Human NgR-Fc Decoy Protein via Lumbar Intrathecal Bolus Administration Enhances Recovery from Rat Spinal Cord Contusion

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Abstract

Axonal growth and neurological recovery after traumatic spinal cord injury (SCI) is limited by the presence of inhibitory proteins in myelin, several of which act via the NgR1 protein in neurons. A truncated soluble ligand-binding fragment of NgR1 serves as a decoy and promotes recovery in acute and chronic rodent SCI models. To develop the translational potential of these observations, we created a human sequence-derived NgR1(310)-Fc protein. This protein is active *in vitro*. When the human NgR1 decoy is administered by continuous intracerebroventricular infusion to rats with a spinal contusion injury at doses of 0.09–0.53 mg/kg/d, neurological recovery is improved. Effective doses double the percentage of rats able to bear weight on their hindlimbs. Next, we considered the half-life and distribution of NgR1(310)-Fc after bolus delivery to the lumbar intrathecal space. The protein is found throughout the neuraxis and has a tissue half-life of approximately 2 days in the rat, and 5 days in the nonhuman primate. At an intermittent, once every 4 day, lumbar bolus dosing schedule of 0.14 mg/kg/d, NgR1(310)-Fc promoted locomotor rat recovery from spinal cord contusion at least as effectively as continuous infusion in open field and grid walking tasks. Moreover, the intermittent lumbar NgR1(310)-Fc treatment increased the growth of raphespinal axons into the lumbar spinal cord after injury. Thus, human NgR1(310)-Fc provides effective treatment for recovery from traumatic SCI in this preclinical model with a simplified administration regimen that facilitates clinical testing.

Key words: contusion; myelin; Nogo; Nogo receptor; pharmacokinetics; translational science; traumatic spinal cord injury

Introduction

TRAUMATIC INJURIES TO THE SPINAL CORD interrupt axonal connectivity between rostral and caudal segments of the central nervous system (CNS) and produce sustained neurologic deficits. Recovery is largely dependent on forming new synaptic connections between surviving neurons. Unfortunately, the growth of axons and dendrites in the adult mammalian CNS after injury is extremely limited, rendering recovery highly circumscribed.

Axonal growth is restricted by both intrinsic and extrinsic mechanisms (reviewed in ^{1,2}). The extracellular environment of the brain and spinal cord contains inhibitory molecules produced by non-neuronal cells. This includes scar tissue composed in part of chondroitin sulfate proteoglycans (CSPG) derived from astrocytes and of other extracellular matrix materials from fibroblasts and pericytes. In addition, the oligodendrocyte produces myelin proteins with potent growth inhibitory properties. Among these myelin

associated inhibitors are Nogo, myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMgp).^{3–8} These three inhibitory molecules are capable of binding with nanomolar affinity to the Nogo-66 receptor, NgR1 (Rtn4R).^{7,9–11} The same three proteins also interact with PirB (LilrB3) as an alternative receptor in some situations.^{12–14} Given the presence of inhibitory factors, one method to promote axonal growth and recovery of neurologic function is to block these negative factors.¹⁵

Several approaches to blocking myelin-associated inhibition of axonal growth have been studied. Antibodies to Nogo or antibodies to MAG can enhance neurological recovery in certain preclinical models^{16–20} and have entered clinical trials for stroke or spinal cord injury (SCI) (ClinicalTrials.gov NCT00406016, NCT00833989, NCT01808261).^{21,22} These approaches, however, neutralize only one of the three myelin inhibitors. In contrast, a soluble fragment of NgR1 that contains the ligand binding domain (aa residue 27–310) can function as a decoy blocking all three myelin inhibitors.^{10,23–25}

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Previous studies have shown efficacy with rat NgR1(310)-Fc protein in a broad range of preclinical experiments. For SCI, intrathecal administration improves neurological function after thoracic dorsal hemisection and after acute thoracic spinal contusion.^{26–28} Most critically, NgR1(310)-Fc is effective in restoring locomotor function in models of chronic spinal cord contusion.²⁹ Treatment with this protein also enhances recovery from dorsal root crush injury and ischemic stroke.^{30,31} Mechanistically, NgR1(310)-Fc increases the sprouting of uncut fibers separate from the injury site, as well as promoting axonal regeneration from cut fibers. Neurological recovery may depend on sprouting and plasticity, at least as much as frank regeneration.³²

With this background, we have sought to provide a translational path to test NgR1(310)-Fc in cases of human SCI. Here, we have developed a human version of the NgR1(310)-Fc protein. It is biologically active, and we have defined a dose response for efficacy in rat spinal cord contusion. We have also considered delivery mechanisms for this protein. Pharmacokinetic studies show that it distributes widely in the CNS and possesses a half-life allowing for intermittent bolus delivery to the lumbar space. Using lumbar intrathecal intermittent bolus delivery, we observed both neurological recovery and raphespinal fiber sprouting. These findings support the potential for clinical translation of NgR1(310)-Fc protein.

METHODS

Human NgR1(310)-Fc production

Human NgR1(310)-Fc was expressed in a stable CHO-S cell line at Axerion Therapeutics. The expression vector encodes aa residues 1–310 of human NgR1 with the Cys266Ala and Cys309Ala substitutions fused to the Fc domain of human IgG1, as described for rat protein previously.³³ After vial thaw, the cells were expanded in protein free, animal origin component-free media. Shake flask cultures were grown in appropriately sized, nonbaffled tissue culture flasks. Cell expansion continued until a sufficient number of cells were attained to inoculate 1-liter bioreactors. The bioreactors were run in fed-batch mode, with commercially available feed plus glucose. The pH and dissolved oxygen were controlled throughout the bioreactor run. Antifoam was added as needed. The bioreactor process ran for approximately 2 weeks. At the conclusion of the bioreactor run, the supernatant was harvested by centrifugation and clarified by sequential filtrations through 0.8 micron and 0.2 micron polyethersulfone membranes.

The purification scheme consisted of three chromatography steps: Protein A (bind-elute mode), Q-Sepharose FF (flow-thru mode), and Q-Sepharose FF (bind-elute mode) chromatography steps. hNgR1(310)-Fc was captured from clarified harvest using Protein A (bind-elute mode) chromatography. After low pH elution, the protein was held at room temperature for 1 hour as a viral inactivation step. After the viral inactivation step, the pH and conductivity of the protein solution was adjusted for load onto an anion exchange chromatography column (Q-sepharose FF). This column was run in flow-thru mode and removed impurities such as host cell protein and DNA. The final column purification step, Q-Sepharose FF (bind-elute mode), was a polishing step to remove additional impurities and to concentrate the protein before dialysis. After the final column chromatography step, the hNgR1(310)-Fc was buffer-exchanged into phosphate buffered saline (PBS) (pH 7.0). The final concentration of the protein was 10 mg/mL.

hNgR1(310)-Fc binding activity

The ability of hNgR1(310)-Fc to bind independently to Nogo-66, MAG, and OMgp was tested in enzyme-linked immunosorbent assay (ELISA)-based binding assays. In this assay, a tagged ligand (biotinylated Nogo-66, MAG-6xHis-StrepTagII, or OMgp-6xHis-StrepTagII) was bound to Streptavidin (Nogo-66) or Strep-Tactin (MAG or OMgp) coated microtiter plates. The plate was blocked with bovine serum albumin (BSA), standards were added in duplicate, and the plate incubated for 16 h. Detection antibody (goat antihuman antibody to the Fc region) was added, and the plate was incubated for 1 hour. Tetramethylbenzidine (TMB) substrate was added and incubated for 30 min before the reaction was stopped with HCl. Biotinylated Nogo-66 (Biotin-RIYKGVIQAIQKSD EGHFPFRAYLESEVAISEELVQKYSNSALGHVNSTIKELRRRLF LVDDLVDLSLK-Amide) was obtained by commercial synthesis. MAG-6xHis-StrepTagII (MAG) and OMgp-6xHis-StrepTagII were produced from stable cell lines developed at Axerion Therapeutics. The background values without ligand coating were subtracted from the values obtained from ligand-coated plates.

Axon regeneration assay

The culture of E18 rat neurons and their exposure to Nogo-22 kDa inhibitory fragment has been described previously.¹³ Here, the purified NgR1(310)-Fc or Nogo-22 or both were added immediately after the scrape injury. Five days later, the extent of axonal regeneration was scored by automated analysis of β III-tubulin staining with an ImageExpressMicro machine (Molecular Devices).

hNgR1(310)-Fc tissue levels

Tissue from rat or nonhuman primate was collected and frozen at -80° before assays. The tissue was homogenized in nine volumes of radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1.0% Triton X-100, 0.5% Na deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) containing enzyme inhibitor cocktail at 4° . After incubation, the homogenates were centrifuged at $15,000 \times g$ for 30 min. The supernatant was assayed for NgR1(310)-Fc level. To detect NgR1(310)-Fc, microtiter plates were coated with Donkey Anti-Human IgG, Fc Fragment Specific (Jackson ImmunoResearch), and then blocked with 1% BSA. Tissue lysates were incubated in these plates for 12–18 h at 4° , and then washed with Tris buffered saline (TBS), 0.1% Tween (TBS-T) before adding goat anti-NgR1 antibody (R&D Systems, #AF1440) followed by biotin-conjugated Bovine Anti-Goat IgG(H+L) secondary antibody. Bound material was detected with DELFIA Eu-labeled Streptavidin (Perkin Elmer) using time resolved fluorescence at excitation at 340 nm and emission at 615 nm. The assay was linear over the range from 0.3–200 ng/mL of NgR1(310)-Fc in samples. Undiluted tissue extracts from untreated rat brain did not alter the standard curve detectably.

hNgR1(310)-Fc pharmacokinetic studies

Animals were housed, dosed, and tissue collected at Northern Biomedical Research, Inc. (Spring Lake, MI). For rat studies, Charles River CrI:CD[®](SD)BR male rats of 250–275 g were anesthetized with isoflurane. A catheter was inserted at the cisterna magna level and advanced 8 cm, past the lumbar enlargement. The proximal end of the catheter was extended through the skin and plugged. Postsurgically, the animals received a single intramuscular dose of ceftiofur sodium (5 mg/kg), butorphanol tartrate

(0.05 mg/kg). After a surgical recovery period of >5 days, a slow bolus dose of NgR1(310)-Fc was administered through the catheter system at a dose volume of 20 μ L followed by 20 μ L of PBS to flush the dose from the catheter system. Animals were sacrificed at 1–168 h after dosing and the brain and spinal cord removed for further analysis.

For multidose pharmacokinetic studies, rats received exactly the same dosing procedure as in the intermittent lumbar intrathecal bolus spinal cord contusion experiments described below, but there was no spinal cord contusion.

For cynomolgus monkeys of 3–5 years of age and 3–4.5 kg body weight, intrathecal catheters were placed under ketamine and isoflurane anesthesia with the tip located at the thoracolumbar junction (IT-L). After a surgical recovery period of >5 days, a slow bolus dose of 2.0 mg NgR1(310)-Fc was administered through the IT-L catheter system at a dose volume of 400 μ L followed by 600 μ L of PBS vehicle to flush the dose from the catheter system. Animals received an additional four 2.0 mg doses of NgR1(310)-Fc given at 3-day intervals. After completion of dosing, animals were sacrificed at 1–168 h after dosing and the brain and spinal cord removed for further analysis.

Rat spinal contusion model

Female Sprague-Dawley rats (10–11 weeks, 220–240 g) were used in this study. Animals were anesthetized with intraperitoneal injection of ketamine (60 mg/kg) and xylazine (10 mg/kg) mixture. A laminectomy was conducted at the caudal portion of T6 and all of T7 spinal levels. A T7 moderate contusion injury (weight of 10 g, height of 25 mm) was produced with the MASCIS impactor as described previously.^{27,29} After the spinal contusion, muscle and skin layers were sutured with 4.0 polyglactin.

Intracerebroventricular (i.c.v.) cannulation and continuous infusion therapy for SCI

Rats received i.c.v. infusion as described.^{27,29,30} Immediately after the spinal contusion injury, rats were placed in a stereotactic frame. A midline, sagittal incision starting slightly behind the eyes through the skull was made on the scalp and the skull was exposed. A right-sided burr hole was drilled and a cannula (Alzet brain infusion kit 2; DURECT) was introduced into the right lateral ventricle at stereotaxic coordinates 0.6 mm posterior and 1.2 mm lateral to bregma and 4.0 mm deep to the pial surface. The cannula was connected to an osmotic minipump (ALZET osmotic pumps, 2ML4; 2 mL volume, 2.5 μ L/h, 28 day continuous delivery; DURECT) containing PBS placed subcutaneously over the scapulae. The cannula was glued in place with cyanoacrylate, and the skin was sutured. Three days after contusion injury, the rats were reanesthetized with isoflurane (5% induction and 1–2% maintenance) and the PBS minipumps were replaced with new osmotic minipumps connected to the same cannula and containing test substance. These pumps were filled with NgR1(310)-Fc in 2 mL PBS or PBS alone. Two sets of experiments were conducted in this study. In the first set of experiments, the osmotic pumps were filled with 0.66 mg of hNgR1(310)-Fc protein in 2 mL PBS (medium dose treatment group 0.09 mg/kg/d, n = 18), 4.0 mg of hNgR1(310)-Fc protein in 2 mL PBS (high dose treatment group, 0.53 mg/kg/d, n = 16), or 2 mL PBS alone (vehicle group, n = 16). In the second set of experiments, osmotic pumps were filled with 0.10 mg of hNgR1(310)-Fc protein in 2 mL PBS (low dose treatment group, 0.014 mg/kg/d, n = 14) or 2 mL PBS alone (vehicle group, n = 6).

Rat intrathecal catheterization and IT-L bolus injection for SCI therapy

For rats receiving lumbar intrathecal infusion, a laminectomy was conducted at the caudal portion of L3 and rostral portion of L4 spinal levels immediately after the spinal contusion injury. The dura was incised using a 25-gauge needle. An ALZET rat intrathecal catheter (item #0007740, DURECT) was introduced into the subdural space over the spinal cord. The catheter was secured to the lumbar muscle to prevent removal. The proximal part of the catheter was introduced subcutaneously through the thoracic area and exited the skin of the interscapular area. The tip was closed with sterile glue. Muscle and skin layers were sutured with 4.0 polyglactin. Three days after SCI, the rats were reanesthetized by inhalation of isoflurane as mentioned above. Fifteen μ L of PBS or hNgR1(310)-Fc protein (10 mg/mL) was delivered through the intrathecal catheter and additional 15 μ L of PBS was injected to flush the catheter after each bolus injection before sealing the catheter tip. The rats were injected at 4-day intervals from the third day after SCI for 4 weeks, with doses at post-injury days 3, 7, 11, 15, 19, 23, and 27. A total of seven different bolus injections were given to each rats with the total amount of 1.05 mg hNgR1(310)-Fc protein (with total dose equivalent to 0.14 mg/kg/d continuous infusion).

Choice of control conditions

We used the PBS vehicle as control in these experiments. In previous studies, we have compared rat NgR-Fc with either PBS or rat IgG control protein, and demonstrated efficacy compared with either control.^{26,27,30,34} There was no obvious difference in the outcome depending on which control is used. The PBS control is of the greatest translational relevance because this is the vehicle and can be considered “no treatment.” The IgG control is a selective control for the effect of NgR domain, a mechanistic question. We have conducted a pilot experiment comparing human IgG (Jackson ImmunoResearch, West Grove, PA, containing the Fc moiety) head-to-head with PBS (Supplementary Fig. 1; see online supplementary material at ftp.liebertpub.com). The two control groups are identical in the primary outcome, the Basso, Beattie, and Bresnahan (BBB) score. Thus, the choice of control protein has no obvious effect in this model at these doses.

Behavioral testing

All behavioral tests were performed by two researchers, both of whom were blinded to the identity of the compound in the osmotic pump. The BBB locomotor scale was used for the rat behavioral testing.³⁵ Tests were run 3 days after SCI and once a week starting from the first week of the injury. The grid walk test was performed at day 56 or 63 in different experiments. In this task, the animals are required to cross a 20 $\times$ 60 cm horizontal wire grid with 2.5 $\times$ 2.5 cm square gaps and successful forelimb steps, successful hindlimb steps (foot does not slip below the grip), and hindlimb faults (foot slips below the grid) were counted from videotapes over a 50 cm distance. Three measures were calculated and scores were recorded as total hindlimb steps, hindlimb missteps, and the ratio of successful hindlimb steps to forelimb steps. To perform well on this examination, the animals must demonstrate normal limb coordination, which is mediated by ventrolateral tracts, have an intact reticulospinal system for initiation of stepping rhythm, and possess voluntary motor control, which is mediated by corticospinal and rubrospinal systems.

Histology and analysis

At the end of the study, animals were perfused transcardially with PBS, followed by 4% paraformaldehyde/PBS solution and analyzed as described.^{8,26,27,29,36} The spinal cord 10 mm rostral to and 10 mm caudal to the lesion center was embedded in a glutaraldehyde-polymerized albumin matrix and cut parasagittally in the thickness of 40 μ m on a vibratome. Transverse sections (40 μ m) were collected from the spinal cord 11–16 mm rostral to and 11–16 mm caudal to the lesion center. Both sagittal and transverse sections of spinal cord were incubated with anti-5-hydroxytryptamine (anti-5HT) antibody (1:10,000; Immunostar, Hudson, WI) and then with Alexa Fluor 568-labeled secondary antibody (Invitrogen) to detect raphespinal fibers. Image analysis was performed with National Institutes of Health (NIH) image version 1.62, as described previously.^{26,27,29,36} For analysis of serotonin innervation, immunoreactive serotonin fibers in the ventral horn of transverse sections caudal to the lesion center were selected by thresholding; then the length of serotonin fiber per area was measured after using the “skeletonize” function.

For the groups receiving intermittent lumbar intrathecal bolus administration after spinal cord contusion, lesion area and tissue sparing were scored in 40 μ m thick parasagittal sections spanning the lesion site after staining with anti-glial fibrillary acidic protein (GFAP) antibody, as described previously.²⁷ Every fifth consecutive section (spaced 200 μ m apart) was stained and scored by an observer unaware of the treatment condition. Areas of high-intensity of GFAP staining, cavity, and GFAP-negative fibrotic scar were measured as the lesion area. The dorsal-ventral width of spared tissue at lesion center and at 10 mm rostral to the lesion were measured. The values from these sections were averaged. All measurements were performed by using Zeiss AxioVision Rel. 4.8 software.

Statistical analysis

Statistical comparisons included one-way analysis of variance (ANOVA) and repeated measures ANOVA with *post hoc* pairwise comparisons, as specified in the figure legends, using SPSS or Prism statistical software.

Results

Production of active human NgR1(310)-Fc protein

The rat NgR1(310)-Fc protein had been produced previously and shown to have activity in binding myelin inhibitors, in blocking the inhibition of these myelin proteins on axonal regeneration *in vitro*, and in promoting recovery from rat SCI.^{26–30,33} Here, we produced a human NgR1(310)-Fc protein. This polypeptide could be purified with high yield from Chinese hamster ovary (CHO) cell culture. The protein migrates as a single species (>97%) by size exclusion chromatography (Fig. 1A), and yields a single primary band at 66 kDa by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Fig. 1B). Without reducing agent, most of the protein migrates at 140 kDa, as expected for disulfide crosslinking in the Fc region (Fig. 1C).

The ability of purified human NgR1(310)-Fc to bind myelin inhibitors was tested in ELISA assays with immobilized Nogo-66, MAG, and OMgp (Fig. 2A–C). Robust dose-dependent binding is observed for all three ligands. In neuronal cultures, Nogo-22 kDa fragment inhibits axonal regeneration after mature cortical cultures are scrape-injured.¹³ The human NgR1(310)-Fc protein blocks this inhibition (Fig. 2D). Thus, we used purified human NgR1(310)-Fc for *in vivo* testing of efficacy and dosing in SCI recovery.

Human NgR1(310)-Fc improves rat recovery from spinal cord contusion

To evaluate the efficacy of the human form of the NgR1(310)-Fc protein in the rat model of spinal contusion injury, we replicated studies we performed previously with the rat version of the protein.^{27, 29} All rats underwent midthoracic contusion with placement of an i.c.v. cannula. The BBB score at 3 days was zero in all groups, meaning none of the rats was capable of any hindlimb movement.³⁵ Rats were randomized to receive either hNgR1(310)-Fc medium dose (0.09 mg/kg/d) and high dose (0.53 mg/kg/d) or PBS starting from the third day and continued for approximately 1 month while the nominal 28-day minipump emptied. Behavioral observation continued through 9–10 weeks (Fig. 3A). The final number of rats studied was $n = 12$ for vehicle (4 rats were lost to mortality), $n = 16$

FIG. 1. Production and purification of human NgR1(310)-Fc. (A) Size exclusion chromatography of NgR1(310)-Fc after purification (lot 15). The absorbance at 280 nm is plotted as a function of elution time from an ACQUITY UPLC BEH200 SEC column using a sodium phosphate/sodium chloride buffer system. The average peak retention times for thyroglobulin (670 kDa), γ -globulin (158 kDa), bovine serum albumin (67 kDa), myoglobin (16.5 kDa), and uracil (0.112 kDa) are indicated. (B) Purified NgR1(310)-Fc protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and stained with Coomassie Blue. The boxed regions were used to calculate a purity for the major band at 66 kDa of 97%. The migration of molecular weight standards is at right. (C) Purified NgR1(310)-Fc protein was analyzed by SDS-PAGE under non-reducing conditions.

FIG. 2. Human NgR1(310)-Fc binds myelin ligands and blocks outgrowth inhibition. (A–C) Streptavidin or Strep-Tactin plates coated with biotin-Nogo-66 (A) or myelin-associated glycoprotein (MAG) (B) or myelin glycoprotein (OMgp) (C) were incubated with the indicated concentrations of NgR1(310)-Fc, washed, and bound human Fc was detected with secondary peroxidase-labeled antibody. The absorbance of the peroxidase reaction is plotted as a function of NgR1(310)-Fc added. (D) Mouse E18 cortical neurons were cultured until a mature lawn of neurites formed. Then the cultures were scrape-injured, and regeneration over 5 days was measured in the presence of Nogo-22 kDa protein, NgR1(310)-Fc protein, or both. The inhibitory effect of Nogo is blocked by an excess of NgR1(310)-Fc. Data are mean $\pm$ standard error of the mean from three separate experiments. Color image is available online at www.liebertpub.com/neu

for medium dose (two rats were lost to mortality), and $n=14$ for high dose NgR1 treatment group (two rats were lost to mortality).

Two weeks after spinal cord contusion, there was a trend toward improved BBB locomotor score in medium and high dose NgR1 decoy protein treatment compared with the vehicle group (Fig. 3A). BBB scores of the medium and high dose hNgR1(310)-Fc treated groups were significantly improved during the interval from 2–9 weeks post-contusion compared with the vehicle group, $p=0.007$ and $p=0.046$, respectively (repeated measures (RM)-ANOVA from 14–63 days, with *post hoc* least significant difference (LSD) multiple comparison correction). At 63 days, the vehicle group BBB score was 8.3 ± 0.3 , while it was 9.7 ± 0.3 in the medium dose group and 9.5 ± 0.2 in the high dose group.

In addition, behavioral improvement was observed when comparing hindlimb nonweight-bearing with hindlimb weight-bearing locomotion. The percentage of medium and high dose hNgR1(310)-Fc treated animals able to bear their weight in the open field was significantly increased compared to vehicle at 35 days after SCI with 80% of rats in medium dose, and 79% of rats in high dose NgR-Fc group, as compared to only 33% of rats in the vehicle group ($p<0.05$ for each treatment group relative to control, ANOVA with *post-hoc* LSD). At 63 days post-injury, the benefit of treatment remained strong with 87% of medium dose and 92% at high dose group compared with 50% in the vehicle group ($p<0.05$ for each treatment group relative to control, ANOVA with *post hoc* LSD, Fig. 4).

Grid walking was used to evaluate the precise control of hindlimbs on a horizontal, square 2.5×2.5 cm mesh wire grid. This task evaluates both sensory and motor system and appraises their connection to each other. By testing more complex behavior, this assay can expose deficits that are not evident in tests of open field behavior locomotion. We analyzed the data in several ways. When we counted successful hindlimb steps over a 50 cm distance, the medium and high dose NgR-Fc protein groups were both about 50% greater than the vehicle group ($p \leq 0.0001$, ANOVA, Fig. 5A). The ratio of successful hindlimb to forelimb steps also revealed significant improvement with NgR-Fc treatment (Fig. 5B). The medium and high dose successful hindlimb to forelimb step ratios of 0.50 are significantly greater than the control value of 0.32 ($P \leq 0.0001$, ANOVA, Fig. 5B). The ratio of hindlimb foot faults to successful hindlimb steps decreased by 50% in the NgR-Fc treatment groups ($p=0.03$ for medium dose and $p=0.006$ for high dose, ANOVA, Fig. 5C). These results show that NgR-Fc protein treatment reduced missteps and increased successful step cycles in this complex neurobehavioral task.

Dose-dependence of human NgR1(310)-Fc protein treatment

Because both hNgR1(310)-Fc treatment groups had similar outcomes in the first experiment, we tested a lower dosage of 0.014 mg/kg/d protein in another cohort. The contusion injury and i.c.v.

FIG. 3. Human NgR1(310)-Fc dose response for locomotor recovery after rat spinal cord contusion. (A) Three days after the contusion and intracerebroventricular (i.c.v.) cannula implantation, rats were assigned randomly to one of three treatment groups. The osmotic pumps filled with 0.66 mg human hNgR1(310)-Fc (0.09 mg/kg/d, medium dose), 4.0 mg human hNgR1(310)-Fc (0.53 mg/kg/d, high dose) or equal volume of 2 mL phosphate buffered saline (PBS) (vehicle). The duration of treatment was 4 weeks, and follow-up was through 9–10 weeks. The Basso, Beattie, and Bresnahan (BBB) scores are plotted as a function of time after injury. Data are mean $\pm$ standard error of the mean (SEM). ** $p = 0.007$ for the effect of medium dose NgR-Fc versus vehicle treatment, and * $p = 0.046$ for the effect of high dose NgR-Fc versus vehicle treatment by repeated measures analysis of variance (RM-ANOVA) from 14–63 days for *post hoc* pairwise comparisons with least significant difference (LSD) correction. At specific time points, one-way ANOVA for *post hoc* comparison with LSD correction of medium dose NgR-Fc versus vehicle, * $p < 0.05$; ** $p < 0.005$. (B) Three days after the contusion and i.c.v. cannula implantation, rats were assigned randomly to one of two treatment groups. The osmotic pumps filled with 0.10 mg hNgR1(310)-Fc (0.014 mg/kg/d, low dose), or equal volume of 2 mL PBS as vehicle group. The duration of treatment was 4 weeks and the follow-up was 8 weeks. Data are mean $\pm$ SEM; $n = 5$ for vehicle and $n = 11$ for low dose NgR1-Fc group, $p = 0.29$, not significant (n.s.) by RM-ANOVA for the effect of low dose NgR1 decoy versus vehicle treatment. (C) The BBB scores at day 56 from A and B are replotted as a function of hNgR1(310)-Fc dose. Data are mean $\pm$ SEM. By one-way ANOVA with pairwise *post hoc* comparisons and LSD correction, the high dose (* $p = 0.025$) and medium dose (** $p = 0.0076$) NgR1-Fc treatment groups had better behavioral outcome compared with the vehicle group. Color image is available online at www.liebertpub.com/neu

catheter placement were identical to the first experiment. The final number of rats studied was $n = 5$ for vehicle (one rat was lost to mortality), $n = 11$ for low dose NgR-Fc treatment group (three rats were lost to mortality). At this dose, hNgR1(310)-Fc had no significant effect on BBB outcome ($p = 0.2876$, by RM-ANOVA, Fig. 3B). Combining the BBB data from the two experiments demonstrates a dose-dependence for human NgR1(310)-Fc treatment of rat spinal contusion by the i.c.v. continuous infusion route (Fig. 3C). A fully effective dose is achieved in the 0.09–0.53 mg/kg/d range.

Pharmacokinetics of single and multiple dose intrathecal bolus hNgR1(310)-Fc

Intermittent bolus dosing of protein to the intrathecal lumbar space provides a simpler means of protein delivery than a contin-

uous infusion with regard to clinical translation. Such delivery, however, necessitates distribution and half-life adequate for efficacy. We examined the half-life of human NgR1(310)-Fc protein in the rat after bolus dosing via a lumbar intrathecal catheter. The protein is detected in both brain and spinal cord tissue using a sensitive sandwich ELISA assay specific for human NgR1(310)-Fc protein (Fig. 6A, B). The time-dependence of tissue levels appears to have a rapid early phase of decay followed by a slower tissue residence phase (Fig. 6B). An exponential curve for the first 12 h has a half-life of about 5 h (Fig. 6C), while during the period from 12–168 h, the half-life is about 2 days in brain and spinal cord (Fig. 6D).

Based on this analysis, we chose to test the efficacy of a bolus dosing regimen on a once every 4 day schedule using a dose amount that equates to 0.14 mg/kg/d based on the continuous infusion

FIG. 4. Recovery of weight bearing after hNgR-Fc treatment of spinal cord contused rats. The percentage of rats capable of body weight support with at least one hindlimb is reported for the cohort described in the figure at 35 or 63 days after contusion injury. * $p < 0.05$ for the indicated comparisons; analysis of variance with *post hoc* pairwise comparisons and least significant difference correction. Color image is available online at www.liebertpub.com/neu

studies. One day after the fifth dose of this schedule, the brain and spinal cord tissue levels of NgR1(310)-Fc protein were 500–1000 ng/g tissue (Fig. 6E), a level expected to provide adequate decoy to block myelin associated protein inhibitors based on affinities measured *in vitro*.

Pharmacokinetic studies were extended to cynomolgus monkeys. Bolus doses of 2.0 mg NgR1(310)-Fc were administered through an intrathecal catheter implanted with the tip in the lower lumbar space. Brain and spinal cord tissues were analyzed 1–168 h after the last dose. The protein distributed widely from the lumbar site even in this larger nervous system. Between 12–72 h after bolus injection in the lumbar region, the tissue hNgR1(310)-Fc levels in the lumbar spinal cord are similar to those in the thoracic spinal cord ($86 \pm 16\%$ of lumbar), cervical spinal cord ($49 \pm 16\%$), and brain ($60 \pm 21\%$). The half-life of NgR1(310)-Fc protein in non-human primate tissue during the period from 24 and 168 h was 5 days. Thus, distribution is broad, and half-life is extended in the monkey relative to the rat.

Lumbar intrathecal bolus injection of human NgR1(310)-Fc improves recovery

Having demonstrated wide and long-lasting distribution of NgR1(310)-Fc protein after intermittent intrathecal lumbar bolus dosing, we sought to test the efficacy under these conditions. Rats received the same midthoracic moderate contusion as for the continuous infusion study, but a lumbar intrathecal cannula was implanted for drug delivery. Beginning on the third day after injury treatment, rats were randomized to receive either 15 μ L containing 150 μ g hNgR1(310)-Fc (0.14 mg/kg/d, $n = 14$) or an equal volume of PBS ($n = 13$). Seven separate bolus injections were then administered, one every fourth day through day 27 post-contusion (Fig. 7). Rats having a BBB score of seven or higher 7 days after SCI were excluded (one from vehicle group and one from NgR-Fc group) from the study because the lesion was deemed incomplete. Three weeks after contusion, there was a trend toward improved BBB locomotor score in NgR1 decoy protein treatment compared with the vehicle group (Fig. 7). BBB scores of the hNgR1(310)-Fc treated groups were significantly improved between 7 weeks to the end of the study, compared with the vehicle group ($p = 0.031$, by RM-ANOVA, Fig. 7).

FIG. 5. Recovery of grid walking after hNgR-Fc treatment of spinal cord contused rats. Spinal cord contused rats from the cohort described in Fig. 4 were assessed for ability to walk a 50 cm distance over a horizontal wire grid at 63 days after injury. (A) Successful hindlimb (HL) steps (steps without the foot slipping below the grid) were counted from walking over a 50 cm distance. Data are mean $\pm$ standard error of the mean (SEM), for vehicle ($n = 11$), 0.09 mg/kg/d ($n = 15$), and 0.53 mg/kg/d ($n = 14$). One-way analysis of variance (ANOVA) with pairwise *post hoc* comparisons with least significant difference (LSD) correction, *** $p < 0.0001$. (B) Grid walking HL versus forelimb (FL) success ratio was evaluated as successful HL step number divided by FL number. Data are mean $\pm$ SEM, for vehicle ($n = 11$), 0.09 mg/kg/d ($n = 15$), and 0.53 mg/kg/d ($n = 14$). One-way ANOVA with pairwise *post hoc* comparisons with LSD correction, *** $p < 0.0001$. (C) Grid walking HL foot fault ratio was scored as the HL foot faults divided by the number of HL steps. Data are mean $\pm$ SEM, for vehicle ($n = 9$), 0.09 mg/kg/d ($n = 14$), and 0.53 mg/kg/d ($n = 14$). Two vehicle rats and one 0.09 mg/kg/d rat were excluded from this analysis because the HL was dragging rather than mis-stepping. One-way ANOVA with pairwise *post hoc* comparisons with LSD correction, * $p < 0.05$; ** $p < 0.01$. Color image is available online at www.liebertpub.com/neu

FIG. 6. Detection of human NgR-Fc protein in central nervous system tissue after intermittent lumbar intrathecal administration. (A) The standard curve for sandwich enzyme-linked immunosorbent assay detection for known concentrations of hNgR1(310)-Fc diluted into brain extract demonstrates detection by europium-conjugated reagent. (B–D) Pharmacokinetics of brain and spinal cord tissue hNgR1(310)-Fc level after a single dose of 100 µg hNgR1(310)-Fc in 20 µL administered via an intrathecal lumbar (IT-L) catheter to the rat. Values for cervical, thoracic, and lumbar spinal cord tissue were similar and averaged together for each rat. Data are mean $\pm$ standard error of the mean (SEM) for $n = 3$ rats for each time. The time course from 1–168 h is plotted in B, the early time period in 1–12 h in C, and 12–168 h in D. (E) Brain and spinal cord levels of tissue hNgR1(310)-Fc after five separate 15 µL IT-L doses of 150 µg hNgR1(310)-Fc or vehicle alone spaced 4 days apart, over a 16-day course. The rats were sacrificed and tissue analyzed 24–28 h after the last dose on the 17th day. Data are mean $\pm$ SEM for $n = 5$ rats. (F) Pharmacokinetics of brain and spinal cord tissue hNgR1(310)-Fc level after administration via an IT-L catheter to nonhuman primate. Individual animals were dosed with 2.0 mg hNgR1(310)-Fc via a catheter placed in the IT-L space, and then the animals were sacrificed and tissue collected at the indicated time points after bolus administration. Values for cerebral cortex and three regions for spinal cord were measured for each animal and averaged together. $N = 1$ monkey at each time point. Color image is available online at www.liebertpub.com/neu

Behavioral improvement was also observed when comparing hindlimb nonweight-bearing with hindlimb weight-bearing locomotion. The fraction of hNgR1(310)-Fc treated animals able to bear their weight in the open field showed a trend to increase at 28 days after SCI (Fig. 8A). At 56 days after injury, the frequency of

weight bearing was more than twice as great in the NgR-Fc than the control group ($p = 0.018$, ANOVA, Fig. 8A).

The precision of hindlimb motor control was evaluated during ambulation over a horizontal, square 2.5 $\times$ 2.5 cm mesh wire grid. We scored successful steps for each hindlimb during a 50 cm grid

FIG. 7. Intermittent intrathecal lumbar (IT-L) administration of hNgR1(310)-Fc improves locomotor recovery after rat spinal cord contusion. Three days after spinal cord contusion and IT-L catheter insertion, rats were randomly assigned to one of two treatment groups and received seven bolus doses spaced 4 days apart (arrows). One group ($n=14$) received hNgR1(310)-Fc (15 μ L containing 0.15 mg protein), and the other group ($n=13$) received an equal volume of phosphate buffered saline vehicle. The locomotor Basso, Beattie, and Bresnahan (BBB) scores are reported as a function of time. Data are mean $\pm$ standard error of the mean. The vehicle and hNgR1(310)-Fc-treated groups are significantly different; repeated measures analysis of variance (RM-ANOVA) from 35–63 days, $*p=0.031$. At specific time points, one-way ANOVA for NgR-Fc versus vehicle, $*p\leq 0.05$. Color image is available online at www.liebertpub.com/neu

walk over three separate trials. hNgR1(310)-Fc treated rats had 50% more successful steps than the vehicle group ($p=0.018$, Student two-tailed t test, Fig. 8B). Thus, by multiple tests of motor performance, the spinal contused rats treated with intermittent bolus infusion of human hNgR1(310)-Fc manifest improved recovery.

Lumbar bolus NgR1(310)-Fc administration enhances raphespinal innervation

Previous studies with rat NgR1(310)-Fc treatment of acute and chronic contusion and hemisection have documented greater caudal 5HT fiber innervation, because of sprouting and/or regeneration of fibers after blockade of myelin inhibitors.^{26,27,29} This mechanism presumably contributes to improvement after intermittent lumbar bolus treatment with human NgR1(310)-Fc. To assess the pathway, we stained spinal cord transverse sections with anti-5HT antibodies and measured neurite length in the transverse plane within the ventral horn. Lower magnification views show fibers in the ventral horn in the vicinity of motoneurons, and higher magnification confocal images highlight individual fibers (Fig. 9A–D). Rostral to the lesion site, raphespinal fiber density was robust and did not differ between the two treatment groups (Fig. 9E). Caudal to the contusion site, innervation was reduced by 90% in the vehicle

FIG. 8. Intermittent lumbar hNgR1(310)-Fc improves weight bearing and grid walking after rat spinal cord contusion. (A) The percentage of rats capable of body weight support with at least one hindlimb (HL) is reported for the cohort described in Fig. 7 at 28 or 56 days after contusion injury. Data are mean $\pm$ standard error of the mean (SEM). $*p=0.020$ for the indicated comparison, analysis of variance. (B) Spinal cord contused rats from the cohort described in Fig. 7 were assessed for ability to walk a 50 cm distance over a horizontal wire grid at 56 days after injury. Successful HL steps (steps without the foot slipping below the grid) were counted. Data are mean $\pm$ SEM. The hNgR1(310)-Fc treated group ($n=9$) had significantly more successful steps than the vehicle group ($n=11$). $*p=0.01$, Student two-tailed t test. Color image is available online at www.liebertpub.com/neu

treated group (Fig. 9F). Density was three-fold greater, however, in the hNgR1(310)-Fc treated cohort ($p=0.002$, Student two-tailed t test, Fig. 9F).

Previous studies with continuous infusion of rat NgR1(310)-Fc showed no effect of treatment on tissue sparing and lesion area.²⁷ Similarly, intermittent lumbar bolus hNgR1(310)-Fc does not alter these metrics of tissue damage (Supplementary Fig. 2; see online supplementary material at ftp.liebertpub.com). We conclude that this intermittent treatment regimen with human NgR1(310)-Fc supports axonal growth to permit neurological recovery.

Discussion

The major findings of the present study support the potential for clinical translation of NgR1(310)-Fc for SCI therapy. We show that human NgR1(310)-Fc is biologically active in binding myelin inhibitors. When administered to rats with spinal cord contusion, the protein improves locomotor performance in a dose-dependent fashion. Pharmacokinetic analysis shows that IT-L doses of the protein distributed throughout the neuraxis in both rat and nonhuman primate. In the CNS tissue, NgR1(310)-Fc protein has a half-life up to 5 days in the monkey. This information allows us to deliver the material intermittently to the cerebrospinal fluid (CSF) and achieve significant functional recovery. Neurological improvement was coupled with sprouting of raphespinal fibers in the caudal spinal cord. Together these observations facilitate the design of clinical trials NgR1(310)-Fc with protein in chronic human SCI.

From the protein engineering point of view, the human and rat NgR1 proteins are highly similar. Thus, it is not surprising that a

FIG. 9. Intermittent lumbar hNgR1(310)-Fc IT-L increases raphespinal serotonergic innervation of the caudal spinal cord after contusion injury. (A–D) Transverse sections of spinal cord for rats treated with vehicle (A, C) or hNgR1(310)-Fc (B, D) as in Fig. 7 were stained with antiserotonin antibodies. Sections containing the ventral horn gray matter are shown at a level 11–16 mm caudal to the contusion site. The images in A and B are standard epifluorescence, and the images in C and D are higher magnification Z-stack confocal images. Note the greater fiber density of B and D versus A and C. Scale bars, 100 μm . (E) Rostral (11–16 mm) 5-hydroxytryptamine (5HT) immunopositive fiber length per unit area ($\mu\text{m}/\text{mm}^2$) in the ventral horn was measured from images similar to the caudal images in C and D for the cohort of rats treated as in Fig. 7. Data are mean $\pm$ standard error of the mean (SEM). (F) Caudal (11–16 mm) 5HT immunopositive fiber length per unit area ($\mu\text{m}/\text{mm}^2$) in the ventral horn was measured from images as in C and D for the rats treated as in Fig. 7. Data are mean $\pm$ SEM. The hNgR1(310)-Fc treated group ($n = 11$) had significantly more serotonergic fibers than the vehicle group ($n = 11$). * $p = 0.002$, Student two-tailed t test. Color image is available online at www.liebertpub.com/neu

human NgR1(310)-Fc protein could be produced with high purity, yield, and stability. The human version produced here includes two alanine for cysteine substitutions near the carboxyl end of the NgR1 peptide fragment. This change prevents disulfide scrambling between the NgR1 segment and the Fc segment.³³ As shown previously for rat protein,^{23,24,26,33} human NgR1(310)-Fc acts as a decoy. It binds the three myelin inhibitors and Nogo, MAG, and OMgp to block biological activity. Because NgR1 may also participate in the inhibitory action of CSPGs,³⁷ it is possible that the NgR-Fc decoy also prevents axon growth inhibition by this class of molecules.

When the decoy protein is administered to rats after thoracic spinal cord contusion, the degree of functional improvement over subsequent weeks is enhanced. Importantly, we designed the experiment so that the majority of control animals were unable to bear weight and made numerous errors when crossing a wire mesh grid. In this setting, the human NgR1(310)-Fc treatment significantly increased motor performance, such that nearly all animals were strong enough to step with their hindlimbs. This beneficial effect outlasts the termination of protein administration, at least for several weeks. Numerous previous studies have documented both axonal sprouting and regeneration after rat NgR1(310)-Fc protein treatment.^{26–31,38} As expected, the human NgR1(310)-Fc protein treatment increase the density of raphespinal fibers in the caudal spinal cord. It is most likely that the majority of this increase is because of sprouting of spared fibers and is consistent with a decoy protein mechanism for NgR1(310)-Fc action.

We also investigated aspects of pharmacokinetics for NgR1(310)-Fc protein. The studies examined the distribution and half-life after administration to the CSF space in the lumbar region. It is clear that the protein rapidly distributes, with brain levels nearly equal to spinal cord levels. This is true both in the rat and in a species with a larger sized nervous system, the cynomolgus

monkey. In the nonhuman primate, brain NgR1(310)-Fc levels are approximately 50% of spinal cord levels after administration to the caudal site.

After administration to the CSF, there appear to be two phases of elimination for the NgR1(310)-Fc protein. During the first several hours, there is a rapid phase, which most likely reflects elimination of free NgR1(310)-Fc. After 12 h, the rate of elimination from tissue is slower, with a half-life of approximately 2 days in the rat and 5 days in the monkey. This second slower rate likely reflects the dissociation of bound tissue NgR-Fc from endogenous myelin ligands. Elimination is expected to be even slower in human subjects, on the order of 1 to 2 weeks half-life, because of the slower rate of CSF clearance in monkey and human compared with the rat, and the lower clearance of therapeutic proteins usually seen in human in comparison with the rat and monkey.^{39,40}

The broad CNS distribution and slow turnover of NgR1(310)-Fc protein allowed us to design a treatment protocol based on intermittent lumbar bolus dosing. This protocol is meant to simulate repeated lumbar puncture for drug administration in human cases. Infusion via lumbar punctures spaced apart by 2 or more weeks has the potential to avoid the need for indwelling intrathecal cannulas or implanted mechanical pump devices. Based on CSF turnover rates, the 4-day intermittent bolus in the rat is expected to be equivalent to infusions at intervals of 2 weeks or longer in humans.^{41–43} With this regimen, the decoy protein treatment was effective in rat spinal cord contusion. As with continuous infusion, the fraction of animals able to bear weight double, and walking was significantly improved with intermittent lumbar bolus delivery.

Together, these studies demonstrate the production of a human decoy NgR1(310)-Fc protein with biological efficacy and a pharmacokinetic profile that facilitates translation to the clinical setting.

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Author Disclosure Statement

Axerion Therapeutics seeks to commercialize the use of NgR-Fc for treatment of SCI. S.M.S. is a cofounder of Axerion Therapeutics. For the remaining authors, no competing financial interests exist.

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