Nanoparticle Estrogen in Rat Spinal Cord Injury Elicits Rapid Anti-Inflammatory Effects in Plasma, Cerebrospinal Fluid, and Tissue

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Abstract

Persons with spinal cord injury (SCI) are in need of effective therapeutics. Estrogen (E2), as a steroid hormone, is a highly pleiotropic agent; with anti-inflammatory, anti-apoptotic, and neurotrophic properties, it is ideal for use in treatment of patients with SCI. Safety concerns around the use of high doses of E2 have limited clinical application, however. To address these concerns, low doses of E2 (25 μ g and 2.5 μ g) were focally delivered to the injured spinal cord using nanoparticles. A per-acute model (6 h after injury) was used to assess nanoparticle release of E2 into damaged spinal cord tissue; in addition, E2 was evaluated as a rapid anti-inflammatory. To assess inflammation, 27-plex cytokine/chemokine arrays were conducted in plasma, cerebrospinal fluid (CSF), and spinal cord tissue. A particular focus was placed on IL-6, GRO-KC, and MCP-1 as these have been identified from CSF in human studies as potential biomarkers in SCI. S100 β , an additional proposed biomarker, was also assessed in spinal cord tissue only. Tissue concentrations of E2 were double those found in the plasma, indicating focal release. E2 showed rapid anti-inflammatory effects, significantly reducing interleukin (IL)-6, GRO-KC, MCP-1, and S100 β in one or all compartments. Numerous additional targets of rapid E2 modulation were identified including: leptin, MIP-1 α , IL-4, IL-2, IL-10, IFN γ , tumor necrosis factor- α , etc. These data further elucidate the rapid anti-inflammatory effects E2 exerts in an acute rat SCI model, have identified additional targets of estrogen efficacy, and suggest nanoparticle delivered estrogen may provide a safe and efficacious treatment option in persons with acute SCI.

Key words: CSF; estrogen; GRO-KC; IL-6; MCP-1; nanoparticle; S100 β ; spinal cord injury

Introduction

THE FIELD OF NANOMEDICINE HAS GROWN tremendously in the past decade and is showing the potential of nanoparticles for targeted drug delivery, improved efficacy, and increased drug safety.¹ This growth, however, has not yet been fully applied to research in acute neurotrauma, specifically spinal cord injury (SCI). Currently, no Food and Drug Administration (FDA)-approved drug exists for the treatment of patients with acute SCI. Methylprednisolone has been used widely, albeit off-label, in the care of acute SCI with limited efficacy and remains a controversial therapeutic that has yet to garner FDA approval.^{2–6}

An estimated 150,000–200,000 worldwide cases of SCI are diagnosed annually, with some 2.5 million persons living chronically with paralysis secondary to SCI. The societal cost of medical and rehabilitative care for these patients is immense, with loss of mobility, independence, and productivity. The cost to the individual is immeasurable.

In the last 30 years, the field of neurotrauma research has seen an abundance of novel and promising pharmacotherapeutic interventions. Many recent reviews summarize the clinical trials conducted thus far.^{7–10} None of these newer therapeutic interventions, however, have found wider clinical application. Thus, a need still exists for effective drug treatment in SCI.

The use of single-agent targeted therapeutics in an enormously complex condition that includes numerous pathophysiologies, such as inflammation, oxidative stress, ischemia, glial scarring, etc., has not proven efficacious. Thus, evaluation of broadly acting drug candidates, including the steroid hormone estrogen, is justified. Also critical to the successful evaluation of new treatments in SCI will be the use of clinically validated biomarkers to better screen and assess the efficacy of potential therapeutics in both preclinical models and clinical trials.

Estrogen (E2) is a highly pleiotropic agent that has been reported to activate transcription of some 137 E2 regulated genes.¹¹ It is known to have anti-inflammatory, anti-oxidant, anti-apoptotic, and

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neurotrophic properties.^{12–18} E2 has exhibited a neuroprotective effect in SCI models.^{17,19–26} In addition, E2 has shown a neuroprotective effect in traumatic brain injury models^{27–32} and stroke models,^{33–37} suggesting E2 warrants clinical evaluation in the setting of neurotrauma. The translation of E2 from preclinical models into clinical practice with the FDA-approved drug Premarin has significant safety concerns, however.³⁸

Long-term E2 treatment (5 years) in post-menopausal women is known to carry adverse risks of cardiovascular disease, cancer, stroke, and pulmonary embolism, as studies conducted by the Women's Health Initiative have reported.³⁹ It has also been reported that shorter term E2 treatment used for contraception is significantly associated with increased risk of venous thromboembolism, with the greatest risk occurring in less than a year of treatment and at the highest dose of E2.⁴⁰ These safety concerns with traditional dosing of E2 pose a hurdle for movement into patients with SCI, but more sophisticated forms of drug delivery, such as nanoparticles, may provide a solution.

Nanoparticle drug delivery systems have been shown to improve the therapeutic index of drugs.⁴¹ By focally delivering E2 to the injured spinal cord directly, maximal efficacy may be achieved while keeping systemic levels of E2 in a physiological range. This technique may effectively increase the therapeutic window of E2. In addition, while much is known about the neuroprotective effects of E2 in subacute and chronic conditions (days to weeks), less is known about its potential to mediate inflammation rapidly in a per-acute setting of SCI (6 h).

To further elucidate the potential for E2 to rapidly modulate inflammation, the cytokines interleukin (IL)-6, growth-related oncogene-keratinocyte-derived cytokine (GRO-KC) (human IL-8), and the chemokine monocyte chemoattractant protein 1 (MCP-1) (these three are candidate biomarkers previously identified in a human study⁴²) were investigated in plasma, cerebrospinal fluid (CSF), and spinal cord tissue of an acute rat model of SCI. The calcium binding protein, S100 β , the most frequently cited biomarker studied in SCI,⁴³ was also investigated in tissue. Because the potential exists for E2 to modulate numerous targets, potentially outside of those previously identified as biomarkers, a cytokine/chemokine array will be used to fully characterize how E2 may function as a rapid anti-inflammatory in SCI.

The focus of this research is to apply a novel drug delivery technique, E2 formulated nanoparticles, in a per-acute setting of SCI and evaluate how E2 may modulate the key biomarkers of injury severity: IL-6, GRO-KC, MCP-1, and S100 β . By sampling the plasma, CSF, and tissue simultaneously, we aim to further understand the dynamics of using plasma/CSF biomarkers to reflect the status of the injured nervous tissue.

In using nanoparticles, we hypothesize that E2 concentration will be greater in tissue than plasma and aim to keep systemic exposure at physiological levels. In addition, by using a multiplex cytokine/chemokine array approach in plasma, tissue, and CSF, we hypothesize identification of novel cytokines/chemokines that are targets of E2 modulation. Collectively, the data presented here may provide additional support for the clinical translation of this novel drug therapeutic into the acute neurotrauma setting.

Methods

Nanoparticles and gel plug delivery system

Nanoparticles. Nanoparticles were formulated at Clemson University (Bioengineering Department) using the nanoprecipita-

tion method as described previously.^{44,45} Briefly, 50:50 poly lactic-co-glycolic acid (PLGA) was mixed with polyethylene glycol-poly lactic acid (PEG-PLA) copolymer, and 17- β estradiol (E2) (Sigma E2758) in a 10:2.5:1 (wt) ratio. The vehicle control batch was loaded with 0.9% saline. This was dissolved in acetone to obtain a 5 mg/mL polymer solution.

This solution was added drop-wise to 20 mL of de-ionized water in an ultrasonic water bath and sonicated for 30 min. The formed particles were then separated by centrifugation (7500 rcf) for 2 h, washed three times with de-ionized water, lyophilized overnight, and then each batch was evaluated for particle size (dynamic light scattering), load efficiency, and zeta potential to ensure consistency among batches. Nanoparticles were stored in sucrose at -20°C .

Gel plug delivery system. Gel plugs (0.6% SeaPlaque agarose in phosphate buffered saline [PBS]) were made by dissolving lyophilized E2 loaded PLGA nanoparticles at either 25 μg or 2.5 μg dose (or saline loaded vehicle control) in 50 μL sterile filtered PBS. The gel (final volume 50 μL) was set in PCR amplification tubes overnight to harden. Before insertion into animals, gels were sterilized under tissue culture ultraviolet lamp for 15 min.

Rat model

All animal experiments were conducted in accordance with the Institutional Animal Care and Use Committee's guidelines and performed under the protocol ARC #2079. Adult male Sprague-Dawley (SD) rats weighing 200–250 g were used. Five groups, each with $n = 6$ animals per group, were included in this study as follows: (1) naïve, (2) sham, (3) injury + nanoparticle vehicle control (Inj + Veh), (4) injury + 25 μg E2 nanoparticle (Inj + 25 μg E2), and (5) injury + 2.5 μg E2 nanoparticle (Inj + 2.5 μg E2). The naïve group was used to establish a baseline for cytokine/chemokine expression. Inclusion of this group was intended to aid in identifying analytes that are changed in neurotrauma, rather than as a result of the laminectomy procedure.

To generate the SCI model, animals were anesthetized with ketamine (100 mg/kg) and xylazine (5 mg/kg), and a laminectomy was performed at thoracic vertebrae 9 and 10 (T9 and 10); the dura mater was left intact. The spine was immobilized with a spinal stereotactic device, and a moderate to severe SCI was induced by the weight-drop model.⁴⁶ A weight of 5 g was dropped from a height of 8 cm onto an impounder (0.3 cm in diameter) placed on the dorsal surface of the spinal cord. Sham animals underwent only a T9 and 10 laminectomy.

Immediately after the injury induction, the sterile gel plug was placed directly on the dorsal surface of the contused spinal cord, filling the space voided by the laminectomy (Fig. 1). The incision was sutured, and animals were monitored for 6 h until euthanasia. At the 6 h time point, animals were deeply anesthetized with isoflurane, and whole blood was drawn via cardiac puncture into ethylenediaminetetraacetic acid (EDTA)-coated tubes for E2 concentration analysis and cytokine profiling. CSF was collected via cisterna magna puncture and flash frozen in liquid nitrogen for cytokine profiling via enzyme-linked immunosorbent assay (ELISA). For CSF analysis, only clear samples completely devoid of blood contamination were analyzed.

Animals were decapitated, and a necropsy was performed to collect a 1 cm section of spinal cord taken centered on the lesion site (0.5 cm caudal to lesion epicenter taken with 0.5 cm rostral to epicenter). It was observed at necropsy that all gel plugs remained in place, and the gel plug was removed and discarded before tissue collection. Tissue was flash frozen in liquid nitrogen and stored at -80°C until processing. Naïve animals were euthanized in identical fashion, and samples were taken at same time of day as in the experimental animals to control for potential circadian effects on cytokine/chemokine levels.

FIG. 1. (A) Polyethylene glycol (PEG) decorated poly D lactide co-glycolide polymeric micelle nanoparticle loaded with 17- β estradiol (E2). (B) E2 loaded nanoparticles embedded into agarose gel plug for implantation onto spinal cord. (C) Contusion injury resulting in lesioned spinal cord. (D) Placement of gel plug on spinal cord lesion. Color image is available online at www.liebertpub.com/neu

Estrogen plasma and tissue concentration

Whole blood drawn via cardiac puncture was collected into EDTA containing tubes (final concentration EDTA 1.5 mg/mL) and placed immediately on ice. Whole blood samples were spun at 10,000 rpm in a Sorvall Legend Micro21 bench top centrifuge, and plasma was collected and stored at -80°C . 17- β estradiol (E2) concentration was determined using a commercially available ELISA kit (Calbiotech Estradiol ELISA ES180S), samples were run undiluted, and kit directions were followed exactly. Spinal cord tissue homogenate (aliquot of identical sample as used for cytokine profiling) was diluted either 1:100 for the 25 μg E2 dose or 1:10 for the 2.5 μg E2 in 6% bovine serum albumin, and samples were run on the same ELISA kit as plasma analysis. Using protein concentration data gathered from Bradford assay, tissue sample E2 concentration was then converted from pg/mL to pg/ μg total protein to account for differences in protein concentrations between samples.

Cytokine profiling

Tissue. Spinal cord tissue samples were homogenized in 400 μL of ice cold PBS with a protease inhibitor cocktail (Sigma P1860, final dilution 1:200). Samples were homogenized with a Polytron handheld tissue homogenizer, set at maximum speed for 30 sec on ice. Samples were then sonicated briefly and spun at 10,000 g for 20 min at 4°C . Supernatants were analyzed via Bradford assay for protein concentration, and samples were then normalized to a final protein concentration of 8 $\mu\text{g}/\text{mL}$ with homogenizing buffer.

Normalized samples were stored at -80°C and then shipped on dry ice to Eve Technologies (www.evetechologies.com) and run undiluted on the cytokine/chemokine 27-plex discovery assay that included: Eotaxin, epidermal growth factor (EGF), fractalkine, IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12(p70), IL-13, IL-17A, IL-18, IP-10, GRO/KC, interferon-gamma (IFN- γ), tumor

necrosis factor (TNF)- α , G-CSF, GM-CSF, MCP-1, leptin, LIX, MIP-1 α , MIP-2, RANTES, vascular endothelial growth factor (VEGF).

Plasma. Plasma samples collected at necropsy were stored at -80°C and then shipped on dry ice to Eve Technologies. Samples were run undiluted on the rat cytokine/chemokine 27-plex discovery assay.

CSF. CSF was taken at necropsy, flash frozen in liquid nitrogen, stored at -80°C , and then shipped on dry ice to Eve Technologies. Samples were run undiluted on the rat cytokine/chemokine 27-plex discovery assay. Three groups (sham, Injury + 25 μg , Injury + 2.5 μg) contained only $n=5$ samples because of blood contamination.

Western Blot S100 β

An aliquot of the spinal cord tissue homogenate that was used for cytokine profiling was used for Western blotting, $n=6$ samples representative of the six animals per group ($n=24$ total samples included in analysis). Using the data from the Bradford assay for protein estimation, samples were diluted to 1.0 $\mu\text{g}/\mu\text{L}$ protein concentration with sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 5 mM β -mercaptoethanol, 10% glycerol) and boiled for denaturation. Then 12.5 μL (12.5 μg total protein) of each sample were loaded into 4–20% precast gels (Bio-Rad Laboratories, Hercules, CA) and resolved at 100 V for 1 and 1/2 h. Immunoblotting was performed as described in a previous laboratory publication.⁴⁷

The resolved gel was transferred to ImmobilonTM-polyvinylidene fluoride microporous membranes (Millipore, Bellerica, MA). The membranes were blocked for 1 h with nonfat dry milk powder in wash buffer (0.1 % Tween-20 in 20 mM Tris-HCl, pH 7.6). Immunoblotting was then performed with primary antibody Anti-S100 β (Abcam rabbit monoclonal #ab52642) at 1:1000, overnight at 4°C , and Anti- β -Actin (Sigma mouse monoclonal #A2228) at 1:10,000 room temperature for 1 h. The membranes were then incubated with horseradish peroxidase tagged secondary antibodies (dilution 1:2000) tagged with (primary goat anti-mouse or primary goat anti-rabbit antibodies) at room temperature.

Chemiluminescent reagents (ECL Prime, GE Healthcare) were used to detect the immunoreactive bands on the membranes, and the images of protein bands were captured via Alpha Innotech FluorChem FC2 Imager. The immunoreactive bands were then quantified using NIH's Image J software. Given multiple gels were used to accommodate all of the samples, the data were analyzed as a percent of control. Sham animals were defined to be 100% and the relative band intensity of the remaining animals was normalized to the average of the sham group per gel.

Statistical analysis

To test our hypothesis that E2 may significantly decrease IL-6, MCP-1, and GRO-KC, a one-way analysis of variance (ANOVA) test was performed using GraphPad Prism 5.0 with a Tukey multiple comparison post-test to determine any differences between sham, Inj + Veh, Inj + 25 μg E2, and Inj + 2.5 μg E2 treated groups. Each test was conducted for each marker in the plasma, tissue, and CSF compartments such that nine one-way ANOVAs were run. Significance was defined as $p < 0.05$.

In addition, as a screening tool to assess the remaining markers, the nonparametric Wilcoxon rank sum test was used to detect differences among naïve, sham, Inj + Veh, and Inj + E2 treated groups. SAS 9.3 software was used to perform the analysis. Each matrix was treated as a separate group, and all of the analytes reported were included in the analysis, such that for plasma, 75 comparisons were made; in CSF, 64 comparisons were made; and in tissue, 75

comparisons were made. Significant differences were defined as $p < 0.05$.

A false discovery rate (fdr) correction was made to account for the multiple comparisons made within each statistical test. Both the uncorrected exact p value and the fdr corrected exact p values are shown for significant p values Inj + Veh vs. Inj + 25 μg / Inj + 2.5 μg in tabular form (Supplementary Tables 1–3; see online supplementary material at ftp.liebertpub.com).

Results

E2 plasma and tissue concentration

The E2 plasma concentration (mean $\pm$ standard error of the mean [SEM]) of animals in the sham group (14.6 pg/mL $\pm$ 0.7) was comparable to the Inj + Veh group (15.8 pg/mL $\pm$ 0.9) (Fig. 2). These values were within range of reported serum concentrations in female rats (during estrous/trough E2 levels) using a radioimmunoassay technique.⁴⁸ E2 concentration in plasma at 6 h post-agarose gel plug delivery to the lesioned spinal cord was nearly 10-fold higher in the 25 μg dose group (725.2 pg/mL $\pm$ 175.2) vs. the 2.5 μg dose group (81.6 pg/mL $\pm$ 18.6) (Fig. 2).

Notably, the E2 concentration in the 2.5 μg dose group was lower than reported E2 concentrations in female rats during proestrous, or peak E2 at 300 pg/mL.⁴⁹ This suggests that the concerns of supraphysiological E2 dosing, as seen in humans taking high dose contraception, may be avoidable using a focal nanoparticle delivery approach that may allow for physiologically relevant plasma E2 levels.

The E2 tissue concentration (mean $\pm$ SEM) of animals in the sham group (1.6 pg/ μg $\pm$ 0.08) was comparable to the Inj + Veh group (0.9 pg/ μg $\pm$ 0.07) (Fig. 2). E2 concentration in spinal cord tissue was also nearly 10-fold higher in the 25 μg dose group (1564 pg/ μg $\pm$ 366) vs. the 2.5 μg dose group (163 pg/ μg $\pm$ 47) (Fig. 2).

FIG. 2. Plasma and spinal cord tissue E2 levels 6 h after injury induction and treatment with E2 nanoparticle gel plugs measured by enzyme-linked immunosorbent assay. Plasma and tissue concentrations in sham and Injury + Vehicle (Inj + Veh) groups showed no difference between groups and were within expected range for male rats. Plasma levels in the Inj + 25 μg E2 group (725.2 pg/mL $\pm$ 175.2) were approximately one log higher than the levels found in the Inj + 2.5 μg E2 group (81.6 pg/mL $\pm$ 18.6), suggesting linear dose response. Tissue levels in the Inj + 25 μg E2 group (1564 pg/ μg $\pm$ 366) and the Inj + 2.5 μg E2 group (163 pg/ μg $\pm$ 47) were also approximately one log higher and were both twice that seen in plasma. $n = 6$ per group

This suggests that given the doses chosen in this study, at this single time point, the nanoparticle E2 release is dose linear. In addition, there is double the concentration of E2 (at both doses) in the tissue compared with plasma. These data provide evidence to support our hypothesis that focal nanoparticle delivery of E2 may allow for increased tissue concentrations over plasma.

CSF cytokine/chemokine profiling

Eighteen of the 27 cytokines/chemokines profiled were assayed at measurable levels. G-CSF, Eotaxin, GM-CSF, IL-5, LIX, TNF- α , MIP-2, RANTES, and MIP-1A were found to be below the levels of quantification (BLQ), and thus not reported. Both IL-6 and GRO-KC were increased with SCI and decreased with E2 treatment, but did not reach significance (Fig. 3). MCP-1, however, was found to be significantly increased with SCI and significantly decreased at both doses of E2 (Fig. 3).

Using a screening approach to evaluate the remaining markers, IL-10, IL-2, IFN- γ , IL-17a, and TNF- α were found to be increased with injury and decreased with E2 treatment (Supplementary Table 1; see online supplementary material at ftp.liebertpub.com). Showing no change with either injury or E2 treatment were IL-4, IL-13, and IL-18 (Supplementary Table 1). IL-1 β , IL-12p70, and leptin showed increased levels with E2 treatment (Supplementary Table 1). The growth factors EGF and VEGF had slightly decreased levels in the 2.5 μg E2 dose group, and the remaining chemokines (IP-10, fractalkine, LIX, and RANTES) exhibited no change with either injury or E2 treatment (Supplementary Table 1).

The factors that showed the greatest change between sham and injury, recapitulating findings in a human CSF biomarker study,⁴² were IL-6, MCP-1, and GRO-KC. In summary, data suggest that E2 treatment at either the 25 or 2.5 μg dose may modulate the following cytokines/chemokines in the CSF: IL-1 α , IL-6, IL-10, IL-2, IL-10, IFN- γ , IL-17a, TNF- α , IL-1 β , IL-12p70, leptin, EGF, VEGF, MCP-1, and GRO-KC.

Spinal cord tissue cytokine/chemokine profiling. All 27 of the cytokines/chemokines profiled were assayed at measurable levels. G-CSF, however, was BLQ for the naïve and 25 μg E2 dose groups. Both IL-6 and GRO-KC were significantly increased with SCI and significantly decreased at the 2.5 μg E2 dose (Fig. 3). MCP-1 was significantly increased with SCI; however, E2 treatment only slightly trended for a decrease (Fig. 3).

Using the screening approach to evaluate the remaining markers, it was found that the majority of cytokines showed no change in level with either injury or E2 treatment. The cytokines not showing any change were IL-4, IL-2, IL-13, IL-12p70, IFN- γ , IL-5, IL-17A, and IL-18 (Supplementary Table 2; see online supplementary material at ftp.liebertpub.com). Showing increased levels with injury and decreased levels with E2 treatment were IL-1 α , IL-1 β , IL-6, IL-10, TNF- α , and leptin (Supplementary Table 2). The chemokines eotaxin, fractalkine, and LIX showed no changes in level with injury or E2 treatment. G-CSF, MIP-1 α , MCP-1, GRO-KC, LIX, MIP-2, and RANTES were increased with injury and decreased with E2 treatment while IP-10 levels were increased with injury but not affected by E2 treatment (Supplementary Table 2).

The growth factors EGF and VEGF were increased with injury and decreased in E2 treated groups (Supplementary Table 2). The factors that showed the greatest change in expression between sham and injury, suggesting their specificity as biomarkers of SCI, were IL-1 α , IP-10, GRO-KC, and MIP-2. These factors showed 4–7-fold increases between sham and injury groups. The marker with the

FIG. 3. IL-6, GRO-KC, and MCP-1 in plasma, spinal cord tissue, and cerebrospinal fluid (CSF) after spinal cord injury measured by multiplex enzyme-linked immunosorbent assay. A significant reduction in IL-6 and GRO-KC in plasma and tissue was found with E2 treatment. A significant reduction in MCP-1 was found in plasma and CSF with E2 treatment. #Significant differences between sham and Injury + Vehicle (Inj + Veh) groups, $p < 0.05$. *Significant difference between Inj + Veh group and Inj + 25/2.5 µg E2 treated group, $p < 0.05$ (one-way analysis of variance with a Tukey multiple comparison post-test). $n = 6$ per group for plasma and tissue; $n = 5$ per group CSF.

largest increase in expression was MIP-1 α , with a 20-fold increase in expression between sham and injury groups. In summary, data suggest that E2 treatment at either the 25 or 2.5 µg dose modulate the following cytokines/chemokines in the spinal cord tissue: IL-1 α , IL-1 β , IL-6, IL-10, TNF- α , leptin, G-CSF, MIP-1 α , MCP-1, GRO-KC, LIX, MIP-2, RANTES, EGF, and VEGF.

Plasma cytokine/chemokine profiling. All 27 of the cytokines/chemokines profiled were assayed at measurable levels. IL-6, GRO-KC, and MCP-1 were all found to be significantly decreased with E2 treatment (Fig. 3). Surprisingly, the levels of IL-6, GRO-KC, and MCP-1 were comparable between sham and Inj + Veh animals. Comparison of these markers in naïve animals reveals that all three markers are increased because of the laminectomy procedure, suggesting that in plasma, these are general markers of inflammation because of the surgical procedure rather than markers specific to injured spinal cord tissue (Supplementary Table 3; see online supplementary material at ftp.liebertpub.com).

Using the screening approach to evaluate the remaining markers, it was found that the majority of cytokines in the plasma showed dynamic responses to sham, injury, and E2 treatment. IL-1 α , IL-4, IL-1 β , IL-2, IL-13, IL-10, IL-12p70, IFN- γ , IL-5, IL-17A, IL-18, and TNF- α were increased with either sham/injury and decreased with E2 treatment (Supplementary Table 3). Both growth factors EGF and VEGF were increased with injury and decreased with E2 treatment, and the chemokines IP-10, fractalkine, MIP-2, and RANTES were unchanged between injury and E2 treated groups (Supplementary Table 3). Eotaxin, GM-CSF, and MIP-1 α were decreased with E2 treatment, but unchanged by injury (Supple-

mentary Table 3). G-CSF and LIX were increased with injury and decreased with E2 treatment (Supplementary Table 3).

A number of factors showed large increases in expression between the naïve and sham groups: IL-1 α , IL-4, IL-1 β , IL-6, IL-13, IL-10, IL-12p70, IFN- γ , IL-18 and GRO-KC (55-fold increase). Given that the sham treatment (laminectomy without contusion injury) does not involve damage to the spinal cord, however, these markers could represent systemic response to trauma. Leptin, however, had a three-fold increase between sham and injury, making it a potential biomarker specific to SCI. In summary, data suggest that E2 treatment at either the 25 or 2.5 µg dose may modulate the following cytokines/chemokines in the plasma after SCI: IL-1 α , IL-4, IL-1 β , IL-2, IL-6, IL-13, IL-10, IL-12p70, IFN- γ , IL-5, IL-17A, IL-18, TNF- α , EGF, VEGF, Eotaxin, GM-CSF, MIP-1A, G-CSF, MCP-1, GRO-KC, and LIX.

Western blot S100 β

There was a significant increase in S100 β protein levels in spinal cord tissue of Inj + Veh group (195% of control) vs. sham (100% of control) (Fig. 4). There was a nonsignificant reduction of S100 β in the Inj + 25 µg E2 vs. Inj + Veh (153% of control vs. 195% of control); however, there was a significant reduction in S100 β protein expression in the Inj + 2.5 µg E2 group vs. Inj + Veh (89% of control vs. 195% of control) (Fig. 4). The Inj + 2.5 µg E2 group had S100 β levels comparable to those seen in sham.

Discussion

Given safety concerns associated with high doses of E2 (thrombus, feminization, stroke, etc.), we have used a nanoparticle

FIG. 4. S100 β expression in injured spinal cord tissue by Western blotting. A significant increase in S100 β was found in the Injury + Veh (Inj + Veh) group compared with sham, and a significant decrease was seen with the 2.5 μ g E2 dose. Two representative bands per group displayed ($n=6$ per group total). #Significant differences between sham and Inj + Veh groups, $p<0.05$. *Significant difference between Inj + Veh group and Inj + 25/2.5 μ g E2 treated group, $p<0.05$ (one-way analysis of variance with a Tukey multiple comparison post-test).

drug delivery system to lessen these concerns while delivering a therapeutic dose directly to the spinal cord. We report that by using an E2 nanoparticle embedded gel approach, there is double the concentration of E2 in the spinal cord tissue compared with plasma (Fig. 2). In addition, the plasma concentration at the 2.5 μ g E2 dose resulted in plasma levels of 81 pg/mL (Fig. 2). These levels are well within physiological range (peak nonpregnant plasma E2 levels in females are 300 pg/mL⁴⁹). Therefore, E2, if delivered focally to damaged tissue, may be efficacious at physiological plasma levels. This approach retains significant translational relevancy, because the majority of patients with acute SCI will undergo spine stabilization surgery, thus providing opportunity for surgical placement of a therapeutic gel patch.

The approach of sampling three biological compartments with naïve controls has allowed for a further understanding of how inflammatory signals correspond between plasma/CSF/tissue in both SCI and in surgical intervention (laminectomy). Notably, the plasma levels of IL-6, GRO-KC, and MCP-1 are comparable between sham and Inj + Veh groups (Fig. 3), while naïve controls are significantly lower (Supplementary Table 3). These results suggest that the laminectomy procedure alone can result in the same response of IL-6, GRO-KC, and MCP-1 in plasma, and suggest that IL-6, GRO-KC, and MCP-1 may be primarily derived from a peripheral source rather than injured nervous tissue.

The pattern of IL-6, GRO-KC, and MCP-1 in tissue and CSF are very different from plasma, however. In tissue and CSF, sham levels are lower than Inj + Veh animals, revealing specificity for injury within these compartments (Fig. 3). Given the specificity of

the CSF for increased IL-6, GRO-KC, and MCP-1 only in the presence of SCI, the data suggest the pool of IL-6, GRO-KC, and MCP-1 measured in CSF is derived, in part at least, from the damaged spinal cord.

Because human studies must extrapolate how the CSF or plasma relates to the state of the injured spinal cord, the data presented here provide a comprehensive profile of acute inflammatory signaling in three compartments to allow for a greater understanding of how plasma and CSF reflect tissue response to injury across 27 cytokines/chemokines (Supplementary Tables 1–3). Because IL-6, GRO-KC, and MCP-1 have been previously identified in human studies as candidate biomarkers in CSF,⁴² these data, in a “reverse translational” approach, help provide evidence that the markers are reflective of the condition of the injured spinal cord.

E2 treatment resulted in rapid anti-inflammatory effects, and these effects were seen at both doses chosen in the present study (25 μ g or 2.5 μ g.) In particular, we focused on IL-6, GRO-KC, MCP-1, and S100 β because these markers have been identified previously in human studies as either being elevated in injury or as potential biomarkers of injury severity.^{42,50,51} E2 treatment significantly decreased IL-6 expression in plasma and tissue, and trended toward a decrease in CSF (Fig. 3). IL-6, a prototypical cytokine, is rapidly secreted at the site of injury by a number of cell types including neurons, glia, T-cells, and macrophages and plays a central role in early neuroinflammatory response.⁵²

E2 pretreatment has been reported to decrease tissue levels of IL-6 at a 24 h time point in a mouse model of SCI.¹⁷ Ritz and Hausmann²¹ reported, however, that a 0.1 mg/kg or 4 mg/kg E2 dose in an acute rat SCI model significantly increased IL-1 α , IL-1 β , and IL-6 in tissue at 6 h post-treatment. Differences in dosages, dose routes, and time points may explain the incongruous results with E2 regulation of IL-6 in acute SCI.

The neutrophil attractant chemokine, GRO-KC (human IL-8 homolog), has been reported to be increased in acute SCI.^{53,54} Neutrophils, one of the first peripheral immune cells to migrate to the lesioned tissue, are thought to be key contributors to the secondary injury seen in SCI⁵⁵ and further, a reduction of infiltrating neutrophils has shown decreased tissue damage in a rat model.⁵⁶ In a traumatic brain injury model, GRO-KC has also been associated with blood–brain barrier dysfunction and nerve growth factor production, making it a potentially important chemokine in neurotrauma and repair.⁵⁷ Thus, GRO-KC (IL-8) may be a critical chemokine to regulate in acute SCI. Here, we report that E2 treatment significantly reduced GRO-KC in the plasma and tissue compartments with a trend for reduction in the CSF (Fig. 3), again highlighting the therapeutic potential of E2 in acute SCI.

MCP-1 (CCL2) is a small chemokine induced by a variety of stimuli (injury, inflammation, oxidative stress, etc.); it is produced by a variety of cell types and functions to recruit monocytes/macrophages to the site of injury or inflammation.⁵⁸ Studies have found a correlation between macrophage infiltration and MCP-1 levels,⁵⁹ and MCP-1 has been found to be upregulated in animal models after SCI.⁵⁹ In a naturally occurring canine SCI, MCP-1 levels in CSF were negatively associated with post-injury outcome.⁶⁰

Here, we report that E2 treatment resulted in a significant decrease in MCP-1 levels in plasma and CSF; tissue levels had a slight trend only for decrease (Fig. 3). A link has been shown between estrogen receptor α (ER α) and MCP-1 expression. In an experimental autoimmune encephalomyelitis (EAE) model, treatment with ER α specific ligand resulted in decreased MCP-1.⁶¹ Given these previous findings, we provide data here to further

support the hypothesis that E2 may decrease inflammation through regulation of MCP-1 signaling.

The calcium binding protein S100 β , most abundantly found in glial cells, is involved in a number of cellular processes including: inhibition of protein phosphorylation, regulation of cell-cycle progression, astrocyte proliferation, and in a concentration dependent manner can act as either a trophic or toxic factor to neurons.⁶² A direct link between E2 and S100 β expression is not yet known.

E2 has been reported to decrease calcium influx through interaction with L-Type voltage gated calcium channels in an *in vitro* setting of glutamate excitotoxicity.⁶³ Given S100 β is a calcium binding protein, a relationship direct or indirect may exist where E2's modulation on calcium influx would influence S100 β expression and/or release.

It should be noted, however, that S100 β is a highly complex signaling protein, with the potential to serve widely varying functions in intracellular or extracellular roles that are also dependent on disease state.⁶⁴ Given the complexity of both E2 and S100 β , it may be overly simplistic to hypothesize calcium as the link between the two because numerous additional pathways may be involved.

Regardless of this complexity as a signaling molecule, the role as a biomarker has been shown in numerous animal models of SCI where S100 β is elevated in serum and/or CSF of injured animals compared with sham.^{65–67} It has been identified in two human SCI studies as a candidate biomarker, capable of predicting injury severity into either a specific American Spinal Injury Association rating⁴² or complete vs. incomplete injuries.⁵⁰ Given the growing evidence that S100 β may be a valid biomarker for injury severity in human SCI, our findings that E2 nanoparticle treatment significantly decreased S100 β expression in spinal cord tissue (Fig. 4) suggest that early intervention with E2 may decrease injury severity. Because of the small volume of CSF that can be collected from a rat, we were unable to perform both cytokine/chemokine screens and S100 β analysis; however, this warrants further investigation.

While IL-6, GRO-KC, MCP-1, and S100 β were the focus of the study, the multiplex assay format allowed for an additional screen to be performed for the potential identification of new E2 targets. Using a Wilcoxon rank sum test to screen all 27 markers evaluated, it was found that E2 treatment exerted widespread effects on cytokines/chemokines/growth factors such as: leptin, MIP-1 α , IL-4, IL-2, IL-10, IFN γ , TNF α , etc. More specifically, 15 analytes were significantly decreased in plasma (Supplementary Table 3), 6 analytes were significantly decreased in spinal cord tissue (Supplementary Table 2), and a number trended to be decreased in CSF (Supplementary Table 1). The lack of significance in the CSF compartment may be attributed to increased variance in these samples compared with tissue or plasma.

These findings further highlight the pleiotropic nature of E2 in the setting of neurotrauma and suggest that the rapid anti-inflammatory signaling exerted by E2 may contribute to the improvement in motor function as reported previously.^{17,19–23} It remains to be seen whether low dose nanoparticle delivered E2 may result in functional improvement. As we continue to evaluate this method of E2 delivery in functional models of SCI, however, we will seek a better understanding of how early anti-inflammatory treatment may modulate late-stage motor recovery. Thus, by using this targeted nanoparticle drug delivery approach, we hope to advance the highly promising therapeutic E2 into clinical studies, ultimately allowing for development of a safe and effective treatment for persons with acute SCI.

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Author Disclosure Statement

No competing financial interests exist.

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