GAS6 Enhances Repair Following Cuprizone-Induced Demyelination

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Abstract
Growth arrest-specific protein 6 (Gas6) activities are mediated through the Tyro3, Axl, and Mer family of receptor tyrosine kinases. Gas6 is expressed and secreted by a wide variety of cell types, including cells of the central nervous system (CNS). In this study, we tested the hypothesis that administration of recombinant human Gas6 (rhGas6) protein into the CNS improves recovery following cuprizone withdrawal. After a 4-week cuprizone diet, cuprizone was removed and PBS or rhGas6 (400 ng/ml, 4 μg/ml and 40 μg/ml) was delivered by osmotic mini-pump into the corpus callosum of C57Bl6 mice for 14 days. Nine of 11 (82%) PBS-treated mice had abundant lipid-associated debris in the corpus callosum by Oil-Red-O staining while only 4 of 19 (21%) mice treated with rhGas6 had low Oil-Red-O positive droplets. In rhGas6-treated mice, SM132-positive axonal spheroids and APP-positive deposits were reduced in number relative to PBS-treated mice. Compared to PBS, rhGas6 enhanced remyelination as revealed by MBP immunostaining and electron microscopy. The rhGas6-treated mice had more oligodendrocytes expressing Olig1 in the cytoplasm, indicative of oligodendrocyte progenitor cell maturation. Relative to PBS-treated mice, rhGas6-treated mice had fewer activated microglia in the corpus callosum by Iba1 immunostaining. The data show that rhGas6 treatment resulted in more efficient repair following cuprizone-induced injury.

Introduction
Gas6, the product of growth arrest-specific gene 6 (Gas6), is upregulated during growth arrest [1–3], and is expressed and secreted by a wide variety of cell types including neurons [3,4]. Gas6 is a vitamin K-dependent growth factor and is the ligand for the Tyro3, Axl, and Mer (TAM) receptor tyrosine kinase family [5,6]. In a concentration-dependent manner, Gas6 induces signaling through TAM receptors and exerts its biological effects [7–12]. Gas6 signaling induces cell survival [13,14], proliferation [15,16], phagocytosis [17], differentiation [18], and prevents cell apoptosis [19,20]. Gas6 binds phosphatidylserine and promotes phagocytosis of apoptotic cells [21]. Shed soluble forms of Axl and Mer bind Gas6 and can function as decoy receptors regulating Gas6 bioactivity [22,23]. In multiple sclerosis (MS) lesions, upregulation of soluble Axl and Mer negatively correlates with Gas6 expression, suggesting that the dysregulation of protective Gas6 receptor signaling may prolong lesion activity [24].

Several studies in vitro and in vivo indicate that an interaction between Gas6 and its receptors has physiologic relevance [4,5]. Our previous studies in vitro demonstrated that Gas6 protected human and wild type (WT) rodent oligodendrocytes against insulin withdrawal and TNF-alpha induced cytotoxicity [19]. This protection was lost in cultured oligodendrocytes isolated from Axl knock-out mice, but not from WT or Tyro3 knock-outs. A protective effect was mediated by Akt activation and the effect was blocked by treatment with a PI3 kinase inhibitor suggesting that Gas6/Axl/PI3 kinase/AKT signaling may be important for maintaining oligodendrocyte survival under conditions of stress. In another study, we showed that Axl knock-out mice had delayed recovery and prolonged axonal damage following cuprizone toxicity, supporting the observation that the Axl receptor was important for recovery [25]. Also, Gas6 knock-out mice had fewer mature oligodendrocytes and greater microglial activation following cuprizone toxicity and recovery, indicating that TAM receptor activation and regulation affected multiple CNS cell types [26].

Cuprizone administration into mouse chow induces oligodendrocyte cell death, demyelination, axonal injury and microglial activation in the corpus callosum 3–4 weeks following administration. Upregulation of cytokines and chemokines occurs, along with gliosis, peaking at 4 weeks of cuprizone treatment [27–32]. In this study, we tested the hypothesis that direct administration of rhGas6 into the corpus callosum by osmotic mini-pump improves recovery following cuprizone toxicity.

Materials and Methods
Animals and cuprizone treatment
C57Bl6J mice were housed in a temperature and humidity-controlled environment with a 12-hr light-dark cycle. Mice were maintained on standard mouse chow and given water ad libitum prior to cuprizone treatment. Male 8-week-old mice were fed 0.2% (w/w) cuprizone (bis-cyclohexane oxalylhydrazine, Sigma; St. Louis, MO) in powdered chow ad libitum for 4 weeks.
Food containing cuprizone was changed every 2 days for 4 weeks. Immediately after cuprizone withdrawal from the diet, rhGas6 or PBS mini-pump treatment was initiated for an additional 14 days.

**RhGas6 or PBS administration**

The Alzet Brain Infusion Kit 3 (Alzet Corp., CA) and the Alzet osmotic mini-pump (Alzet 1002, Alzet Corp., CA), were used to deliver rhGas6 or PBS (vehicle) directly into the corpus callosum. Sterile rhGas6 diluted in PBS was kindly provided by Amgen (Thousand Oaks, CA). The assembly and pre-incubation of the mini-pump were performed according to the manufacturer's instructions. Briefly, one day before surgery, mini-pumps were connected to the brain infusion cannula by catheter tubing and filled with either rhGas6 or PBS. The filled infusion assembly with attached osmotic mini-pump was incubated overnight in sterile saline at 37°C.

RhGas6 was diluted in PBS and three doses, 400 ng/ml (5.2 nM); 4 μg/ml and 40 μg/ml, were administered by mini-pump. PBS was infused as a control. For treatment, mice were anesthetized with a mixture of Ketamine (30mg/kg)/Xylazine (6 mg/kg)/Acepromazine (1 mg/kg), and placed in a stereotaxic frame with non-traumatic ear-bars to hold the skull in place. A 23G needle was used to create a hole above the entry site for the sterile stainless steel mini-pump cannula and the stereotaxic arm holding the cannula was lowered into place unilaterally targeting the corpus callosum. The coordinates of the hole for the cannula were 1.0 mm anterior to the bregma, 0.8 mm lateral, and 2.3 mm ventral to the skull surface. The cannula was implanted and glued to the skull surface using veterinary skin adhesive (Loctite 454, Global Test Supply, NC). Thereafter, the cannula was covered by orthodontic powder diluted in acrylic resin liquid (Lang Dental Inc., IL). The mini-pump was inserted beneath the skin at the right orthodontic powder diluted in acrylic resin liquid (Lang Dental Inc., IL). The mini-pump was inserted beneath the skin at the right

**Tissue preparation, immunohistochemistry and Oil-Red-O staining**

After 2 weeks of rhGas6 or PBS treatment, mice were euthanized by cardiac perfusion with 4% paraformaldehyde (PFA). The brains were fixed in 1% PFA for 24 h, cryoprotected in 30% sucrose, and 20 μm frozen sections were prepared and used for analysis from the area corresponding to sections 230–250 of Sidman’s Atlas of the mouse brain and spinal cord [33]. To confirm that a significant reduction in myelin occurred in the corpus callosum of mice on the cuprizone diet at 4 weeks, and to examine the extent of remyelination at 14 days following cuprizone withdrawal and rhGas6 or PBS treatment, multiple non-serial coronal sections of mouse brain were stained with myelin basic protein (MBP) monoclonal antibody, SMI99. For immunohistochemistry, all sections were incubated for 30 min with 1× Tris-buffered saline pH 7.4 (1× TBS), containing 0.25% Triton X-100 and 3% H2O2, followed by a 1 h incubation in 5% goat serum and 5% nonfat dry milk in 1× TBS. Antibodies were diluted in 5% nonfat dry milk in 1× TBS and sections were incubated overnight at 4°C. Following incubation, the sections were washed 3 times in 1× TBS containing 0.1% Triton X-100 and incubated with biotinylated secondary antibody, as detailed in the Vectastain Avidin-Biotin complex kit (Vector Laboratories, Burlingame, CA), and were visualized by 3,3′-diaminobenzidine (DAB, Sigma). The MBP-positive immunostaining was evaluated by Gray value using ImageJ software, with images photographed at a magnification of ×400. This method quantified the extent of MBP expression. Multiple non-serial sections per mouse were used. Data were expressed as mean ± SD.

SMI32, APP, Iba1 and Olig1 immunostaining was performed as described above for MBP. SMI32 and APP are markers of axonal damage [34-36]. SMI32-positive axonal swellings and APP-positive deposits were visualized and counted at a magnification of ×400 in the midline and lateral regions of the corpus callosum of the fornix. Three to six non-serial sections per mouse were used and more than 3 mice per group were examined. Data were expressed as mean ± SD. APP positive deposits were counted and expressed as mean ± SD for multiple non-serial sections per mouse and four mice per group. Iba1 was used as a microglial cell marker [37]. Olig1 was used as a marker of oligodendrocyte progenitor maturation. Cytoplasmic Olig1-positive immunostaining was used as a marker of mature oligodendrocytes [38]. Iba1- and Olig1-positive cells were visualized and counted as above. The number of Iba1- and Olig1-positive cells was expressed as mean ± SD for four mice per group.

The lysosome dye, Oil-Red-O, was used to demonstrate triglycerides and cholesterol esters which associate in particular with lipid deposits in cells and tissues after injury [39]. Frozen sections were incubated in distilled water for 1 min, followed by a 2 min incubation in 100% propylene glycol (Polyscience; Bay Shore, New York), and transferred to Oil-Red-O (0.5% solution in propylene glycol, Polyscience; Bay Shore, New York), for 36 h at room temperature. Sections were incubated for 1 min in 85% propylene glycol, lightly stained with hematoxylin and mounted with glycerine jelly mounting medium (Polyscience; Bay Shore, New York). Oil-Red-O positive deposits were visualized by light microscopy. Multiple, non-adjacent sections per mouse within regions 230–250 of the corpus callosum were evaluated [33].

Once the positive effect of 400 ng/ml and 4 μg/ml of rhGas6 were revealed following Oil-Red-O staining, SMI32 and MBP immunostaining, we focused the remainder of our analysis on the two lower rhGas6 doses. All comparative analyses of MBP, Olig1 and Iba1 immunostaining and for EM focused on the corpus callosum at the midline [40]. Four mice per group were evaluated for APP, Iba1 and Olig1 immunostaining.

**Electron Microscopy (EM)**

Following cardiac perfusion with 4% PFA, brain samples were fixed with 1% uranyl acetate, dehydrated through a graded series of ethanol and embedded in LX112 resin (LADD Research Industries, Burlington VT). Ultrathin (80 nm) sections were cut on a Reichert Ultracut UCT, stained with 0.01% uranyl acetate followed by lead citrate, dehydrated through a graded series of ethanol and embedded in LX112 resin (LADD Research Industries, Burlington VT). Ultrathin (80 nm) sections were cut on a Reichert Ultracut UCT, stained with 0.01% uranyl acetate followed by lead citrate and viewed in a JEOL 1200EX or an Hitachi HS6000. Brain sections within regions 230–250 were taken and oriented such that cross sections of axons within the corpus callosum were obtained. Non-serial sections were evaluated from each mouse (3 mice per group) and at least four fields per mouse were taken at a magnification of ×5000. Image J software was used to calculate axonal diameter, myelin thickness and g-ratios. Non-serial sections were evaluated and 100 axons per mouse were counted. The g-ratio represents the ratio of the diameter of the axon to the diameter of the axon plus myelin. Approximately 14 fields (per animal) with dystrophic axons and axonal spheroids were taken randomly at ×5000 and their sizes were measured in both PBS- and rhGas6-treated mice. All data were reported as the mean ± SD.

**Antibodies.** Both SMI99 (MBP, 1:1500) and SMI32 (1:1000) were purchased from Covance Inc, Emeryville, CA. Mouse anti-
Alzheimer precursor protein A4 antibody (APP, 22C11, 1:500), was purchased from Millipore Corp., CA. Iba1 antibody for immunohistochemistry (1:400) was purchased from WAKO, Richmond, VA. Olig1 antibody (DF389, 1:1000) was kindly provided by Dr. John Alberta from Dana-Farber Cancer Institute, Boston, MA.

Statistical analysis

The Kruskal-Wallis statistical analysis was applied (Systat 7.0). P<0.05 was considered significant.

Results

rhGas6 reduces Oil-Red-O positive deposits post-cuprizone treatment and withdrawal

Following treatment with three doses of rhGas6 for 14 days, the corpus callosum of 15 of 19 mice (79%) was completely negative for Oil-Red-O (Fig. 1G-L). Only 4 of 19 mice (21%) treated with 400 ng/ml and 4 μg/ml of rhGas6 had low or moderate staining. By contrast, following PBS treatment, only 2 of 9 mice (18%) were Oil-Red-O negative, whereas 9 of 11 mice (82%) were strongly positive (Fig. 1A–F). Mice fed powdered chow and receiving PBS were Oil-Red-O negative while mice treated with cuprizone for four weeks had extensive positive staining within the corpus callosum (data not shown). These results demonstrated that all rhGas6 doses had a beneficial effect on the clearance of cellular and myelin debris following cuprizone toxicity.

rhGas6 reduces SMI32+ and APP+ axonal swellings

Brain sections from both PBS- and rhGas6-treated mice were evaluated for evidence of axonal damage using the monoclonal antibody SMI32 that recognizes a non-phosphoepitope on NF proteins H and M, and an APP monoclonal antibody which detects accumulation of APP due to failed axonal transport. The number of positive axonal swellings was counted as a marker of axonal damage. Representative examples are shown of SMI32 positive axonal swellings in PBS-treated mice (Fig. 2A and E), and in rhGas6-treated mice at the various doses (Fig. 2B–D and F–H). The number of axonal swellings in PBS-treated mice was 27.4±8.8 (n = 11). In mice treated with rhGas6 the number of axonal swellings was 14.9±11.3 (400 ng/ml, p<0.02, n = 7), 10.7±12.1 (4 μg/ml, p<0.01, n = 9), and 14.3±3.7 (40 μg/ml, p<0.05, n = 3). Also, APP immunoreactivity was significantly reduced in rhGas6-treated mice relative to PBS-treated mice (Fig. 2I–K). In PBS-
treated mice (n = 4), there were 18.5 ± 6.9 APP positive deposits while in rhGas6-treated mice it was 7.0 ± 2.9 (400 ng/ml, p < 0.05, n = 4) and 7.5 ± 3.7 (4 µg/ml, p < 0.05, n = 4). Thus, these results showed that all tested doses of rhGas6 significantly reduced the number of axonal swellings relative to the PBS-treated group.

RhGas6 enhances remyelination after cuprizone toxicity

MBP immunoreactivity was used to estimate the extent of demyelination in the corpus callosum following cuprizone intoxication and to evaluate remyelination after cuprizone withdrawal in the PBS- and rhGas6-treated groups. At 4 weeks post treatment with cuprizone, there was visible demyelination relative to the untreated mice maintained on powdered chow (Fig. 3A–C). The gray value analysis of the intensity of MBP expression confirmed demyelination 4 weeks after cuprizone treatment: 164.4 ± 1.3 (untreated mice, n = 2) vs. 127.4 ± 10.7 (cuprizone-treated mice, n = 3). At 14 days after cuprizone removal and treatment with either PBS or rhGas6, remyelination was observed (Fig. 3D–G). The gray value of PBS-treated mice was 25.0 ± 18.2 (400 ng/ml, NS, n = 4) and 39.0 ± 7.1 (4 µg/ml, p < 0.05, n = 4) while in PBS-treated mice it was 12.0 ± 5.3, n = 4 (Fig. 4). Also, the percentage of cells with Olig1 positive cytoplasmic localization relative to the total number of Olig1 positive cells was increased in rhGas6-treated mice. In PBS-treated mice, the mean percentage was 72.8% ± 7.4. The mean percentage was 89.2% ± 3.7 in 400 ng/ml rhGas6-treated mice and 96.6% ± 1.8 in 4 µg/ml rhGas6-treated mice. Therefore, these findings showed that the number of cells with Olig1-positive cytoplasmic localization was increased in rhGas6-treated mice.

Number of myelinated axons is increased in mice treated with rhGas6

EM was performed to determine whether the increased MBP immunoreactivity and enhanced maturation of oligodendrocytes in the rhGas6-treated groups of mice reflected more myelinated axons in the corpus callosum. More structured tissue architecture, organized axons as well as more myelinated axons in rhGas6-treated mice relative to PBS-treated mice were observed (Fig. 5). While administration of 400 ng/ml rhGas6 increased the number of myelinated axons (Fig. 5B), it was not statistically significant relative to PBS treatment (Table 1). The number of myelinated axons was significantly increased (p < 0.02) in mice treated with 4 µg/ml rhGas6 (Fig. 5C). Axonal diameter was smaller in mice treated with rhGas6- versus PBS-treated mice, whereas only 4 µg/ml rhGas6 was statistically significant. The g-ratio was significantly decreased in rhGas6- versus PBS-treated mice, indicating
increased myelin thickness in rhGas6-treated mice. These data showed that relative to PBS treatment, administration of rhGas6 enhanced remyelination in the corpus callosum 14 days following treatment.

To determine whether axonal swellings detected by SMI32 immunostaining constituted dystrophic axons or spheroids (10–50 μm), we measured the size of axonal swellings in mice treated with PBS and rhGas6. Dystrophic axons ranged from 3.0 μm to 9.5 μm in diameter while axonal spheroids were greater than 10.0 μm in diameter (Fig. 6). The percentage of axonal spheroids and dystrophic axons was calculated from the total number of injured axons from approximately 14 randomly selected fields at a magnification ×5000. The percentage of smaller dystrophic axons was higher in rhGas6- versus PBS-treated mice; 77% (400 ng/ml rhGas6); 86% (4 μg/ml rhGas6) compared to 42% (PBS). By contrast, the percentage of axonal spheroids was greater in PBS-treated relative to rhGas6-treated mice: 23% (400 ng/ml rhGas6); 14% (4 μg/ml rhGas6) compared to 58% (PBS). In addition, EM revealed more myelin and lipid debris in PBS-treated relative to rhGas6-treated mice. Representative micrograph (Fig. 6A) shows myelin debris and lipid deposits in PBS-treated mouse. Therefore, EM supported our observation that rhGas6 administration had a positive effect on the clearance of debris.

Number of activated microglial cells not significantly altered after PBS or rhGas6 treatment

We used Iba1 as a marker of microglial cell activation to determine whether following direct delivery of rhGas6 versus PBS, there was a reduction in Iba1-positive immunoreactivity. Representative Iba1-positive microglia immediately following cuprizone treatment for 4 weeks is shown (Fig. 7). Abundant Iba1-positive microglia (92.0 ± 4.2, n = 4) was seen immediately following 4 weeks of cuprizone treatment. Significant reduction in Iba1-positive microglial cells at 14 days following cuprizone withdrawal.
and treatment with either PBS or rhGas6 was observed. The number of Iba1-positive cells in the rhGas6-treated mice was 38.0 ± 14.6, 400 ng/ml, (n = 4), and 34.0 ± 3.7, 4 μg/ml, (n = 4) relative to PBS-treated mice 48.0 ± 17.7, (n = 4), the differences were not statistically significant.

Overall, these results suggest that compared to PBS, rhGas6 treatment resulted in more efficient repair of the corpus callosum following cuprizone toxicity. This conclusion is based upon enhanced clearance of cellular and myelin debris and remyelination, increased axonal survival and integrity as well as OPC maturation.

**Discussion**

Gas6 is a survival factor for neurons and glial cells involved in myelination as well as a growth and anti-apoptotic factor for Schwann cells [41]. Gas6 and the TAM receptors are widely expressed in the rodent nervous system [4]. In this study, we show that compared to mice receiving PBS, rhGas6 administration directly to the corpus callosum by osmotic mini-pump for 14 days enhanced the clearance of lipid-laden debris, remyelination and reduced axonal damage that resulted from 4 weeks of ingestion of cuprizone damage. In another study, in which lysolecithin was injected directly into the cervical spinal cord, large deposits of Oil-Red-O were observed, correlating with areas of demyelination 7–14 days after injection. Despite the observation that the density of the lipid-laden droplets inside the lesion area decreased over the time course examined, it did not disappear entirely [42]. Perhaps, rhGas6 can be considered as a potential enhancer of debris clearance and remyelination following spinal cord injury.

Although the number of activated microglia in PBS- relative to rhGas6-treated mice was not significantly different, rhGas6 likely was more effective for clearance of debris. A positive effect of Gas6 on dying cell clearance in vivo has been reported for multiple cell types [43–46], and the ability of rhGas6 to affect phagocytosis in murine microglia by acting on Axl/Mer has been shown previously in vitro [47].

The clearance of cellular and myelin debris might be an initial and important early step for recovery in the corpus callosum. Efficient clearance of damaged cells and myelin debris likely impacts upon remyelination and cell survival. In this study, a significantly greater number of SM32-positive axonal spheroids were observed in PBS- versus rhGas6-treated mice. After treatment with different doses of rhGas6, all doses noticeably reduced the number of SM32 positive axonal swellings relative to PBS treatment. SM32-positive immunoreactivity in axons as well as the presence of axonal swellings or spheroids is considered pathologic and was previously shown to occur during cuprizone treatment and during MOG-induced experimental autoimmune encephalomyelitis [34,35,48,49].

Increased SM32-positive immunoreactivity correlates with axonal injury due to dephosphorylation of neurofilament H [25,50]. Further, SM32 immunoreactivity is found in chronic lesions from patients with MS [51]. Similarly, increased APP immunoreactivity within axons represents a defect in axonal transport and serves as a marker of axonal injury resulting from cytoskeletal breakdown and calcium influx that interrupts axoplasmic flow and subsequent accumulation of organelles [36]. By EM, we showed that the percentage of small dystrophic axons

Table 1. Increased number of myelinated axons is observed in mice treated with rhGas6.

| Parameters                      | PBS                  | rhGas6 400 ng/ml | rhGas6 4 µg/ml |
|--------------------------------|----------------------|------------------|----------------|
| Number of myelinated axons, mean ± SD a | 48.0±20.1           | 80.6±6.6         | 100.5±18.6**   |
| g-ratio, mean ± SD b            | 0.877±0.004         | 0.853±0.007**    | 0.836±0.001**  |
| Axonal diameter, mean ± SD b    | 0.868±0.063         | 0.752±0.057      | 0.70±0.067**   |

a- data was collected from four photomicrographs, ×5000; 3 mice/group.

b- 100 randomly selected axons were measured per mouse.

* g-ratio of cuprizone untreated mice was 0.802±0.01 (mean ± SD).

** P<0.05.

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Figure 5. EM shows more myelinated axons in rhGas6-treated mice. Relative to PBS (A), the number of myelinated axons was significantly increased in mice treated with 400 ng/ml (B) and 4 μg/ml (C) rhGas6. Black arrows denote myelinated axons; white arrows show demyelinated, naked axons, ×5000.

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was greater in mice treated with rhGas6 whereas the percentage of large axonal spheroids was higher in PBS-treated mice relative to rhGas6. Thus, data from EM support our conclusion that mice treated with rhGas6 have a reduction in axonal spheroids. The results obtained demonstrate a beneficial effect of rhGas6 on axonal survival and the maintenance of axon integrity following cuprizone toxicity. In this context, we previously showed in vitro that 400 ng/ml (5.2 nM) rhGas6 had a maximal positive effect on human oligodendrocyte survival using oligodendrocytes isolated from human fetal spinal cord. This suggests that rhGas6 serves as a survival factor for oligodendrocytes during development [52].

In the present study, we evaluated how rhGas6 affected remyelination following cuprizone withdrawal by MBP immunostaining. We used gray value analysis to distinguish the intensity of MBP expression between PBS- and rhGas6-treated mice. Extensive demyelination in the corpus callosum (Fig. 3B and C), was observed after 4 weeks of cuprizone treatment. The extensive myelin loss had the lowest gray value which correlated with weak MBP immunoreactivity. Our data are consistent with other studies demonstrating demyelination in the corpus callosum 3–4 weeks after cuprizone treatment [53,54]. Quantification of MBP staining revealed that gray values gradually increased after cuprizone
removal in both PBS- and rhGas6-treated mice. The increase in gray values likely reflected progressive remyelination consistent with the MBP expression. Comparative analysis of gray values in rhGas6- versus PBS-treated mice demonstrated that 4 μg/ml and 40 μg/ml of rhGas6 were more effective for remyelination compared to the 400 ng/ml rhGas6. Although the dose of 400 ng/ml of rhGas6 did not produce statistically significant results, these mice also had more MBP immunoreactivity than PBS-treated mice, indicating that enhanced clearance of debris and reduced number of axonal spheroids was associated with successful remyelination. EM showed increased numbers of myelinated axons and a decreased g-ratio in rhGas6-treated mice indicative of increased myelin thickness. Thus, the data from EM are consistent with results obtained by MBP immunostaining. It was shown that following acute demyelination after cuprizone intoxication, many small caliber axons become preferentially remyelinated [30]. We also found that the number of small diameter myelinated axons was significantly increased in rhGas6-treated mice. Thus, remyelination of small axons was an important index of corpus callosum recovery.

As OPCs mature, they synthesize myelin proteins that contribute to remyelination. We used Olig1 immunostaining as a marker of OPC maturation to determine if direct administration of rhGas6 affected OPC development. Olig1, a closely related homolog to Olig2, is co-expressed with Olig2 in many cells of the oligodendrocyte lineage. As OPCs mature, there is a shift in Olig1 immunostaining from a nuclear to a cytoplasmic localization [38]. Analysis of the corpus callosum showed that the number of cells with Olig1-positive cytoplasmic localization was increased in rhGas6-treated mice but only the dose of 4 μg/ml was statistically significant. Further, the percentage of cells with Olig1-positive cells with cytoplastic localization relative to total number of Olig1-positive cells was increased in mice treated with 4 μg/ml of rhGas6.

Taken together, these data provide compelling support for rhGas6 having a beneficial effect on corpus callosum recovery following cuprizone toxicity. Although 400 ng/ml rhGas6 was a therapeutic dose for the clearance of cellular debris, maintenance of cell survival and axonal integrity, 4 μg/ml of rhGas6 was more effective for remyelination. To our knowledge this is the first report of a beneficial effect of rhGas6 administration in vivo. These results open new avenues for rhGas6 as a treatment modality, in addition to the study of mechanisms by which rhGas6 exerts this effect.

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Author Contributions

Conceived and designed the experiments: BZ VS XL. Analyzed the data: VT CSR BSZ. Wrote the paper: VT CSR BSZ.

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