exhibited more consistent plantar stepping and coordination of front and hind limbs than rats in the human fibrin-treated (BBB score ~ 11) or control groups (BBB score ~ 11.5). We tested the efficacy of salmon fibrin treatment for dorsal hemisection SCI in two additional experiments and in all three experiments the group treated with salmon fibrin maintained higher BBB scores than the other two groups through the end of the experiment (70 days for experiment 1, 50 days for experiment 2, and 50 days for experiment 3) (Supplemental Fig. S1).

BBB subscore analysis was performed on the highest functioning animals in all groups (minimum BBB score of 11) to provide a more detailed analysis of locomotor behavior. The subscores indicate that salmon fibrin-treated rats exhibited more complex locomotor function such as increased trunk control, greater coordination, improved paw position, and consistent plantar stepping than rats in the other groups (Fig. 2B; BBB subscores on day 56 after treatment were salmon fibrin-treated 7.9 ± 1.3 , human fibrin-treated 3.1 ± 0.8 , untreated 4.3 ± 1.2). Differences in the BBB subscores of the three groups were initially evident at 2 weeks post-treatment. Improved BBB subscores of the salmon fibrin-treated groups were seen at the endpoints of all three experiments, demonstrating the reproducibility of this effect (Supplemental Fig. S1).

Salmon fibrin-treated rats experience better bladder function

Spinal cord injuries occurring rostral to the lumbosacral region result in urine retention in the rat that, in contrast to human injury-induced bladder dysfunction, eventually resolves over time (de Groat and Yoshimura, 2006). Regaining bladder, bowel, and sexual function are some of the highest priorities for people living with SCI, so urinary function should be monitored as an outcome measure in animal injury models (Anderson, 2004, Estores, 2003). We assessed whether treatment with fibrin improved bladder function by measuring the volume of urine retained in the bladder of SCI animals. Greater volumes of retained urine indicate more severe bladder dysfunction.

Animals treated with salmon fibrin consistently exhibited less urine retention than animals treated with human fibrin or untreated controls (Fig. 3A). Human fibrin-treated and untreated animals retained 2–3 times as much urine on day 4 as animals treated with salmon fibrin (Fig. 3A, volume retained urine (ml) on day 4 salmon fibrin-treated 1.2 ± 0.5 , human fibrin-treated 3.8 ± 0.4 , untreated 2.8 ± 0.6). We assessed how rapidly the groups of rats recovered normal bladder function by noting the time point at which most of the animals (75% or more) retained less than 1 ml of urine. The salmon fibrin-treated group reached this milestone by 7 days after treatment whereas the untreated control and human fibrin-treated groups did not reach this point until 11 days after treatment (data not shown). We tested whether the concentration of salmon fibrin affected recovery of bladder function and found no differences between animals treated with 3 mg/ml or 6 mg/ml concentrations of salmon fibrin (data not shown), demonstrating no additional benefit with a 2-fold increase in salmon fibrin in the lesion site.

We hypothesized that there may be a link between recovery of bladder function at early time points (day 4 post-injury) and locomotor function at later times (BBB scores at day 70 post-injury) and found that they were indeed correlated (Fig. 3B). For the animals in our experiments, early measures of bladder function were reasonable predictors of eventual locomotor function.

Treatment with salmon fibrin does not alter glial scar formation, lesion volume, or Schwann cell infiltration

Improvements in functional recovery could be due to a variety of underlying anatomical changes in response to salmon fibrin treatment. One hallmark of SCI lesions is formation of a glial scar due to proliferation of astrocytes surrounding the lesion and a variety of implanted matrices including fibrin have been shown to decrease the glial scar (Johnson, et al., 2009, Taylor, et al., 2006). We measured the effects of fibrin treatment on glial scar formation by quantifying immunoreactivity for the astrocyte marker GFAP in the tissue surrounding the lesion site. Visual inspection of the immunostained sections showed no obvious distinctions in the amount of glial scar formation among the three groups (untreated, human fibrin-treated, and salmon fibrin-treated) (Fig. 4A). Quantitative analysis of GFAP immunoreactivity revealed that glial scar formation was similar between salmon fibrin-treated animals and untreated controls and elevated in human fibrin-treated animals (Fig. 4B). Since this result was unexpected, we pooled data for animals from all groups and tested for a correlation between the degree of glial scar formation and locomotor function (BBB score endpoints). The degree of glial scar formation does not correlate with locomotor ability, suggesting that the beneficial effects of salmon fibrin on functional recovery are not due to modification of glial scarring (Fig. 4B). While fibrin treatment did not alter glial scar formation in the dorsal hemisection SCI model, results may differ with other lesion models or treatment paradigms. For example, an early assessment of the effects of salmon fibrin in a contusion model of SCI in which lesioned animals were treated one week after injury suggested a reduction of glial scarring associated with salmon fibrin treatment (Supplemental Fig. S2). Further studies will be necessary to clarify the key similarities and differences between salmon fibrin treatments in these two SCI models.

Scaffold treatment has been suggested to affect the volume of the lesion cavity, which may also be influenced by glial scar formation. We measured lesion volumes from H&E stained spinal cord sections and found that the size of lesions in salmon fibrin-treated animals was similar to that of untreated controls while human fibrin-treated animals had larger lesion volumes (Fig. 4C). Much as seen for glial scar formation, when all animals were pooled together the size of the lesion did not correlate well with functional recovery as measured by BBB scores (Fig. 4C). The similarity in lesion size between the salmon fibrin-treated group and untreated controls confirms that a difference in the extent of the initial lesion is not responsible for the differences between the locomotor and bladder function of these groups at early time points.

Schwann cells, the myelinating cells of the peripheral nervous system, can migrate into the lesion site after SCI and improve recovery of locomotor behavior (Takami, et al., 2002). Mammalian fibrin has been proposed as an inhibitor of Schwann cell migration after peripheral nerve lesion (Akassoglou, et al., 2003), suggesting that there might be differences in Schwann cell infiltration into the lesion sites due to the presence of fibrin. We assessed the presence of Schwann cells in the lesion sites of animals treated with salmon fibrin, human fibrin, or untreated controls by immunostaining for the low affinity nerve growth factor receptor, p75. While there was variation in the degree of Schwann cell infiltration among the spinal cords, there was not a significant difference based on treatment group (Supplemental Fig. S3). The beneficial effects of salmon fibrin are therefore not primarily due to increased infiltration of Schwann cells into the lesion site.

Rats treated with salmon fibrin exhibit increased serotonergic fibers caudal to the injury site

Serotonergic neuronal circuits play a vital role in locomotor behavior (Kim, et al., 2004, Li, et al., 2004, Saruhashi, et al., 1996, Schmidt and Jordan, 2000), so we measured the level of

serotonergic fibers caudal to the lesion site in spinal cords of fibrin-treated and untreated animals by immunostaining for serotonin (5-hydroxytryptamine, 5-HT). The density of 5-HT fibers caudal to the lesion site appeared greater in spinal cord sections from animals treated with salmon fibrin than those from animals treated with human fibrin or untreated controls (Fig. 5A). Quantitative analysis of the percentage of 5-HT signal in the region of the cord immediately caudal to the lesion site revealed higher levels of 5-HT in the salmon fibrin-treated group (Fig. 5B). Salmon fibrin-treated animals retained ~30% caudal 5-HT innervation, which was significantly greater than the values for animals treated with human fibrin (~14%) or untreated controls (~18%).

While neither GFAP staining nor lesion volume correlated well with recovery of locomotor function, there was a positive correlation between the percentage of caudal serotonergic fibers and locomotor function when animals from all groups were analyzed individually (Fig. 5B). Confocal microscopic analysis of spinal cord sections at the lesion site shows association of neurofilament H (NFH)-positive neuronal axons with salmon fibrin (Fig. 5C), indicating the potential for direct protective effects of salmon fibrin on neurons and neuronal circuitry. The early beneficial effects of salmon fibrin on locomotor and bladder function and the greater density of serotonergic fibers in salmon fibrin-treated animals suggest salmon fibrin provides neuroprotection at the time of treatment.

Salmon fibrin treatment does not increase pain and decreases autophagia

SCI can lead to the development of chronic pain that manifests in animal models as mechanical allodynia (perception of innocuous stimuli as painful) of the paws. Some treatments for SCI that augment locomotor function are also associated with increased sprouting of fibers that contribute to allodynia, which limits their potential usefulness as therapies (Hofstetter, et al., 2005). Since treatment with salmon fibrin enhanced locomotor ability and fiber density, we tested the reactions of the animals in our studies to mechanical stimuli using von Frey filaments. These tests showed that the responses of animals treated with salmon fibrin did not significantly differ from those of animals treated with human fibrin and untreated controls several months post injury (Fig. 6A), suggesting that while salmon fibrin-treated rats have improved locomotor and bladder functional recovery, the anatomical underpinnings responsible for recovery do not lead to greater mechanical allodynia.

Rats with dorsal hemisection lesions sometimes exhibit autophagia (gnawing of the paws) that leads to significant tissue damage and is thought to be due to paresthesia, or numbness, of the paws (Das, et al., 1989). Since treatment with salmon fibrin improved recovery of locomotor and bladder function (motor systems) we wondered whether salmon fibrin treatment would also affect sensory systems. We monitored the incidence of autophagia among rats treated with human or salmon fibrin and found that fewer rats treated with salmon fibrin exhibited autophagia than those treated with human fibrin (Fig. 6B). These data suggest that salmon fibrin treatment preserves sensory as well as motor neuronal systems.

Discussion

The data presented herein demonstrate that rats with a dorsal hemisection SCI treated with injectable salmon fibrin show improved recovery of both hindlimb locomotor function and bladder control. Salmon fibrin treatment results in greater density of serotonergic innervation caudal to the lesion site, and the early amelioration of bladder and locomotor function make it likely that this is due to axonal sparing rather than regeneration. Salmon fibrin injected as a liquid forms a solid in the lesion site and is found in close association with axons. Injured rats treated with salmon fibrin may also experience better sensory

function since in comparison to untreated or human fibrin-treated rats they engage in less autophagia and do not suffer from enhanced allodynia. In contrast with results of other scaffold treatments, rats treated with salmon fibrin did not manifest differences in glial scar formation or lesion size and these parameters did not correlate well with functional recovery. The beneficial effects of salmon fibrin were long-lasting, persisting after fibrin degradation. Species-specific differences in salmon and mammalian fibrins likely underlie their distinct effects on CNS injury.

Scaffold-induced functional recovery

Over the past ~30 years, many studies have investigated the utility of scaffolds in animal models of SCI. However, relatively few have tested whether scaffold treatment is associated with functional improvement. Our study measured two independent readouts of functional recovery, bladder function as measured by urine retention and locomotor function as assessed by the BBB scale. Treatment with salmon fibrin at the time of injury significantly improved recovery of bladder function of rats after dorsal hemisection SCI and recovery of bladder function correlates well with locomotor recovery in individual animals (Fig 3). We could find no other studies in which bladder function was measured after scaffold treatment of SCI. Testing of locomotor function showed that BBB scores of salmon fibrin-treated animals were on average 2–3 points higher than untreated controls or animals treated with human fibrin (Fig. 2). Furthermore, BBB subscore analysis demonstrated that animals treated with salmon fibrin recovered complex motor functions to a greater degree than animals treated with human fibrin or untreated controls.

Treatment of SCI with preformed solid scaffolds has yielded mixed functional recovery results in animal models. While many scaffolds are supplemented with cells, growth factors, or adhesion peptides, we will limit our discussion to scaffolds without added components in order to make the clearest comparison to our unsupplemented fibrin scaffolds. A solid PLGA scaffold containing linear guidance channels aligned with the white matter tracts of the spinal cord has been shown by several groups to promote axonal growth into the channels (Rauch, et al., 2009, Teng, et al., 2002, Yang, et al., 2009). An early publication reported improvements in locomotor function of rats due to treatment with this type of scaffold even in the absence of embedded stem cells (BBB scores of scaffold treated animals 9 and untreated controls 7)(Teng, et al., 2002). However, later studies by two separate groups failed to find differences in BBB scores between animals treated with a similar PLGA scaffold and untreated controls (BBB scores all groups 10 (Yang, et al., 2009), or 11 (Rauch, et al., 2009)). The inconsistency in the functional recovery of rats treated with PLGA scaffolds may be in part related to toxic effects of the scaffold on host tissue since PLGA generates an acidic environment when it degrades (Park, et al., 1995).

Similarly mixed results have been reported for the use of solid scaffold large-bore single channel guidance tubes for the treatment of SCI. In one case, the treatment of a complete transection SCI with a porous poly(2-hydroxyethyl methacrylate-co-methyl methacrylate) (PHEMA-MMA) tube resulted in BBB scores of 7 for the treatment group compared to 1.4 for untreated controls (Reynolds, et al., 2008). Another study using reinforced PHEMA-MMA tubes documented a more modest improvement in the treatment group (BBB scores of ~4 compared to ~2 for the control group) and the channels caused complications: treated animals developed syringomyelia in the rostral stump of the cord, which continued to expand and led to caudal migration of the rostral stump within the channel (Nomura, et al., 2006). A number of other studies using similar single channel guidance tubes to treat complete transections have demonstrated growth of axons into the channel but no augmentation of BBB scores relative to control groups (Nomura, et al., 2008, Nomura, et al., 2008, Tsai, et al., 2004). For both PLGA and single channel solid scaffolds, the growth of axons into the linear channel does not necessarily translate into better locomotor recovery.

Injectable scaffolds of several different materials have been tested in rodent models of SCI to determine effects on locomotor function recovery. Rats with a dorsal hemisection SCI treated with human fibrin two weeks post-injury did not exhibit improved BBB scores whether fibrin was injected as a liquid and polymerized in situ or pre-polymerized and implanted as a soft scaffold (Johnson, et al., 2010, Johnson, et al., 2010). These data are consistent with our findings that rats treated with human fibrin did not manifest improved BBB scores, although the timing and method of scaffold implantation differed. A self-assembling peptide amphiphile that displays a portion of the laminin sequence has been tested as an injectable nanofiber scaffold in SCI rodent models (Tysseling, et al., 2010, Tysseling-Mattiace, et al., 2008). BBB ratings of rats with a T13 contusion injury showed that animals treated with injection of the peptide amphiphile 0.5 mm rostral and caudal to the lesion site 24 hours after injury scored 12.7 while untreated controls scored 9.3 (Tysseling, et al., 2010). The same group has reported similar improvements in the locomotor function of SCI mice with peptide amphiphile treatment (Tysseling, et al., 2010, Tysseling-Mattiace, et al., 2008). Similar to rats injected with salmon fibrin, rats treated with peptide amphiphile experienced sustained improvements in locomotor function and enhanced serotonergic innervation caudal to the lesion site (as discussed further below). Finally, poly(laminin), which is a polymeric form of laminin generated under acidic conditions, has been tested in a partial dorsal transection (80–90% of the cord severed) rat model of SCI. Poly(laminin) was injected rostral and caudal to the lesion site in an acidic buffer 30 minutes after injury and locomotor analysis revealed that the BBB scores of treated animals were 17 while untreated controls were 13.2 (Menezes, et al., 2010). These BBB values are similar to those of our second experiment in which the final BBB scores of salmon fibrin-treated animals were 17 while control scores were 13.5 (Supplemental Fig. S1). Similar to treatment with salmon fibrin, the beneficial effects of poly(laminin) were evident at 1 week post-treatment. Improved locomotor function has been demonstrated with a variety of injectable scaffolds, suggesting that linearly oriented guidance channels are not necessary for enhanced neuronal connectivity and function after SCI.

The enhanced locomotor recovery associated with salmon fibrin treatment is similar to that found with treatment by peptide amphiphile or poly(laminin) although the means by which these scaffolds act are likely different. Salmon fibrin was injected directly into the lesion site, rather than into the tissue rostral and caudal to the lesion site, and may provide neuroprotection by efficiently establishing hemostasis. Salmon fibrin acts as an effective haemostatic in aortic rupture and skin lesion models in swine (Rothwell, et al., 2005, Rothwell, et al., 2009). By rapidly contributing to hemostasis, salmon fibrin would decrease the infiltration of mammalian (in this case, rat) fibrinogen and limit infiltration of immune cells, both of which may contribute to neuroprotection and functional recovery (Ryu, et al., 2009). It will be of interest in future studies to test salmon fibrin in a contusion model of SCI in which treatment can be implemented at various time periods after the initial injury to determine the relative contributions of haemostatic and other effects of salmon fibrin on locomotor recovery.

Serotonergic innervation caudal to the lesion

Salmon fibrin treatment is associated with early but sustained amelioration of locomotor function. Although salmon fibrin degrades within the first week after treatment, its positive effects on BBB scores are stable for more than two months, which was the longest time point tested (Fig. 2). A probable neuronal correlate underlying better locomotor function is the greater serotonergic innervation caudal to the lesion site in animals treated with salmon fibrin (Fig. 5). Serotonergic innervation has been clearly linked to locomotor function. Serotonergic fibers originate in the brain stem nuclei and descend throughout the spinal cord (raphespinal projections) and, even in the absence of treatment, recovery of locomotor

function after SCI correlates well with density of serotonergic innervation (Powers and Binder, 2001, Saruhashi, et al., 1996, Schmidt and Jordan, 2000). Consistent with this, the locomotor improvements of individual animals in our study, without regard to treatment group, correlated well with density of serotonergic innervation caudal to the lesion (Fig. 5). A variety of treatments that boost functional recovery after SCI, such as administration of growth factors or agents that block the Nogo receptor, lead to increases in caudal serotonin-containing fibers (Coumans, et al., 2001, Li, et al., 2004, Piantino, et al., 2006, Tobias, et al., 2003). Supplementation of serotonin after SCI, either by intrathecal injections of serotonin or transplantation of embryonic raphe cells, ameliorates the locomotor function of treated animals (Feraboli-Lohnherr, et al., 1999, Ribotta, et al., 2000). Serotonin treatment initiates the rhythmic pattern of hindlimb locomotion in animals with complete transection of the spinal cord (Barbeau and Rossignol, 1991).

In addition to salmon fibrin, a peptide amphiphile and polylaminin injectable scaffolds are associated with increased caudal serotonergic fiber density and improved locomotor ability (Menezes, et al., 2010, Tysseling, et al., 2010). Although the site of implantation and initial mechanism of action are likely different for these three scaffolds, they appear to all finally affect function of serotonergic neurons. Augmentation of serotonergic systems appears to be common for injectable scaffolds that boost locomotor function, showing that neuronal function can be augmented without linearly oriented guidance channels.

Salmon fibrin's early but lasting effects on rat locomotor function (Fig. 2), rapid improvement of bladder function (Fig 3), close association with neurofilament-positive axons (Fig. 5C), and ability to enhance the density of serotonergic fibers caudal to the lesion (Fig. 5) make it likely that although salmon fibrin *in vitro* enhances neurite extension (Ju, et al., 2007), its effects *in vivo* are neuroprotective rather than regenerative. Consistent with this interpretation, there was no increase in pain sensation of salmon fibrin-treated animals as has been reported with some therapies that encourage axonal sprouting (Hofstetter, et al., 2005). Protection may occur when neuronal axons not damaged by the initial injury are shielded by salmon fibrin from the harmful secondary effects of the lesion (Beattie, et al., 2000, Tator and Fehlings, 1991). Salmon fibrin's activation of cell surface integrins stimulates extension of neuronal processes (Ju, et al., 2007) and integrin ligation can also increase cell survival (Stupack and Cheresch, 2002). The close association of salmon fibrin with neurons adjacent to the lesion site may provide stimulation of neuronal integrins to enhance post-injury axonal integrity and neuronal survival.

Glial scar and lesion volume

Formation of the glial scar is a complex process that may provide beneficial functions early by repairing the blood-brain barrier, restricting inflammation, and limiting the size of the lesion cavity but hinder regeneration later via the deposition of molecules inhibitory to axonal growth such as chondroitin sulfate proteoglycans (Faulkner, et al., 2004, Silver and Miller, 2004). Glial scar development has been linked to the eventual size of the lesion cavity since manipulations that reduce gliosis lead to expansion of the cavity boundaries (Faulkner, et al., 2004). Our quantitative analysis of glial scar formation and lesion volume demonstrated that rats treated with salmon fibrin had improved functional recovery without alteration in the extent of glial scarring or lesion size (Fig. 4). In contrast, lateral hemisection SCI animals treated with implantation of a preformed solid PLGA scaffold with linear guidance channels had less glial scar formation than controls but did not experience behavioral improvements (Rauch, et al., 2009). Furthermore, while human fibrin treatment provides early reductions in glial scar formation it does not alter lesion size or ameliorate locomotor functional deficits (Johnson, et al., 2009, Johnson, et al., 2010, Johnson, et al., 2010, Taylor, et al., 2006). Conversely, animals injected with peptide amphiphile exhibited reduced glial scar formation and significantly ameliorated locomotor recovery (Tysseling-

Mattiace, et al., 2008) and those treated with poly(laminin) scaffold appear to have decreased lesion size and enhanced locomotor recovery (Menezes, et al., 2010). The inconsistent correlation of glial scar formation or lesion volume with functional recovery in scaffold treated animals is most likely due to the mode of action of the scaffold or the manner in which the scaffold affects the tissue. Functional recovery in our study was more directly related to neuroprotection since the locomotor function of individual animals showed an association with density of serotonergic neuronal axons (Fig. 5) but not with glial scar formation or lesion size (Fig. 4). The functional improvements associated with salmon fibrin treatment were not due to incomplete lesions within that group because there was no difference in the lesion sizes of salmon fibrin-treated and untreated control rats (Fig. 4). Although animals treated with some scaffolds, including other injectable materials, manifest reduced glial scar formation or lesion volume, rats treated with salmon fibrin sustain improved functional recovery without alteration of these elements of the dorsal hemisection injury.

Unique characteristics of salmon fibrin

Although salmon and human fibrin function similarly in coagulation cascades (Wang, et al., 2000), differences in salmon and mammalian fibrinogen can be detected by anion exchange chromatography (Ju, et al., 2007) and 2D gel electrophoresis (Laidmae, et al., 2006). These differences are likely to underlie the distinct effects of salmon and mammalian fibrin on cells. While salmon fibrin stimulates elongation of neuronal dendrites and axons *in vitro*, mammalian fibrin is inhibitory (Ju, et al., 2007, Schachtrup, et al., 2007). Effects of both fibrins on neurons are integrin dependent, making it likely that inherent differences exist in the fibrinogen-integrin linkage or the intracellular signaling cascades that are stimulated by binding. A comparison of the amino acid sequences of fish and mammalian fibrinogen indicates differences in the location and number of integrin-binding RGD sequences (Ju, et al., 2007). The differences in RGD sequences may affect accessibility for binding to cellular integrins and may be involved in differential integrin activation by salmon and mammalian fibrin. Activation of cellular integrins affects cell survival, making it possible that direct interaction of neurons with salmon fibrin via integrin binding promotes greater neuroprotection than binding to mammalian fibrin.

Fibrin from divergent species may differentially influence additional cell types after injury. Endogenous mouse fibrinogen is thought to serve as a signal to recruit astrocytes after injury (Schachtrup, et al., 2010) and if all fibrinogens acted similarly, we would predict that treatment of SCI with fibrin would serve to increase the local concentration of fibrinogen and enhance gliosis. However, while there is increased glial scar formation in animals treated with human fibrin there is not a similar expansion in animals treated with salmon fibrin (Fig. 4). Salmon fibrin appears to have different effects than mammalian fibrin on two key cell types of the CNS, astrocytes (Fig. 4) and neurons (Fig. 5, (Ju, et al., 2007)). Furthermore, salmon and mammalian fibrin may differ in their effects on migratory cells that impact neurons near the injury site, including immune modulators such as neutrophils and macrophages/microglia (Adams, et al., 2007).

Conclusion

In conclusion, salmon fibrin provides several key characteristics of a scaffold to treat CNS injury since it is an injectable matrix that forms a solid in the injury site, can be compliance-matched to CNS tissue, does not contain infectious agents found in mammalian fibrin, and clearly has effects distinct from those of mammalian fibrin. Treatment of the injured spinal cord with salmon fibrin led to greater locomotor and bladder functional recovery and enhanced serotonergic innervation caudal to the injury site without altering formation of the glial scar or the size of the lesion cavity. An injectable scaffold can therefore have

significant functional consequences without linear regeneration of axon tracts and obvious modulation of the glial scar. Thus, injectable matrices that target recovery of neuronal populations rather than glial scarring may provide greatest functional improvements after SCI.

Highlights

Unique injectable scaffold, salmon fibrin, implanted for spinal cord injury.
 Rats treated with salmon fibrin significantly improve locomotor and bladder function.
 Greater serotonergic innervation occurs caudal to the lesion but no increase in pain.
 No change found in glial scar formation or lesion volume.
 Injectable scaffolds lead to functional improvement without linear guidance channels.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Salmon fibrin gels form in lesion sites. (A) An anti-salmon fibrinogen antibody specifically detects salmon fibrinogen on Western blots. Analysis of total protein (left panel) shows that similar amounts of salmon, human, and bovine fibrinogen chains were present on the blot. The salmon fibrinogen chains are strongly detected by the antibody while human chains were not detected and only minimal reactivity of the higher molecular weight bovine A α chain was evident. (B) Anti-salmon fibrinogen antibody robustly stains salmon fibrin gel cryosections but does not stain rat fibrin gel cryosections above background levels. Scale bar = 100 μ m. (C) Rats were subjected to dorsal hemisection spinal cord lesions and injected with salmon fibrin in the lesion site. Cryosections of spinal cords stained with the antibody to salmon fibrinogen show intact fibrin gel in the lesion site 2 days after treatment (red-salmon fibrin; green-GFAP to detect astrocytes; blue-cell nuclei). Scale bar top panel = 300 μ m, bottom panel = 100 μ m.

Figure 2.

Salmon fibrin treatment of dorsal hemisection SCI leads to improved locomotor function. (A) Locomotor behavior was assessed by BBB testing beginning one day after surgery and continuing until ~10 weeks post-surgery (salmon fibrin $p < 0.05$ repeated measures ANOVA, error bars represent s.e.m., $*p < 0.05$ and $**p < 0.01$ by Bonferroni post-test). (B) BBB subscore analysis applied to the highest performing animals in each group shows that the salmon fibrin-treated animals exhibited greater fine motor control, for example paw position, toe clearance, tail position, and trunk stability, than animals in the other groups (salmon fibrin $p < 0.05$ repeated measures ANOVA, error bars represent s.e.m., $*p < 0.05$ by Bonferroni post-test).

Figure 3.

Salmon fibrin treatment improves bladder function after SCI. (A) The volume of urine retained in the bladders of injured animals was measured for several days post-injury. Salmon fibrin-treated animals retain less urine than animals treated with human fibrin or left untreated (salmon fibrin $p < 0.05$ repeated measures ANOVA, error bars represent s.e.m., * $p < 0.05$ compared to controls and ** $p < 0.01$ compared to human fibrin-treated rats by Bonferroni post-test). (B) Analysis of all rats without regard for group shows that bladder function measured 4 days after injury and locomotor function at 70 days post-injury correlate with each other ($R = 0.53$, each point represents data from a single animal).

Figure 4.

Functional improvements of salmon fibrin-treated animals are not due to effects on astrocytes or lesion volume. (A) Immunostaining for GFAP reveals similarities in the glial scar along the edges of the lesion cavities in sagittal sections of cords from untreated controls and animals treated with human or salmon fibrin. Scale bar = 300 μ m. (B) Glial scar formation in the salmon fibrin-treated animals was not different from that in control animals, but human fibrin-treated animals had greater glial scarring (left panel, human fibrin $**p < 0.01$, $*p < 0.05$, error bars represent s.e.m.). The amount of glial scarring did not correlate with improvements in locomotor function for any of the animals, suggesting that this parameter of the injury was not directly linked to the observed functional improvements (right panel, $R = 0.14$, each point represents data from a single animal). (C) Volume of the lesion cavity in salmon fibrin-treated animals was not different from that in control animals while human fibrin-treated animals had larger lesions than untreated control animals (left panel, human fibrin $*p < 0.05$, error bars represent s.e.m.). Lesion volume for animals in all groups did not correlate with functional improvement in locomotor ability (right panel, $R = 0.21$, each point represents data from a single animal).

Figure 5.

Salmon fibrin-treated animals have more serotonergic fibers caudal to the lesion site, which correlate with locomotor improvements. (A) Sagittal spinal cord sections immunostained for 5-HT reveal a greater density of serotonergic axons caudal to the lesion site in animals treated with salmon fibrin compared to animals treated with human fibrin and untreated controls. Boxed areas highlight regions of the cords caudal to the lesion site. Scale bar = 300 μ m. (B) Quantitation of the 5-HT-positive tissue rostral and caudal to the lesion site revealed that salmon fibrin-treated animals had a greater percentage of caudal 5-HT than animals in the other groups (left panel, $**p < 0.01$, $*p < 0.05$, error bars represent s.e.m.). The extent of caudal 5-HT axons for animals in all groups positively correlates with locomotor improvements (right panel, $R = 0.54$, each point represents data from a single animal). (C) Salmon fibrin colocalizes with axons near the lesion site. Confocal microscope images show salmon fibrin marked by an anti-salmon fibrin antibody (green) directly apposed to axons stained with an antibody to NFH (red) 2 days after treatment. Some areas of colocalization (yellow) are marked by arrows.

Figure 6.

Animals treated with salmon fibrin experience no increase in pain and engage less in autophagia. (A) Assessment by von Frey hairs shows no difference in the stimulus threshold responses of rats in the three groups 68 days after injury and treatment (forelimbs: human fibrin $p=0.6$, salmon fibrin $p=0.6$; hindlimbs: human fibrin $p=0.3$, salmon fibrin $p=0.4$, error bars represent s.e.m.). (B) Animals treated with salmon fibrin experience less autophagia than animals treated with human fibrin. At 6 weeks post-injury almost 40% of animals treated with human fibrin exhibited severe autophagia whereas none of the animals treated with salmon fibrin were affected.