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PEGylated interferon-β modulates the acute inflammatory response and recovery when combined with forced exercise following cervical spinal contusion injury

Harra R. Sandrow-Feinberg[1], **Victoria Zhukareva**[1], **Lauren Santi**[1], **Kassi Miller**[1], **Jed S. Shumsky**[1], **Darren P. Baker**[2], and **John D. Houle**[1]

[1]Department of Neurobiology and Anatomy Drexel University College of Medicine

[2]Biogen Idec Incorporated 14 Cambridge Center Cambridge, MA 02142

Abstract

Secondary degeneration leads to an expansion of the initial tissue damage sustained during a spinal cord injury (SCI). Dampening the cellular inflammatory response that contributes to this progressive tissue damage is one possible strategy for neuroprotection after acute SCI. We initially examined whether treatment with a PEGylated form of rat interferon-beta (IFN-β) would modulate the expression of several markers of inflammation and neuroprotection at the site of a unilateral cervical level 5 contusion injury. Adult female Sprague-Dawley rats were injured using the Infinite Horizon Impactor at a force of 200Kdyne (equivalent to a severe injury) and a mean displacement of 1600–1800 μm. A single dose (5×10^6 units) of PEGylated IFN-β or vehicle was administered 30 minutes following SCI. Here we demonstrate temporal changes in pro- and anti-inflammatory cytokine levels and the expression of heat shock proteins and iNOS (involved in neuroprotection) at the lesion epicenter and one segment caudally after SCI and PEG IFN-β treatment. The results suggested a potential therapeutic treatment strategy for modulation of secondary damage after acute SCI. Therefore, we examined whether acute treatment with PEG IFN-β would improve forelimb function alone or when combined with forced exercise (Ex). Animals began the Ex paradigm 5 days post SCI and continued for 5 days per week over 8 weeks. Locomotion (forelimb locomotor scale [FLS], hindlimb BBB, and TreadScan) and sensorimotor function (grid walking) was tested weekly. Additional outcome measures included lesion size and glial cell reactivity. Significant FLS improvements occurred at 1 week post SCI in the PEGylated IFN-β-treated group but not at any other time point or with any other treatment approaches. These results suggest that this acute neuroprotective treatment strategy does not translate into long term behavioral recovery even when combined with forced exercise.

INTRODUCTION

Spinal cord injuries (SCI) compromise the integrity of the central nervous system (CNS) by damaging motor and sensory pathways. Tissue immediately affected by a mechanical impact readily degenerates as a result of multiple factors including physical trauma, ischemia, hypoxia,

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Corresponding Author: John D. Houle, Department Neurobiology and Anatomy, Drexel University College of Medicine, 2900 Queen Lane, Philadelphia, PA 19129, Phone: (215) 991-8295, Fax: (215) 991-8307, jhoule@drexelmed.edu.

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hemorrhage, and edema (reviewed by Schwab, 2006). This is followed by a cascade of secondary degeneration events (Schwab and Bartholdi, 1996) initiated by neutrophils recruited to the CNS from the circulation, while, at the same time, microglia and astrocyte activation occurs in a spreading zone of injury (Popovich et al., 1997; Beattie, 2004). Activated immune cells release many molecules (cytokines, chemokines, free radicals, matrix metalloproteinases, growth factors, and proteolytic enzymes) that are both beneficial and damaging to spinal cord tissue (Bethea, 2000; reviewed by Donnelly and Popovich, 2007). The rapid increase in local levels of interleukin-6 (IL-6), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) initiate a neuroinflammatory process, which can last from weeks to months. Inflammation within the central nervous system (CNS) can also be beneficial, in that exogenous phagocytic cells and endogenous microglia and astrocytes recruited to the lesion site aid in removing necrotic debris and initiate formation of a glial scar which has some protective features apart from its limitation of axon regeneration. Therefore, maintenance of this delicate balance should be taken into consideration when implementing treatment designed to curb the destructive events caused by neuroinflammation without hindering the beneficial effects of the immune response.

Anti-inflammatory pharmacotherapy is a strategy used in the clinic to treat non-SCI neurodegenerative conditions. Interferon-β (IFN-β) is an immunomodulatory cytokine which alters the expression of pro-and anti-inflammatory cytokines to decrease destruction of neural tissue occurring in multiple sclerosis (MS) (Chabot and Yong, 2002; Ersoy et al., 2005). A study by Gok et al. (2007) investigated the effect of human IFN-β (1×10^7 International units given immediately after injury followed by 5×10^6 International units given 4 h post-injury) after a thoracic contusion in rats, and showed significant functional changes 24 hours post-injury with decreasing levels of myeloperoxidase and lipid peroxidation, thereby limiting the extent of tissue damage. However, caution in interpreting the results of this study is advised since the authors used Betaferon®, a form of human IFN-β that is approved for the treatment of multiple sclerosis, and which is formulated in mannitol and human serum albumin. Since IFNs-β show varying degrees of species specificity (see for example, Arduini et al., 2004), and since the authors did not run a mannitol and human serum albumin control group (at the 1×10^7 and 5×10^6 International unit doses, the rats also received 15 and 7.5 mg of human serum albumin), an independent assessment of the efficacy of the IFN-β cannot be confirmed. More relevant, however, is a study by Garg et al. (2006) in which rat IFN-β was used after a thoracic spinal cord contusion injury in rats. In this case, an increase in white and grey matter sparing, as well as the presence of significantly more preserved Neu+ neurons at both 1000 and 2000 μm from the lesion epicenter, were observed when single doses of 5×10^3 units or 5×10^4 units of IFN-β were given subcutaneously 2 h post-injury. These results led us to complete a dose response study with rat IFN-β as an acute treatment for a unilateral cervical contusion injury in rats. Five daily intraperitoneal doses (5×10^4– 5×10^6 units per 0.1mL dose) were administered beginning 30 minutes post SCI. We examined the injured forelimb over a 6 week period using the Forelimb Locomotor Scale and found that the group receiving the highest dose (5×10^6 units per 0.1mL dose) of IFN-β regained extensive movement of one joint (mean FLS= 5.01 ± SEM 1.91) while the vehicle and moderate dose (5×10^5 units per 0.1mL dose) group could slightly move one joint 2 days post SCI (mean FLS= 1.80± SEM 0.20 for both the vehicle and moderate dose unpublished results). The recovery seen two weeks after injury was not sustained over the 6 week period, possibly due to the fluctuating levels of the drug since it has an elimination half-life of 2.9 h following intraperitoneal administration (Arduini et al., 2004).

A major drawback with IFN-β drug therapies has been the short half life of the protein, making frequent administration necessary to maintain an effective dose. Polyethylene glycol (PEG) added to the N-terminal end of either human or rat IFN-β (PEG IFN-β) increases its effective half life (Arduini et al., 2004; Baker et al., 2006; Basu et al., 2006). When determining the

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pharmacokinetics of the modified protein, Baker and colleagues found by intravenous administration and serum sampling at hourly time points in adult rats that the PEGylated form of human IFN-β had a half-life of 13 hours compared to less than 1 hour for the unmodified protein (Baker et al., 2006). In our study, we chose to use the PEGylated form of rat interferon-β (PEG IFN-β), a molecule with a 7 h intravenous half-life in rats as compared to 0.8 h for the unmodified protein (Arduini et al., 2004), to increase the exposure of the drug and to decrease the number of administrations. Indeed, Baker et al. (2006) have shown that a single administration of 1×10^6 units of PEGylated human IFN β-1a was as effective as nine daily doses of 1×10^6 units of the unmodified protein in a human melanoma angiogenesis xenograft model in *nu*/*nu* mice. Due to the prolonged exposure of the compound, the second phase of inflammation, the so-called reparative phase, may also be affected by possible increases in the levels of anti-inflammatory cytokines. We hypothesized that administering PEG IFN-β shortly after SCI would alter the pattern of inflammatory events occurring at the molecular level. To test this hypothesis we measured the cytokine response at the lesion site and one segment caudal at several time points after injury: 6 hours, 24 hours, 3 days, and 5 days.

The same tissue samples were used to identify the biochemical profile of several heat shock proteins (HSPs) and inducible nitric oxide synthase (iNOS) after the contusion injury and anti-inflammatory treatment with PEG IFN-β. HSPs are highly conserved and made up of ubiquitous components they are induced by a number of conditions stressful to the CNS including ischemia and injury. A number of HSPs are constitutively expressed in the CNS and their upregulation is tightly linked to neuroprotection. Although HSPs primarily function as molecular chaperones to facilitate proper protein folding and stabilization in normal and pathological conditions, stress-induced HSPs also have significant roles in inflammatory signaling pathways, immune responses, and in regulation of apoptosis (Moseley, 1998; Chen et al., 2004; van Noort, 2008). The role of HSPs in SCI-mediated intracellular signaling is not well understood, although the over-expression of HSPs in response to injury is suggested to be an important mechanism in neuroprotection (Sharma et al., 2006).

Upregulation of pro-inflammatory cytokines after SCI triggers a significant production of nitric oxide (NO), leading to possible neurotoxic effects during the secondary lesion phase (Beckman et al., 1994). NO synthesis is regulated by several nitric oxide synthases (NOS), and an inducible form of the enzyme, iNOS, is implicated in the pathogenesis and inflammatory reaction after SCI (Conti et al., 2007). Activation of iNOS occurs in damaged tissue at the transcriptional level within several hours after the injury and it progressively increases over several days (Xu et al., 2001; Conti et al., 2007). We examined whether this anti-inflammatory drug treatment would reduce NO-elicited damage after SCI by decreasing the expression of iNOS.

Many studies have focused on modulating the destructive aspects of the central immune response or on amplifying its reparative effects (reviewed by Tator, 2006; reviewed by Schwartz and Yoles, 2006), but therapeutic approaches targeting a single facet of SCI have yet to provide substantial functional or anatomical recovery. Combination therapies targeting different elements of the injury are currently being proposed as a more beneficial approach. Neuroprotective strategies aim at preventing spared but damaged cells from succumbing to secondary degeneration. Two examples of neuroprotective therapies previously used to treat SCI are the acute delivery of anti-inflammatory drugs and physical rehabilitation (reviewed by Baptiste and Fehlings, 2007).

Interferon-β (IFN-β) is an agent that has been shown to decrease the progression of multiple sclerosis (MS), a chronic neuroinflammatory disease (Javed and Reder, 2006). During the course of this disease, myelin sheaths of brain and spinal cord axons are damaged by a local inflammatory response that is similar to the destruction of white matter during secondary

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degeneration after SCI. Studies investigating the mechanistic actions of IFN-β indicate decreased activation of monocytes and macrophages, reduced proliferation of T-cells in the periphery (Noronha et al., 1993; Javed and Reder, 2006), and modified expression of both pro- and anti-inflammatory cytokines (Chabot and Yong, 2000; Ersoy et al., 2005). In vitro T-cell treatment with IFN-β followed by interactions with microglia resulted in increased levels of the anti-inflammatory cytokine, interleukin-10 (IL-10) (Chabot and Yong, 2000). An increase in IL-10 has important downstream effects by reducing macrophage expression of tumor necrosis factor-α (TNF-α), a potent inflammatory signaling molecule (Bethea et al., 1999; Brewer et al., 1999). By diminishing the recruitment of immune cells and production of pro-inflammatory cytokines it may be possible to reduce tissue loss resulting from neuroinflammation.

Rehabilitative strategies, including exercise (Ex) of limbs affected by SCI, may contribute to recovery by increasing the production of neurotrophic factors (NTF) in muscle and spinal cord, thereby preventing muscle atrophy and promoting reorganization of spinal circuitry (Gomez-Pinilla et al., 2002;Vaynman and Gomez-Pinilla, 2005). Transcription and translation of proteins involved in cell protection and plasticity, such as c-fos, heat shock proteins, and synapsin also are increased by Ex (Dupont-Versteegden et al., 2004; Vaynman et al., 2003; Sandrow et al., 2008). A previous study from our lab investigated the effects of Ex after a cervical contusion and found an acceleration in the early phase of recovery in the FLS and grid tests (Sandrow-Feinberg et al., 2009). Thus Ex can improve function and aid in neuroprotection, by a mechanism different from PEG IFN-β.

In the present study, we describe biochemical approaches to quantitatively evaluate early post-SCI changes in the expression of pro- and anti-inflammatory cytokines, HSPs and iNOS in rat spinal cord tissue after cervical contusion injury and acute administration of PEG IFN-β. We also tested the hypothesis that using PEG IFN-β as an acute intervention after SCI in conjunction with long term forced Ex would prolong and enhance the neuroprotective effects displayed by these two therapies. Absence of an additive outcome may be due to either the short exposure to PEG IFN-β or the more dramatic improvements with forced Ex.

MATERIALS AND METHODS

Spinal Cord Injury

Acute Biochemical Study—Adult (225–250 g; n=45) female Sprague-Dawley rats (Charles River) were randomly assigned to vehicle or PEG IFN-β groups. Four time points post treatment were evaluated: 6 hour, 24 hour, 3 day, and 5 day. Each rat was anesthetized with a ketamine (60 mg/kg) and xylazine (6 mg/kg) mixture. A partial C5 laminectomy exposed the right side of the spinal cord and forceps, fixed to the base of an Infinite Horizon Impact Device (Precision Systems and Instrumentation Lexington, KY), were used to stabilize the cord by clamping the C2 and C6 vertebral bodies. A unilateral contusion injury was created using a mean impact force of 200 Kdyne with tissue displacement to a depth of 1600–1800 μm. After injury, animals were released from the clamping forceps and overlying muscles were sutured. For the 6 hour time point, a sham-operated group with laminectomy only was included as a control. The skin incision was closed with wound clips. Animals were given ampicillin (100 mg/kg) and the analgesic buprenorphine (0.05 mg/kg) for 3 days post operatively, and were maintained on food and water *ad lib*.

Chronic Behavioral Study—Adult (225–250 g) female Sprague-Dawley rats were randomly assigned to one of four groups: vehicle only (n=7), PEG IFN-β (n=8), vehicle + Ex (n=7) or PEG IFN-β + Ex (n=8). SCI was performed in the same manner as described for the acute study. PEG IFN-β or vehicle (0.1mL, ip) was administered 30 min following SCI. All procedures were performed in accordance with protocols approved by the Drexel University

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College of Medicine Institutional Animal Care and Use Committee and followed National Institutes of Health guidelines for the care and use of laboratory animals.

Preparation of PEG IFN-β and Vehicle-Matched Control

PEGylated rat IFN-β (Arduini et al., 2004) was prepared as a 3.75×10^6 units/mL solution in phosphate buffered saline pH 7.0 containing 3 mg/mL rat serum albumin (Sigma) as a carrier protein. 170 μL (~6.3×10^5 units) was administered as a single dose. Vehicle-treated animals were administered with 170 μL of phosphate buffered saline pH 7.0 containing 3 mg/mL rat serum albumin. Endotoxin analysis of the dosing solutions using the *Limulus* amebocyte lysate Endosafe PTS cartridge system (Charles River Laboratories) showed that the 170 μL dose of vehicle control and PEG IFN-β contained 0.22 and 0.21 endotoxin units, respectively. Both vehicle and PEG IFN-β were supplied by D. Baker as blinded samples with the blind revealed only after the in-life and analysis phases of the study were completed.

Acute Biochemical Study

Cytokine measurements and Western blot analyses—Animals were overdosed with Euthasol (390 mg/kg sodium pentobarbital and 50 mg/kg phenytoin, ip) and separate 3 mm long spinal cord samples incorporating the lesion epicenter (C5) and caudal (C6) spinal cord were harvested, weighed, and immediately placed in ice cold extraction buffer (50 mM Tris buffer, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.2 % Tween-20) in the presence of protease inhibitors (Roche) and 10 mM phenyl-methyl sulphonyl-fluoride (PMSF). Samples were sonicated and spun at 14,000 rpm (Eppendorf centrifuge, model 5810R) for 30 min at 4°C. Supernatants were collected and aliquots from each sample were used for cytokine measurements using the rat cytokine multiplex kits (Millipore) for the BioPlex instrument (BioRad, Hercules, CA) and for Western blot analyses of HSPs and iNOS.

Cytokine levels (IL-6, TNF-α, IL-1β, IL-1α, IL-18, IL-4, and IL-10) were measured in spinal cord tissue extracts 6 hours post sham operation and 6 hours, 24 hours, 3 days, and 5 days post SCI in PEG IFN-β and vehicle groups (n=5 per group). All data, in pg/mL and/or mean fluorescent intensity (MFI) were normalized to control values to eliminate variability between different experiments. According to the manufacturer, intra-assay and inter-assay variability for different cytokines is reported as between ~5% and ~20%. Millipore cytokine detection kits were originally developed to analyze plasma, serum, and tissue culture medium. Tissue extract analyses should be adjusted for the individual samples/buffers. A standard curve (Std) was generated using known concentration of each cytokine. BioPlex analysis is based on the software generated conversion of MFI to pg/mL. However, one of the assay limitations is the sensitivity of the minimum detectable concentration (MinDC) that varies from ~1 pg/mL to ~50 pg/mL for different analytes (similar to ELISA assay). When MFI values are below Std values, the output reading is "below the level of detection". Therefore, we used MFI values for some analyses that fell into this category, and final data was normalized to control samples. Despite some limitations of the assay, BioPlex analysis of multiple analytes in a single sample is a reliable method for cytokine detection.

Quantitative Western blot analyses were performed on samples resolved on 10% polyacrylamide gels and transferred onto PVDF membrane (BioRad). Each membrane replica was blocked in 5% non-fat milk/Tris buffer saline/0.1% Tween-20 (TTBS), and was sequentially probed with rabbit polyclonal antibodies against HSP27 (dilution 1:1000; Cell Signaling), HSP70 (dilution 1:1000; Cell Signaling), HSP32/HO-1 (dilution 1: 1000, Stress Gene), and iNOS (dilution 1:400; Santa Cruz). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin (IgG, Jackson Laboratories) was used as a secondary antibody. Finally, each membrane was probed with mouse monoclonal anti-actin antibodies (dilution 1:5000, Sigma). The enhanced chemi-luminescent kit (ECL, Amersham) was used to detect

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protein bands. Densitometry analyses of immunopositive bands were performed using Syngen software (Frederick, MD). To account for variability in sample loading and transfer efficiency, all data were normalized to densitometry values of actin for each sample. Final data (mean ± SEM) are presented as a ratio to values from the sham operated control group (dotted line in Fig. 2) for each time point.

Statistical analysis—Two-way ANOVA was used to determine the significant differences between experimental groups receiving PEG IFN-β or vehicle, and sham or normal controls. If significance was found, post-hoc analysis was completed with Mann-Whitney U tests. T-tests determined whether significance ($p<0.05$) existed between groups at each time point. All analysis was completed with SPSS version 16.0 (SPSS Inc., Chicago, IL).

Chronic Behavioral Study

Forced Exercise Paradigm—One week prior to SCI, animals in the Ex rehabilitation groups were acclimated to the forced exercise wheel system (Lafayette Inst., Lafayette, IN) used to increase forelimb and hindlimb activity (as described in Sandrow-Feinberg et al., 2009). Rungs of the wheel were covered with a cloth material to create a relatively smooth yet graspable surface to reduce the possibility of additional injury from limbs falling through the rungs during early stages of Ex. Animals were placed in the wheels for 10 minutes before Ex began, then had a running session of 20 minutes/day, 5 days/week. Rats began Ex 5 days after injury with a wheel speed beginning at 2.5 meters/minute, which was increased daily according to the capabilities of individual animals to a maximum speed of 14.0 meters/minute (comparable to a treadmill training speed).

Behavioral testing—Animals were maintained on food and water *ad lib* with a 12 hour light-dark cycle (lights on at 07:00 AM). After acclimation to the testing apparatus over a 1 week period, baseline scores for forelimb and hindlimb open field locomotion, TreadScan (Clever Sys Inc., Reston, VA), as well as grid walking performance (see below) were obtained for each animal. Two days later, animals were subjected to the unilateral C5 contusion injury. Open field forelimb and hindlimb evaluation began 2d post operatively but most animals had difficulty performing the grid task at this time after injury. Animals were evaluated for all tests beginning 1 week after injury for 8 weeks total. All behavioral testing was started at least 30 minutes following the Ex session and completed between 08:00 and 11:00 AM.

Open Field Locomotion—Forelimb and hindlimb function was evaluated in an open field measuring 2.5 × 3 feet and rats were observed for 4 minutes by two individuals blinded to the treatment condition. The forelimb locomotor scale (FLS), devised in our behavioral core facility from observation of recovery patterns in cervically injured rats, is an 18 point scale that defines deficits based on range of motion, level of weight support, and whether the paw is placed parallel to the body (Cao et al., 2007; Sandrow et al., 2008; Sandrow-Feinberg et al., 2009). The FLS is thus similar in assessment style to the hindlimb BBB (Basso et al., 1995) rating scale. Each animal was scored during direct observation in the open field for 4 minutes and videotaped for later reference if necessary.

Forced locomotion (TreadScan)—The TreadScan device, made up of a clear treadmill with a camera and appropriate lighting to allow for recording of step cycles, was used to obtain and evaluate several aspects of gait. A background image was taken prior to each test day. Forelimb and hindlimb locomotor capabilities were assessed during a 20 second period of forced locomotion. Speed was closely monitored to allow for a consistent walk without causing further impairment. The recorded AVI file was converted to MPEG file and analyzed by the CleverSys TreadScan software.

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Grid-walk (Sensorimotor) Test—Paw placement of forelimbs and hindlimbs was assessed as animals walked on an elevated plastic coated wire mesh grid (36 cm × 38 cm with 3 cm^2 openings). Animals were placed on the grid for 2 min and allowed to walk freely across the platform. Each limb was scored for the total number of steps and the percentage of correct steps and missteps. A misstep occurred when the entire foot fell through the grid. The grid-walk test has been validated as an assessment of sensorimotor function (Grill et al., 1997).

Statistical Analysis—Behavioral data were analyzed by two-way ANOVA between groups and time, with time taken as a repeated measure. The FLS and BBB scores covered a sufficient range over time to approximate a normal distribution and therefore could be analyzed in this manner. However, post hoc analysis for the FLS and BBB data did not meet conditions of normality and therefore were analyzed with the Mann-Whitney U test to examine the differences between: vehicle and PEG IFN-β, vehicle+EX and PEG IFN-β+EX, vehicle and vehicle+Ex, PEG IFN-β and PEG IFN-β+Ex. Post hoc analysis for the grid test was performed using the Bonferroni test. All statistics were conducted using SPSS (version 16.0, SPSS Inc, Chicago, IL).

Immunocytochemistry & Image analysis—At eight weeks post injury, animals were overdosed with Euthasol and perfused transcardially with 4% paraformaldehyde in 0.1M Sorenson's phosphate buffer. C3–C6 spinal cord was removed, post-fixed in paraformaldehyde at 4°C for 4 h, and then immersed in 30% sucrose for 36 h at 4°C. Four series of alternating sections at 25 μm were prepared with a cryostat in a transverse plane through the rostral to caudal extent of the lesion or in a horizontal plane through the dorsal to ventral extent of the lesion. Sections from a single series were mounted on a glass slide and stained for Nissl-myelin to measure lesion size and extent of grey and white matter sparing.

For immunocytochemical labeling, free-floating sections were washed in phosphate buffer and blocked for non-specific reactivity with an appropriate 5% normal serum (goat, NGS or rabbit, NRS), 1% bovine serum albumin and 0.1% Triton-X 100 in phosphate buffer at room temperature for 1 hour. Primary antibody against glial fibrillary acidic protein to detect astrocytes (GFAP, 1:500, Dako, Carpinteria, CA) or the lysosomal membrane of phagocytic cells (ED1 clone, 1:1000, Chemicon International, Temecula, CA) for incubation overnight at 4°C. Sections were washed, incubated with appropriate fluorescently-tagged secondary antibody overnight at 4°C, washed, mounted and cover slipped with VectaShield mounting medium (Vector Laboratories, Burlingame, CA). Sections were examined under with a Zeiss Axioskop microscope bright field for Nissl-myelin or fluorescent light for GFAP and ED1.

To detect changes in lesion size and amount of spared tissue after SCI, the area of contralateral white and grey matter of the spinal cord and spared grey and white matter on the ipsilateral side was measured. Every section that contained the lesion cavity was included in these measurements. MetaMorph (Molecular Devices, Downingtown, PA) software was used to manually trace the damaged area of white and grey matter and to calculate the area of each trace. For densitometric analysis of reactive astrogliosis and phagocytic presence, three representative sections from the lesion epicenter, as well as the rostral and caudal areas, were evaluated for each animal. Each of the sections was thresholded and the density was determined by selecting 4 areas of interest on the labeled ipsilateral tissue from 3 different areas with the MetaMorph software. All of the data was then imported into Microsoft Excel. The densities of reactive tissue were averaged together and combined with their representative group. Statistical significance was determined by calculating an ANOVA (SPSS version 16.0; SPSS Inc, Chicago, IL) followed by post-hoc Students t-tests ($p<0.05$).

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RESULTS

To monitor the effects of PEG IFN-β treatment following SCI, we measured the levels of the pro-inflammatory cytokines IL-6, TNF-α, IL-1β, IL-1α and IL-18, and the anti-inflammatory cytokines IL-4 and IL-10 in normal, sham operated, and injured animals that received vehicle or PEG IFN-β. At the injury epicenter (C5), all of the pro-inflammatory cytokines showed a significant ($p<0.05$) upregulation following injury, although differences were observed in the time to peak upregulation and the time for levels to return to pre-injury levels (Figure 1A). Significant differences between vehicle and PEG IFNβ-treated animals were observed for IL-6 and IL-18. At 6 and 24 h post-injury, the levels of IL-6 were significantly lower in the PEG-IFNβ-treated animals compared to the vehicle-treated animals, and at 5 days post-injury, the level of IL-18 was significantly higher in the PEG IFNβ-treated animals. In the caudal (C6) region, the only pro-inflammatory cytokine to show a significant upregulation following injury was IL-1α, although no significant difference was observed between the levels in the vehicle- or PEG IFNβ-treated groups. TNF-α levels 5 days post-injury were lower in the PEG IFNβ-treated animals than in the vehicle control group even though there was no significant effect of either the vehicle or PEG IFN-β compared to normal or sham operated animals in the C6 region post injury.

For the anti-inflammatory cytokines IL-4 and IL-10, the only significant difference between the injured and normal and sham operated controls was for IL-10, for which levels at both the C5 and C6 regions were higher only in the PEG-IFNβ-treated group (Figure 1B). HSP expression in injured rat spinal cord was spatially and temporally regulated in both vehicle control and PEG IFN-β treated groups. HSP27 levels increased in the epicenter (C5) starting between 24 hours and 3 days after the contusion and reached ~10-fold increase at 5 days post-injury in the PEG IFN-β treated group compared to the sham operated group, which was significantly higher than the vehicle treated group (Figure 2A). The response in samples from the caudal (C6) segment was similar to levels in the epicenter.

The expression profile of the inducible form of HSP70 was upregulated earlier when compared to HSP27 (Figure 2B). An increase in HSP70 after SCI was detected starting at 24 hours through 3 days after injury compared to the sham operated group; with no differences between levels in the lesion epicenter and caudal regions. In animals that received PEG IFN-β after SCI, the level of HSP70 was significantly elevated at 6 hours in the spinal cord followed by no difference between vehicle and PEG IFN-β at 24 hours, and a decrease ($p<0.05$) at 3 days post SCI compared to the vehicle treated group. At 5 days, HSP70 levels in both vehicle and PEG IFN-β treated groups approached levels similar to the sham operated animals.

Upregulation of HSP32 was confined to the lesion epicenter, beginning at 24 hours post injury in vehicle and PEG IFN-β treated animals (Figure 2C). Only minor immunoreactive bands were found in the adjacent caudal region at 24 hours, which were undetectable at later times. PEG IFN-β treatment significantly increased levels of HSP32 at 3 days and the ~90-fold increase was maintained out to 5 days after SCI ($p<0.05$).

Upregulation of iNOS protein began 24 hours post injury in the segment caudal to the injury. Levels in the PEG IFN-β treated tissue both at the injury and caudal levels were significantly decreased compared to the vehicle tissue on days 3 and 5 ($p<0.05$; Figure 2D). Interestingly, the level of expression appeared lower at the epicenter compared to the caudal segment at most time points studied. No significant difference occurred between the C5 and C6 regions.

Behavioral Assessments

Open Field Locomotion (FLS)—Locomotor function of the affected forelimb was evaluated beginning 2 days following the C5 unilateral contusion and weekly thereafter for 8

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weeks (Figure 3A). All groups showed similar deficits at 2 days, with an average score of 2 indicating extensive movement of 1 joint (primarily the shoulder) and slight movement of another, usually the elbow. The similar scores across groups indicated consistency of the injury. At 1 week post SCI, all groups improved although the PEG IFN-β group performed better compared to the vehicle only and PEG IFN-β + Ex groups ($p<0.05$). All groups used the affected forelimb while locomoting in an open field and had recovered plantar placing (FLS: 12 & 13) while the PEG IFN-β group also recovered occasional toe clearance (FLS: 14.25 ± 0.16) at week 8. Thus PEG IFN-β treatment alone showed recovery that was incrementally greater than the other treatment or vehicle.

Open Field Locomotion (BBB)—Evaluation of the ipsilateral hindlimb in an open field began 2 days post SCI and continued weekly for 8 weeks (Figure 3B). Two days following injury all groups plantar placed with weight support in stance only (BBB score around 9). Both Ex groups had significantly higher BBB scores at week 3, indicating forelimb-hindlimb coordination which was not present in either the vehicle or PEG IFN-β only groups ($p<0.05$). The Ex groups also exhibited significant ($p<0.05$) improvements in the affected hindlimbs during weeks 5 through 7 compared to the non-Ex groups. The Ex group scores indicated consistent weight supported steps, and forelimb-hindlimb coordination with paw rotation. The non-Ex groups showed frequent to consistent weight supported plantar placements with occasional forelimb-hindlimb coordination. In this assessment, the Ex provided clear improvements in hindlimb locomotor performance.

Quantitative Locomotion—Both the affected right and unaffected left fore paws were examined to determine placement of the right fore paw onto the treadmill during locomotion and degree of compensation implemented by the left paw. Groups trained in the wheels one week prior to surgery may have altered their gait pattern as depicted by the baseline right fore paw print areas. The forced Ex only and PEG IFN-β + Ex groups had significantly larger print areas compared to the non-Ex groups prior to injury (Vehicle: 628.87 pixel area ± 32.22; Ex: 788.40 pixel area ±50.81; $p<0.05$; Fig. 4A). One week following SCI, there was a decrease in the average print area exhibiting the functional impairment in response to the injury. Over the next eight weeks all treatment groups completed steps with comparable print areas (ranged between 600–750 pixel area). The left fore paw print area of the PEG IFN-β + Ex group was significantly greater at baseline compared to the remaining 3 groups (Figure 4B; $p<0.05$). One week following SCI the average print area was significantly increased in the Ex groups compared to the non-Ex groups (Vehicle: 433.59 pixel area ± 20.85: Ex: 576.56 pixel area ± 39.18; $p<0.05$), suggesting a high level of compensation. A separation between PEG IFN-β and the remaining groups began during week 4 and became significant during weeks 6 through 8 ($p<0.05$).

Grid-Walk—This assessment examined the sensorimotor capabilities of the affected fore and hindlimb over a challenging terrain. The total number of foot placements (correct + missteps) was determined and the percentage of correct placements was calculated. All of the groups exhibited similar sensorimotor function by completing 75–85% correct foot placement by week 4 of the study (Figure 5A). At 1 week, the hindlimb of the animals treated with PEG IFN-β made more correct foot placements compared to the vehicle, Ex, and PEG IFN-β + Ex groups (Figure 5B: PEG IFN-β: 73.58% ± 2.32; Vehicle: 43.98% ±6; Vehicle + Ex: 58.22 ± 9.47; PEG IFN-β + EX: 43.48 ± 15.1; $p<0.05$). Over the last 4 weeks, all groups displayed similar sensorimotor function, and by week 8 all performed ~80–90% correct steps.

Anatomical Assessment

Immunocytochemical Analysis—Spinal cord tissue ipsilateral to the injury was subjected to computer assisted image analysis (Figure 6). Tissue rostral to the injury, at the epicenter,

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and caudal to the injury was evaluated, in 3 regions of the white matter and 1 in the ventral horn of the cord, to determine the extent of secondary tissue reaction. Figure 6 displays the area density of GFAP and ED1 caudal to the injury site (rostral not shown but not different). No significant differences were found in either the grey or white matter for reactive astrogliosis or presence of phagocytic cells (Figure 6A and 6B).

Tissue Sparing & Lesion Size—The amount of grey and white matter sparing was quantified and compared among groups at distances rostral and caudal to the injury site (Figure 7A). No significant differences were found for the rostral or caudal regions among each treatment groups. All groups exhibited similar tissue sparing at the lesion epicenter and at rostral or caudal directions from the injury site. Rostral-caudal lesion length was quantified and was comparable in all groups (Figure 7B).

DISCUSSION

Acute Biochemical Study

In this report we first demonstrated that the administration of a single dose of the PEGylated form of IFN-β exerts multiple effects in the injured spinal cord as measured by changes in the expression of pro- and anti-inflammatory cytokines, HSPs, and iNOS. The reduction in levels of IL-6 and TNF-α at 24 hours after injury may be advantageous since these modulators of inflammation are among the first cytokines to be upregulated in response to spinal cord trauma (Donnelly and Popovich, 2007). Diminishing the levels of major contributors to the pro-inflammatory cascade during the acute response phase injury could interfere with the progression of secondary injury. Following this early response there was an increase in the level of IL-18 at the lesion epicenter beginning 3 days post SCI in both vehicle and PEG IFN-β groups but at 5 days the drug treated group showed a significant increase of IL-18 beyond the vehicle treated group. IL-18 is a pleiotropic cytokine that can have dual actions by amplifying the pro-inflammatory response of microglia and astrocytes as well as decreasing the potentiation of its own activities via binding to its endogenous antagonist, IL-18 binding protein (reviewed in Felderhoff-Mueser et al., 2005). The mechanism of action of this immunomodulator is complex and differs from other pro-inflammatory cytokines in that its expression in the injured spinal cord was delayed for several days after SCI. Whether IL-18 is functioning as a pro- or anti-inflammatory cytokine at these time points is not known from the present experiments. A study had previously demonstrated that systemic administration of IL-10 thirty minutes after spinal cord contusion had beneficial effects (Bethea et al., 1999). The presence of IL-10 decreased TNF-α production and aided in functional recovery. In combination, the marked decreases in pro-inflammatory cytokine expression, a rapid increase in the anti-inflammatory IL-10, and a delayed increase of IL-18 may indicate mechanisms for a neuroprotective action of PEG IFN-β in SCI. Although these acute biochemical changes indicate possible neuroprotection effects they did not translate into long term tissue sparing or recovery of function. Therefore we chose to implement an additional therapy, forced exercise, which has previously shown to aid in recovery when used alone (Sandrow-Feinberg et al., 2009).

The first stage of neuroinflammation involves activation and recruitment of cells to the area of damaged tissue. In our model, most changes in cytokine expression after SCI occurred in the lesion epicenter, while caudal segment levels were comparable to the sham operated group. This indicates a regionally specific response to the injury and treatment. One of the mechanisms might involve IFN-β-mediated negative regulation of matrix metalloproteinases (MMPs). MMP production is upregulated immediately following SCI, it remains elevated for several days following trauma, and the molecules act by degrading the extracellular matrix to facilitate migration of immune cells through the blood brain barrier (Yushchenko et al., 2003; Yong et

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al., 2007). Reduction of several pro-inflammatory stimuli with PEG IFN-β treatment may provide additional neuroprotection to spared neurons surrounding the lesion site, thus preventing further spread of degeneration.

The induction of adaptive responses to oxidative stress includes the upregulation of HSPs in neurons and glia. Cytoprotective effects of HSPs have been attributed to the inhibition of pro-inflammatory cytokines and upregulation of anti-inflammatory cytokines via a nuclear factor-kappa B (NF-κB) controlled gene expression pathway (DeMeester et al., 2001). Interaction between HSPs and oxidative stress response genes in cellular adaptation to stress was postulated as an important protective mechanism (Chen et al., 2007). Based on studies of the mammalian brain, HSPs are transferred between cell types in the CNS providing a fast delivery of neuroprotective molecules from glia to neurons, therefore maintaining neuronal viability (Brown, 2007). In our model three major HSPs; (HSP27, HSP70, and HSP32), were upregulated in rat spinal cord after a contusion injury. Each of the proteins had its own temporal profile of expression suggesting a unique role in modulation of the intensity and duration of events following spinal cord injury and response to treatment with PEG IFN-β. Five days post SCI, animals that received PEG IFN-β had significantly higher levels of HSP27 both at the level of the lesion and below. This dramatic rise in HSP27 was seen in our previous study in animals that received 5 days of forced exercise (Sandrow-Feinberg et al., 2009). Potent neuroprotective effects of HSP27 have been demonstrated after excitotoxic insult in cultured neurons (reviewed by Latchman, 2005) and in a transgenic animal model over expressing HSP27 (Akbar et al., 2003). The variable pattern of HSP70 expression after SCI and after PEG IFN-β treatment likely reflects the complex dual function of this protein. Significant upregulation of HSP70 after focal spinal cord trauma was reported but it is unclear whether the underlying mechanism reflects cell stress or an adaptive response (Sharma et al., 2006). It is possible that an initial increase in HSP70 expression after injury reflects the cellular response to oxidative stress after profound upregulation of pro-inflammatory cytokines TNF-α, IL-1α, and IL-1β. A decline in HSP70 expression after several days post SCI coincides with a reduction in cytokine production, thus lowering cellular demand for HSP70-dependent neuroprotection, and possibly turning on other stress genes. The significant reduction of HSP70 level 3 days post SCI and administration of PEG IFN-β provides an additional link between inflammation-induced oxidative stress and HSPs in the injured spinal cord. Expression of HSP32 in the CNS is normally very low and its expression is transiently upregulated mainly in astrocytes and reactive microglia after injury. This upregulation has been linked to increased production of the pro-inflammatory cytokines TNF-α and IL-1β (Dwyer et al., 1996; Mehindate et al., 2001), and a robust upregulation in HSP32 restricted to the lesion epicenter in this study was similar to a temporary increase in HSP32 reported in a thoracic SCI model (Liu et al., 2002). HSP32 function is not limited to an anti-oxidative role as it also serves as an anti-inflammatory immunomodulator in ischemia/reperfusion injuries (Katori et al., 2002). In our study, PEG IFN-β-induced HSP32 production at the injury site provides additional evidence for a beneficial effect of acute anti-inflammatory treatment on activation of potent neuroprotective pathways. Although the levels of HSP32 were substantially increased at the lesion epicenter 3 and 5 days post SCI, translation to function and tissue sparing was not found in our subsequent long term study.

Among other free radicals, the detrimental effect of NO on the injured spinal cord depends upon the concentration and time of release, as NO is necessary for normal cellular homeostasis. After SCI, the initial upregulation of NO is controlled by neuronal NOS (nNOS), while a late robust increase of NO is due to transcriptional activation of iNOS (Conti et al., 2003). In the present study, an increase of iNOS expression was found in the injured spinal cord at 3 days and 5 days post injury but PEG IFN-β administration significantly impaired this increase, suggesting that acute systemic anti-inflammatory treatment can modulate multiple aspects of the secondary response to SCI.

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The addition of the polyethylene glycol moiety to IFN-β enables a more constant level of drug to remain within the blood serum (Arduini et al., 2004). The increased exposure of the PEGylated form of IFN-β may also provide sustained induction of neuroprotective signals within the CNS compared to use of an unmodified form that exhibits peaks and valleys in circulating levels and requires more frequent administration.

In summary, changes in the acute expression of several pro- and anti-inflammatory markers in tissue extracts after cervical contusion injury in rats were identified. After a cervical contusion injury, a single injection of a PEGylated form of IFN-β diminished potentially damaging aspects of the cytokine response and promoted production of potentially neuroprotective molecules. Such an acute anti-inflammatory treatment could be considered beneficial if used in combination with other therapeutic strategies in the management of acute spinal cord injury.

Chronic Behavioral Study

Spinal cord injury is a multifaceted disorder that requires attention from a variety of approaches and it is apparent that no single intervention will be adequate (Bunge, 2008). In this study, we combined an acute anti-inflammatory treatment for neuroprotection followed by a long term rehabilitation strategy to promote neuroplasticity. We used a variety of behavioral tests to evaluate recovery. The severity of injury manifested as an initial deficit and a partial recovery within the first week. This pattern suggested that these tests could reveal intervention-based improvements.

Functional improvements from these therapies produced several significant changes in the assessments of motor and sensory behavior which were not permanent, rather they were observed only at specific time points. Examination of the affected right forelimb in the open field (FLS) revealed an increase in function by the PEG IFN-β-treated group 1 week post SCI compared to vehicle treated animals. The groups given daily forced Ex were able to locomote to the same degree with or without PEG IFN-β throughout the 8 week period. The same functional impairments in those exposed to forced Ex suggests a training effect which has been demonstrated to be beneficial in both gross and fine skilled behaviors (Smith et al., 2006; Girgis et al., 2008; Sandrow-Feinberg et al., 2009). An effect of Ex was also observed in the FLS test during the early phase of recovery and the ipsilateral hindlimb in the BBB. Coordinated stepping that was consistent in the exercised animals, implies changes in neuronal circuitry, possibly due to reorganization of propriospinal connections (Bayrere et al., 2004; Courtine et al., 2008). We analyzed locomotion quantitatively with the TreadScan device to detect changes in specific skills that were not detectable by the FLS or BBB scoring.

The Ex groups had similar print areas for both the right and left forelimb, indicating that the left forelimb did not over compensate for the affected side. For non-Ex groups there was more variability in print area, with the left forelimb of the PEG IFN-β group being larger than the right forelimb print area, demonstrating compensation for diminished function in the right fore paw. Clearly, Ex contributes to reestablishing patterns of gait that are detected by FLS, TreadScan, grid, and grooming tests. The use of forced Ex as a rehabilitation strategy aided recovery by diminishing the degree of impairment over the 8 week period.

Sensorimotor function was evaluated with the grid-walk test. The ipsilateral hindlimb of the PEG IFN-β only group showed a higher percentage of correct foot placements 1 week post SCI compared to the remaining groups ($p<0.05$). This increase in proper paw placement declined by week 2 but remained slightly improved during the remainder of the study. This short lived change could be due to the decrease in inflammation shortly after injury with PEG IFN-β alone. Interestingly the combination group did not exhibit the same increase in correct placements. The grid test was not sensitive enough to detect a difference since all groups had similar levels of recovery.

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Immunostaining for reactive astrocytes and microglia/macrophages did not show a difference with respect to presence in the white or grey matter or rostrocaudal extent. All groups exhibited similar labeling patterns, with more labeling rostral and caudal compared to the epicenter. Despite the early functional changes in each of the behavioral tests examined, the effects of PEG IFN-β was not capable of decreasing tissue loss. Tissue sparing was equivalent at the lesion epicenter among all groups, indicating the consistency of the injury and the absence in effects of intervention. The combination group did not differ significantly from the other groups. Since most of the behavioral changes were seen early after injury and cannot be explained by difference in lesion size or sparing the acute application of the therapies alone may be best described as having an adjunct effect on spontaneous recovery that is normally observed after a contusion injury. The absence of a long term effect indicates that PEG IFN-β and forced exercise is not the most appropriate approach for a combination treatment strategy.

Conclusion

Neuroprotection continues to be a major objective when designing therapies for SCI. In this study, a PEGylated form of IFN-β was investigated as a possible neuroprotective agent since unmodified IFN-β has had success battling chronic neuroinflammation in the context of MS. Since inflammation contributes to secondary injury, targeting this reaction early after injury may diminish the extent of damage. Biochemical results indicated that application of this agent influenced the production of both pro- and anti-inflammatory cytokines, heat shock proteins, and iNOS within 6 hours and lasted out to 5 days post injury. These changes could have profound effects on function if tissue was in fact being spared from secondary degeneration. Therefore we completed a long term study evaluating the effects of PEG IFN-β alone or in combination with forced exercise. A previous study in our lab had shown that forced exercise was capable of accelerating functional recovery during the early weeks post injury (Sandrow-Feinberg et al., 2009). Combining the two therapies in this study addressed whether they work synergistically to promote long term recovery.

The behavioral and anatomical results from the combination therapy indicated that they did not work in concert to aid in recovery but each intervention was capable of working separately as an acute therapy. Our results do not agree with the long term positive results found by others (Garg et al., 2006, Gok et al., 2007). The lack of tissue sparing in our study compared to the Garg et al. (2006) data could be attributed to a multitude of technical variations between the studies; different IFN-β formulation (PEGylated vs. unmodified), route (intraperitoneal vs. subcutaneous) and delivery time post SCI (30 minutes vs. 2 hours). The injury models used in each study were also different; here a severe unilateral cervical contusion versus moderate bilateral thoracic contusion. Finally, we used Sprague-Dawley rats while Garg et al. used Lewis rats. Previous work has demonstrated that although an immune response is activated in all rat species following injury, the cascade of events can be altered temporally or spatially (Popovich et al., 1997). Despite all of the differences mentioned the presence of short term incremental behavioral changes in response to injury and treatment gave good insight into the use of the PEGylated formation and the length of treatment needed to produce long lasting changes. This study along with previous work in our lab has shown that use of forced Ex alone was capable of producing accelerated recovery four weeks post injury followed by a plateau, indicating both the high level of spontaneous recovery and the limitations of providing rehabilitation without another therapy. The results do not dissuade from the original hypothesis of pairing an acute neuroprotective strategy with a long term treatment, but the right combination and course of treatments remains to be found.

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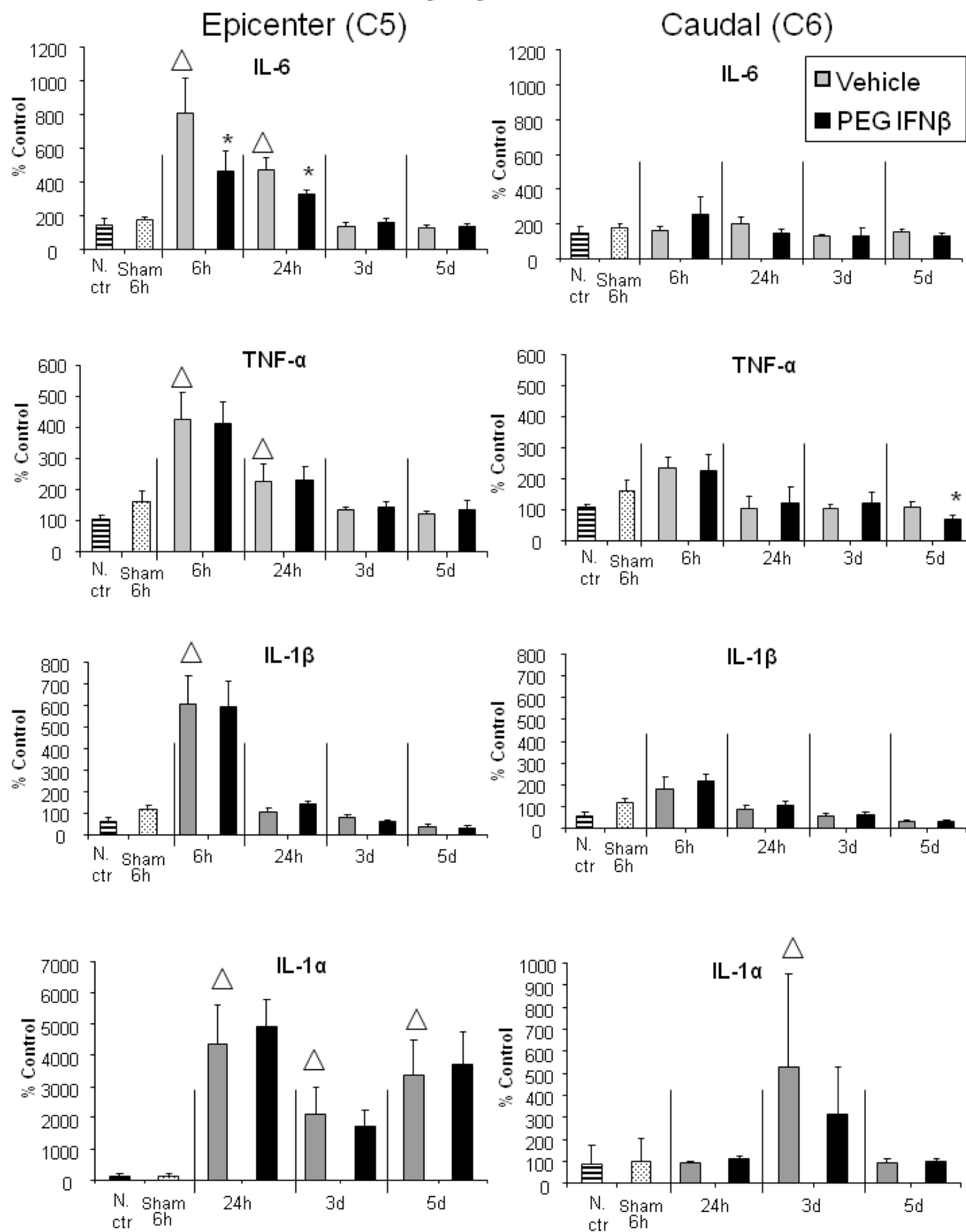

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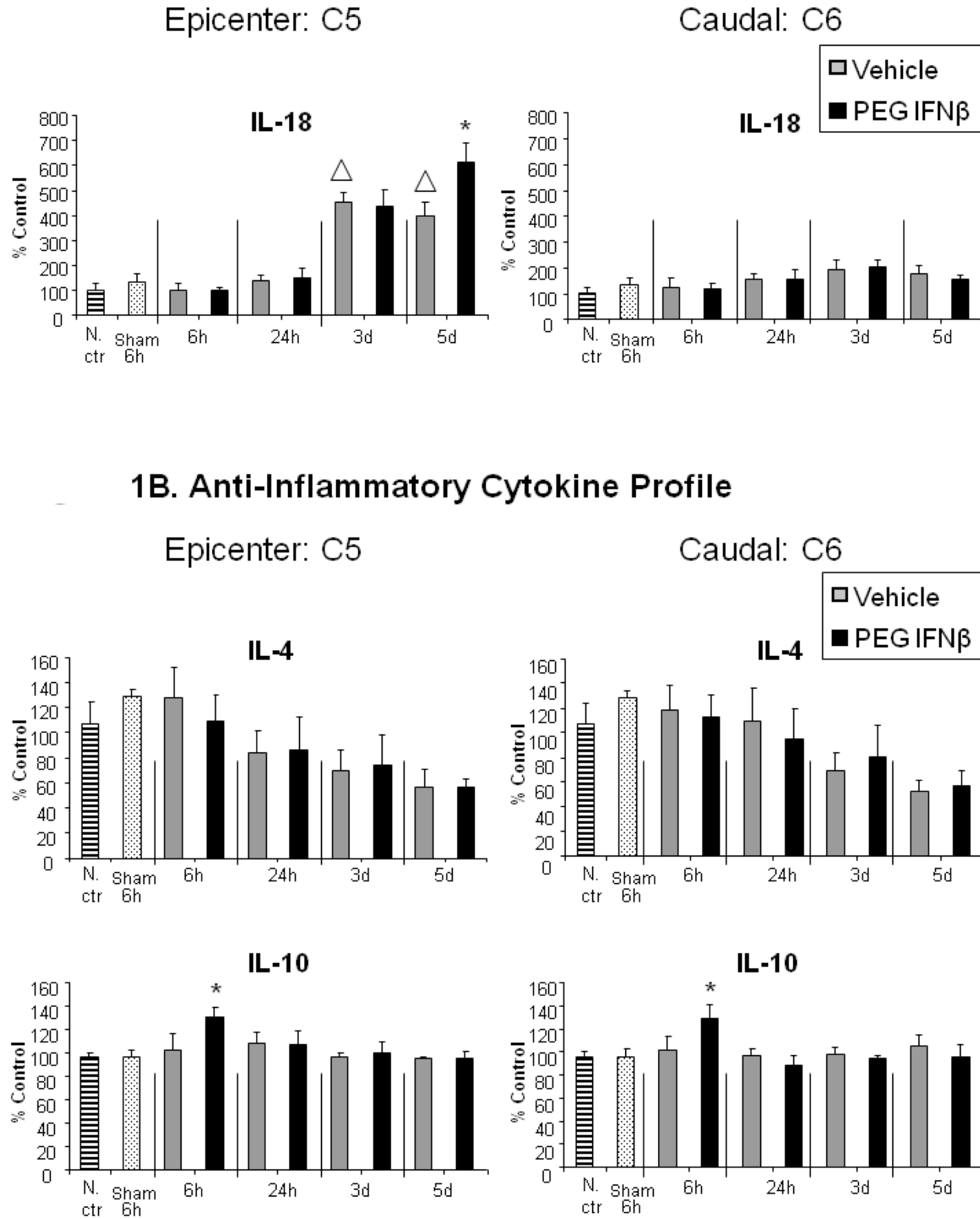

Figure 1.

Temporary changes in pro- and anti-inflammatory cytokine expression in the injured rat spinal cord after acute PEG IFN-β treatment.

A. Spinal cord tissue at the lesion epicenter (C5) and caudal segment (C6) from each animal was used to detect levels of the pro-inflammatory cytokines IL-6, TNF-α, IL-1β, IL-1α, and IL-18 (means ± SEM).

B. Anti-inflammatory cytokines IL-4 and IL-10 regulation.

Δ indicates significant difference from 6 hour sham control.

* indicates significant difference between PEG IFN-β and vehicle.

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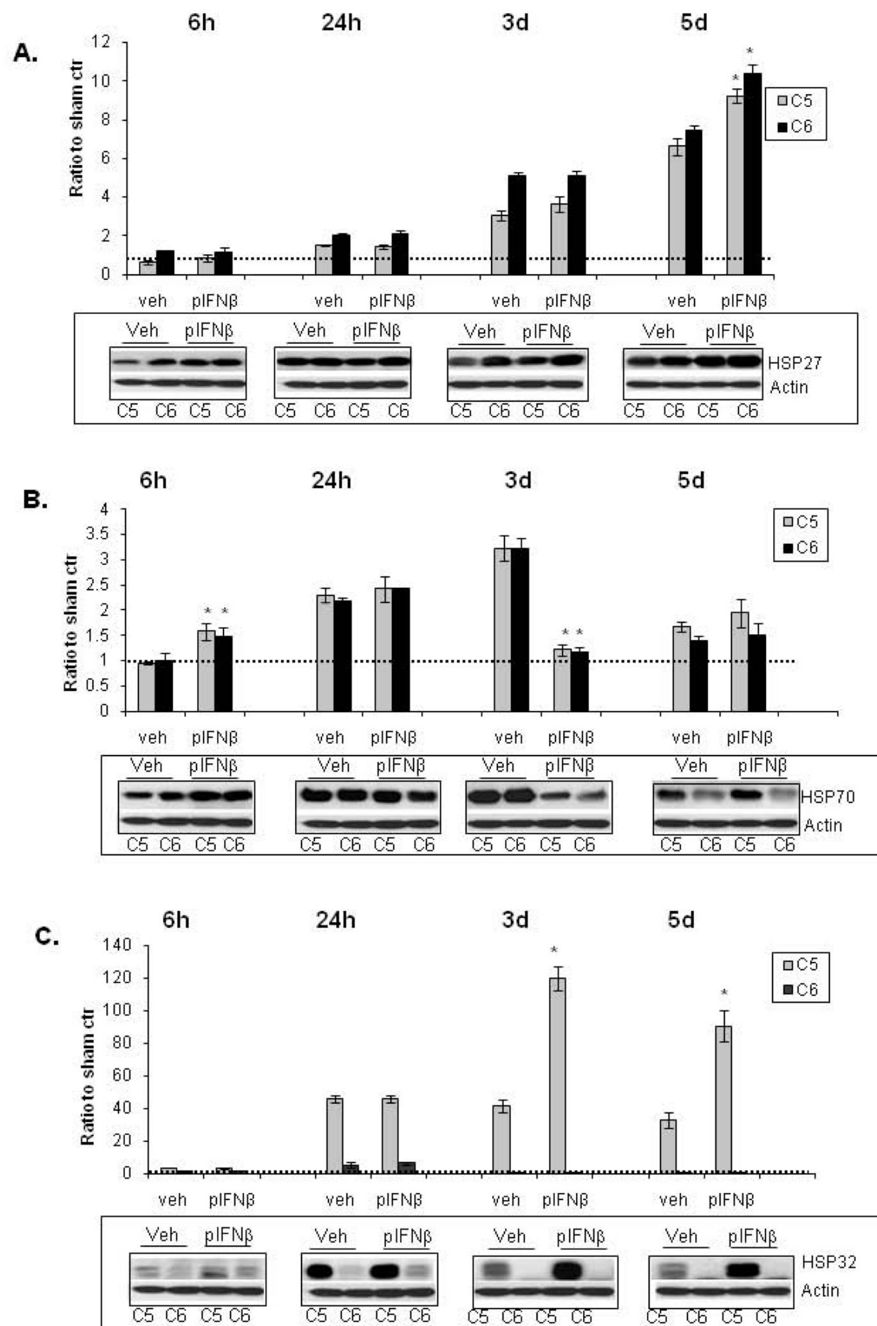

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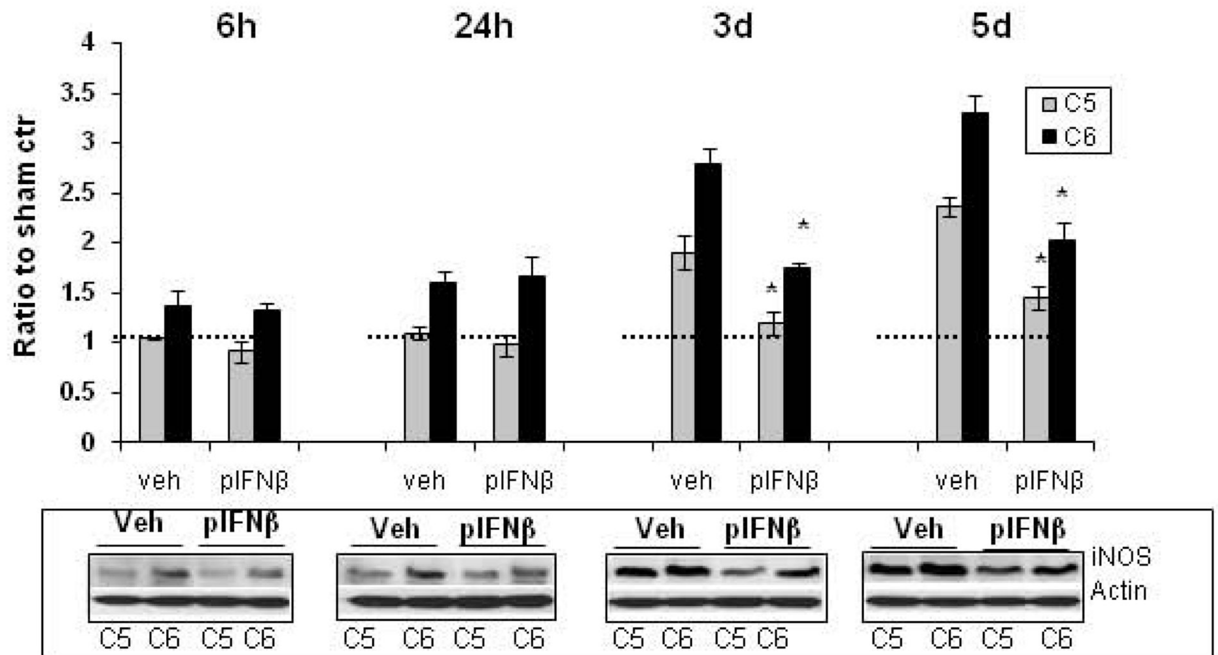

Figure 2.

Heat shock protein and iNOS expression profile in the injured rat spinal cord after acute treatment with PEG IFN-β.

(A. HSP27; B. HSP70; C. HSP32; D. iNOS) Quantitative Western blot analysis was performed on rat spinal cord extracts from the injury site (C5) and caudal region (C6) in vehicle (Veh) and PEG IFN-β (pIFNβ) treated groups at 6 hours, 24 hours, 3 days, and 5 days after the injury. For quantitative analysis, densitometry values of immunopositive bands were determined using Syngene software, and final data were normalized to the densitometry values of actin. Final data are presented as a ratio to the 6 hour sham operated control group (dotted line) for each time point. Each bar represents the average measurements between three blots (means ± SEM). * $p < 0.05$ indicates significant difference between PEG IFN-β and vehicle treated groups. Representative images of Western blots for each protein are shown in the box below each graph.

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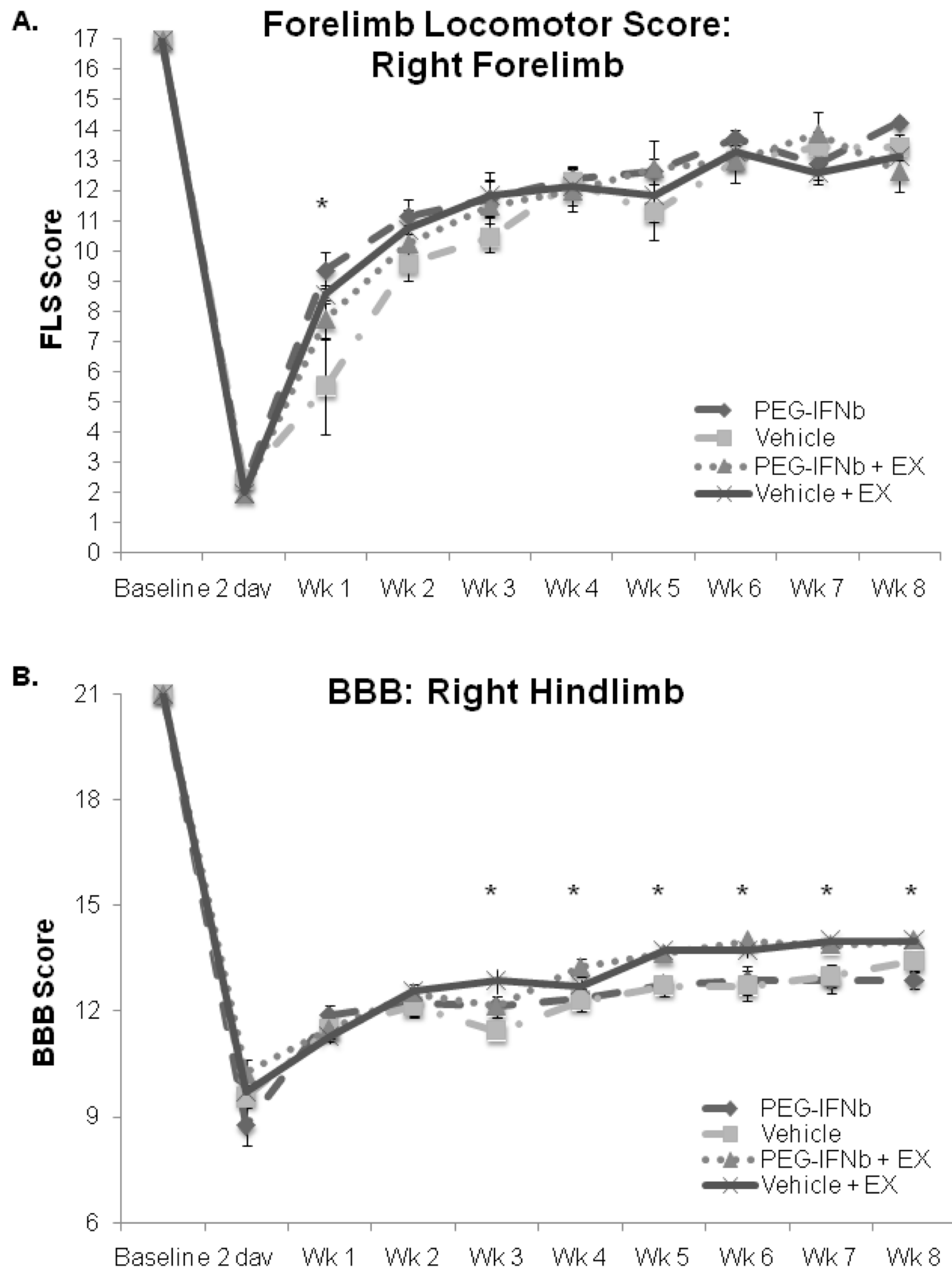

Figure 3.

Locomotor assessment of forelimbs and hindlimbs after forced Ex. A. Forelimb locomotor score (FLS) was evaluated over the 8 week period (Vehicle, pink; PEG IFN-β, blue; PEG IFN-β+Ex; yellow; Vehicle+Ex, turqoise) (mean ± SEM). Testing began 2 days post SCI. A significant difference between PEG IFN-β and vehicle occurred during week 1 (*$p<0.05$). B. BBB assessment over the 8 week time period (mean ± SEM). Injured hindlimbs from each group (Vehicle, pink; PEG IFN-β, blue; Vehicle+Ex, turquoise; PEG IFN-β+Ex; yellow) were examined. A significant (*$p<0.05$) separation between Vehicle+Ex and Vehicle alone occurred during week 3. Weeks 4–8 revealed a significant difference in the groups exposed to Ex compared to no Ex (*$p<0.05$).

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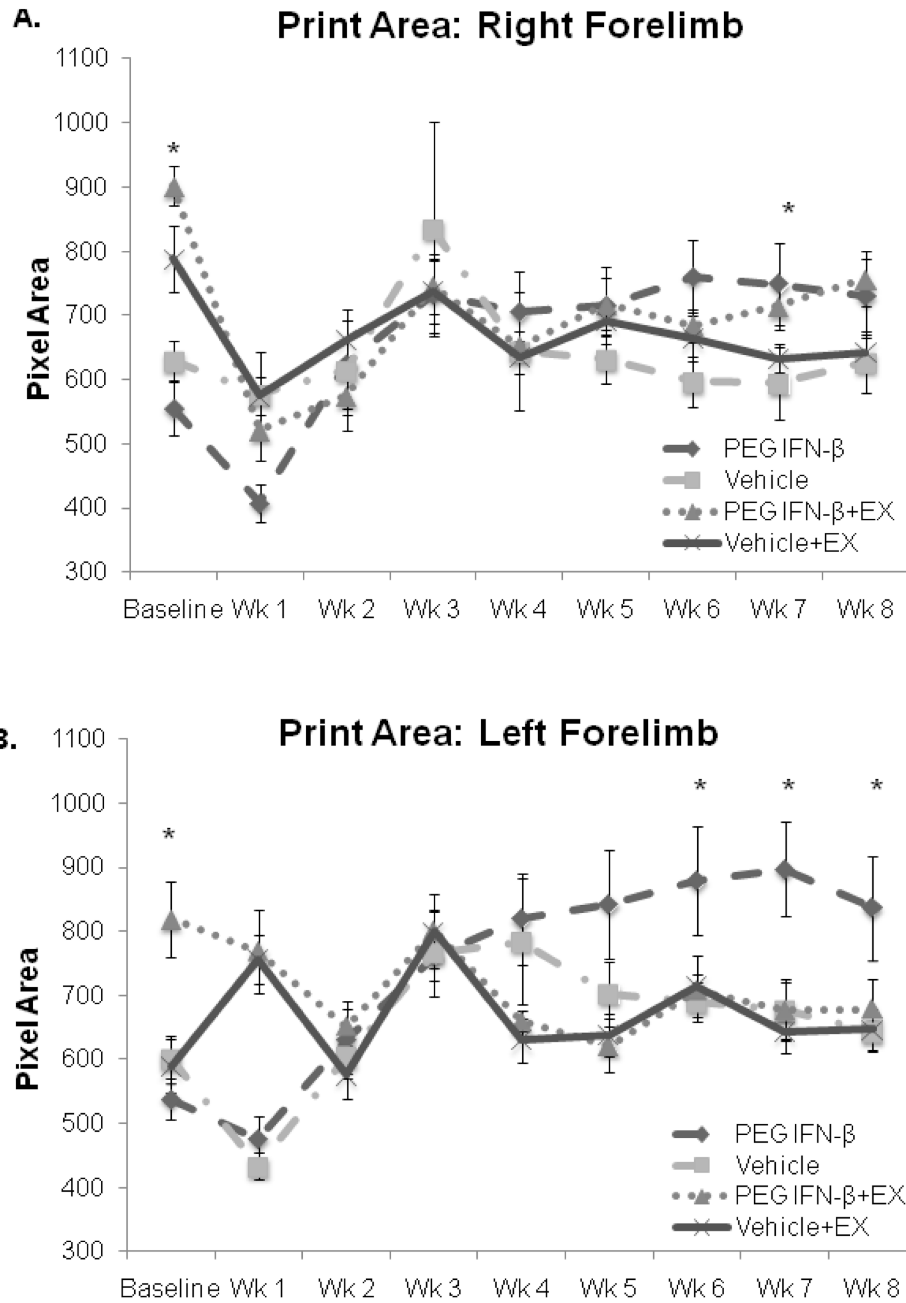

Figure 4.

TreadScan Assessments (mean ± SEM).. A. Print area of the right forelimb. Print area decreased 1 week post SCI in all groups. A significant increased print area was seen during week 7 between PEG IFN-β and Vehicle (*p<0.05). An early increase in print area plateaued by week 5. B. Print area of the left forelimb. One week post SCI, Ex alone and Ex + PEG IFN-β had an increased print area compared to groups not exposed to Ex. The Ex groups revealed a decreased print area towards the end of the study while the PEG IFN-β had an increase in print area which became statistically significant during weeks 6, 7, and 8 (*p<0.05).

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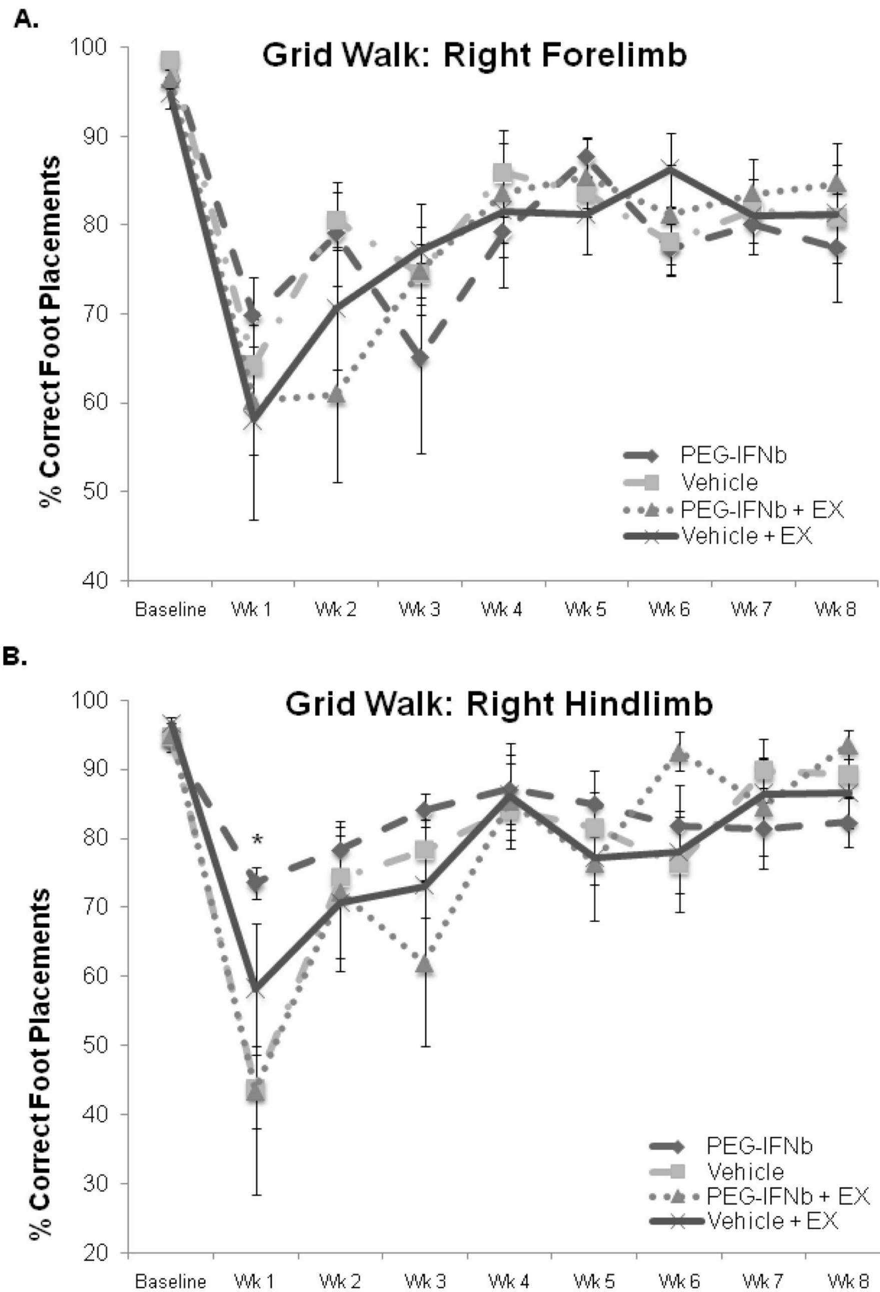

Figure 5.

Grid walk performance (mean ± SEM). Affected limbs from each group (Vehicle, pink; PEG IFN-β, blue; Vehicle + Ex, turquoise; PEG IFN-β+Ex; yellow) were examined. A. Average percentage of correct foot placements on the grid by the right forelimb. The 8 week period displays an initial deficit during 1 week post SCI in all groups to ~65% of baseline. Throughout the remaining weeks both groups perform in a similar manner reaching ~85% by 8 weeks. B. The percentage of correct foot placements completed by the ipsilateral hindlimb. PEG IFN-β alone (square) made significantly more correct placements compared to the remaining groups during week 1 (*$p<0.05$). During the rest of the 8 week period all groups performed similar correct foot placements.

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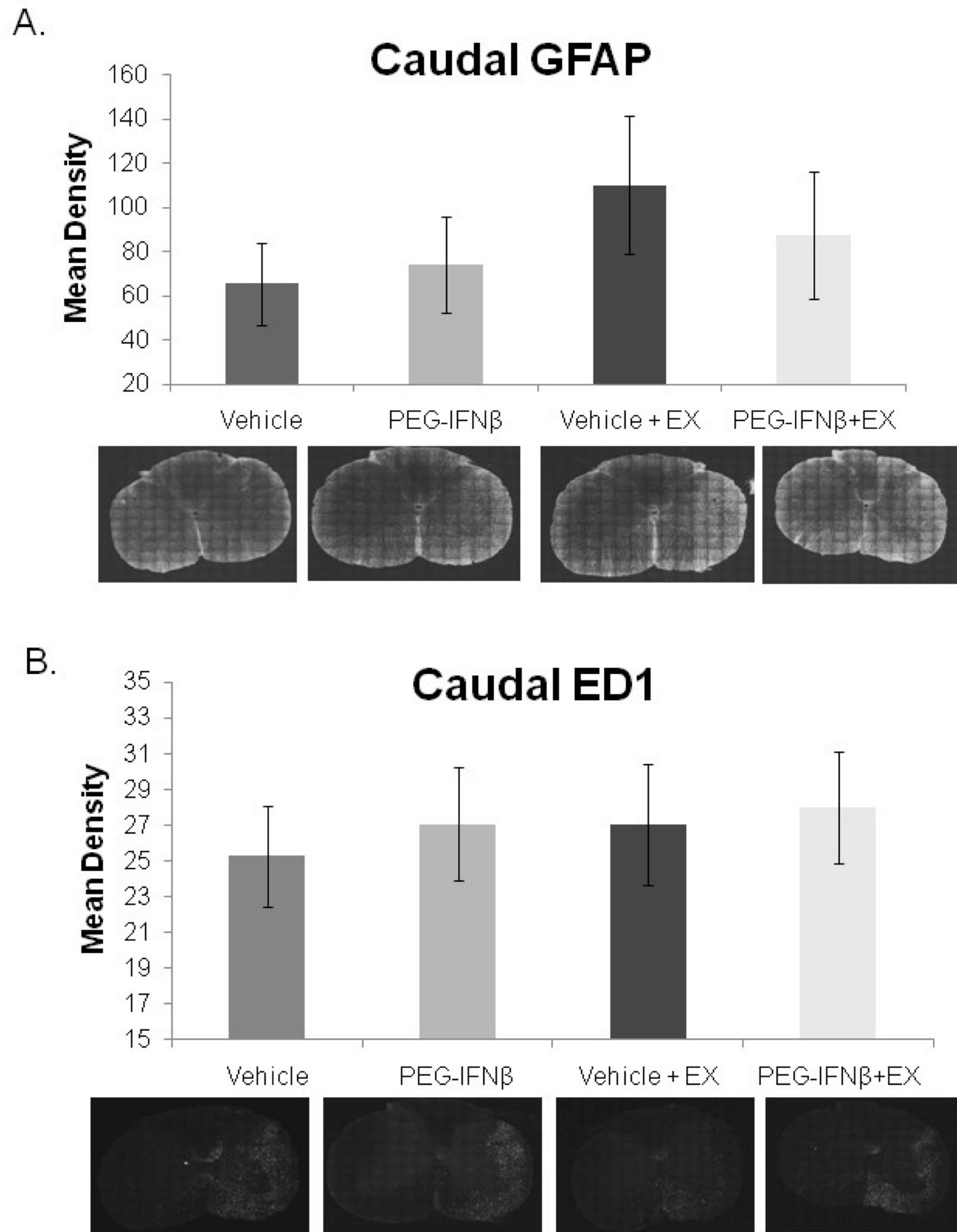

Figure 6.

A. Immunocytochemical Evaluation of Reactive Astrocytes (GFAP label) 8 weeks post SCI (mean ± SD). The rostral, epicenter, and caudal regions of the spinal cord were analyzed to determine presence of reactive astrocytes. Only the caudal area is shown as a representation since no significant difference was found. **B.** Immunocytochemical Evaluation of Macrophage Presence (ED1 label) 8 weeks post SCI (mean ± SD). The graph displays tissue positive for macrophage presence caudal to the lesion site. Both white and grey matter were evaluated. There were no significant differences or trends with respect to treatment. Beneath each graph are representative images from each group.

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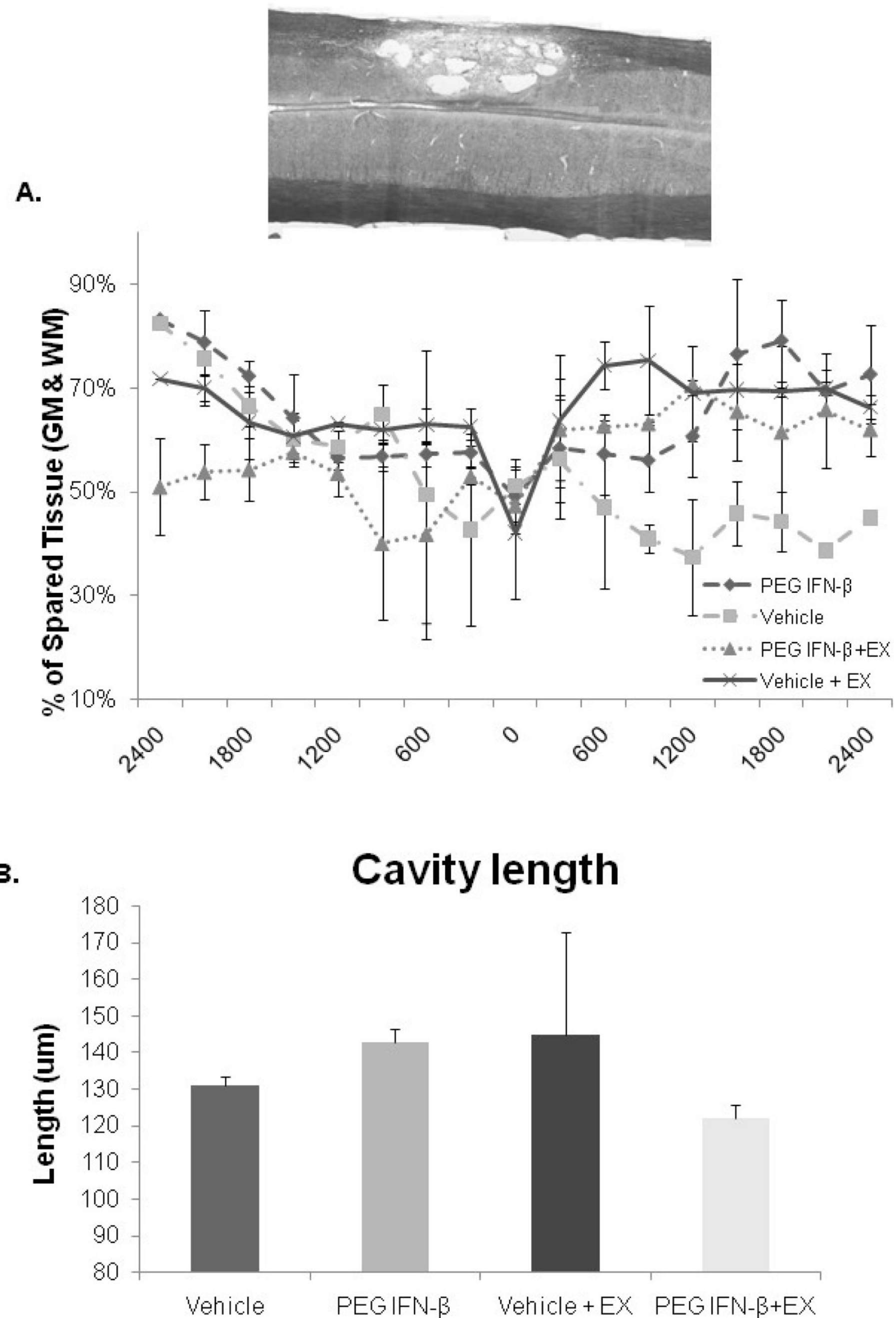

Figure 7.

Histological Evaluation of Lesion 8 weeks post SCI (mean ± SD). Top image is longitudinal representative section of SCI. A. Amount of Spared Tissue. All groups had similar tissue loss at the lesion epicenter and varying tissue sparing in both rostral and caudal directions. There was a trend towards a decrease in tissue sparing in the vehicle group caudal to the lesion site and the PEG IFN-β + Ex group had a trend towards a decrease in tissue sparing rostral to the lesion site. B. Lesion Length (mean ± SD). All groups exhibited similar lesion lengths.