Antiapoptotic Effect of Granulocyte-Colony Stimulating Factor After Peripheral Nerve Trauma

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■ BACKGROUND: **Granulocyte-colony stimulating factor (G-CSF) has been observed to have direct protective effects on neurons after stroke in experimental models and in humans. In the present study, the antiapoptotic effects of G-CSF on spinal α-motoneurons after inducement of peripheral sciatic nerve lesions were evaluated in a rat model.**

■ METHODS: **Of 48 rats, 24 were treated with G-CSF and 24 were treated with glucose 5% solution (control group). The spinal cord of 6 rats in each group were removed at days 1, 4, 7, and 14. The α-motoneurons of spinal cord section L4–L6 were counted and investigated for the expression of choline acetyltransferase (ChAT), G-CSF receptor (G-CSFR), and Bcl-2 and Bax proteins. Additionally, α-motoneuron fluorescence double staining was performed for ChAT/Bcl-2, ChAT/Bax, and ChAT/G-CSFR.**

■ RESULTS: **Without G-CSF treatment, the number of ChAT-positive α-motoneurons on the lesion side was significantly decreased ($P < 0.001$). The number of α-motoneurons with Bcl-2 and G-CSFR positivity on the lesion side was significantly decreased ($P < 0.05$). In contrast, the number of α-motoneurons with Bax positivity was significantly greater ($P < 0.05$). After G-CSF treatment, the differences in the number of α-motoneurons on the 2 sides were not statistically significant. Fluorescence double staining of α-motoneurons was positive for ChAT/Bcl-2, ChAT/Bax, and ChAT/G-CSFR.**

■ CONCLUSION: **The results indicated that G-CSF has neuroprotective properties in spinal α-motoneurons and contributes to antiapoptotic effects after peripheral nerve lesions. The relevance of G-CSF, its precise mode of action, and the effect of these findings in clinical situations remains to be elucidated and require examination in further studies.**

INTRODUCTION

The granulocyte-colony stimulating factor (G-CSF) is a membrane-bound glycoprotein composed of 207 amino acids.[1-3] Together with the interleukins and tumor necrosis factor-α, G-CSF is a member of the cytokine family.[4] It stimulates the proliferation, differentiation, and survival of hematopoietic progenitor cells, in particular, neutrophil granulocyte precursors.[5-7] Thus, G-CSF has been applied for many years to treat severe neutropenia—especially in conjunction with stem cell transplantation and leukemia.[3,5,7-10] In general, the growth factor has been safe and well tolerated, with low toxicity.

In addition to the expression of G-CSF and the G-CSF receptor (G-CSFR) in hematopoietic cells, the evidence for positive expression of G-CSF and G-CSFR has been verified in neurons and glial cells.[1,3,4,10,11] In the past years, G-CSF has been reported as an important neurotrophic factor.[3,12] A variety of experimental in vitro and in vivo studies has shown that G-CSF induces neuroprotective and neuroregenerative properties. Additionally, it has been demonstrated that G-CSF diminishes neuronal destruction and neuronal death and improves the recovery of sensorimotor and cognitive function after induced ischemia.[3,12-15] This has also been reported in humans with acute stroke.[16,17] In addition to its

Key words
- G-CSF
- Nerve regeneration
- Peripheral nerve lesion
- Traumatic nerve injury
- Waterjet dissection

Abbreviations and Acronyms
ALS: Amyotrophic lateral sclerosis
BW: Body weight
ChAT: Choline acetyltransferase
CNS: Central nervous system
G5%: glucose 5%
G-CSF: Granulocyte-colony stimulating growth factor
G-CSFR: Granulocyte-colony stimulating factor receptor
mNCV: Motor nerve conduction velocity
SFI: Sciatic functional index

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antiapoptotic effects, G-CSF enhances angiogenesis after ischemia and promotes neurogenesis.[3,12] Furthermore, the application of G-CSF reduced the cell death of α-motoneurons in an experimental amyotrophic lateral sclerosis (ALS) model and of dopaminergic neurons in an experimental model of Parkinson's disease.[11,18] In different experimental studies, the use of G-CSF had a positive effect on neurological recovery after inducement of experimental direct spinal cord lesions.[2,19]

In contrast, compared with the effects on pathologic entities of the central nervous system (CNS), less information regarding the potential neuroprotective and neuroregenerative effects of G-CSF after peripheral nerve lesions exists. Injury of spinal or cranial nerves often results in permanent functional loss of different severity, even if their continuity has been maintained. The manipulation of nerves during surgical procedures often leads to distinct functional loss despite morphological preservation. Despite the regenerative capacity of nerves, a persistent deficit can lead to prolonged patient morbidity.

In the present study, the potential antiapoptotic effects of G-CSF on spinal α-motoneurons after inducement of sciatic nerve lesions was investigated in an experimental rat model. The emphasis in the present study was on histological evidence regarding the possible antiapoptotic properties of G-CSF. Neuronal apoptosis after peripheral nerve trauma occurs in the first hours after trauma and persists for a period of 14 days.[20-22] To date, no final agreement has been reached regarding the exact time course of neuronal programmed cell death, onset, and duration after a peripheral nerve lesion. In several studies investigating cell death after dissection of peripheral nerves in rat models, the greatest proportion of cell death was observed within the first week.[20,21,23] In other studies, the greatest proportion of cell death in the rats' α-motoneurons was observed in the second week after axotomy.[22,24] Thus, in the present study, histological analysis was performed at various times, starting quite early at day 1 and concluding with analysis at day 14.

In the present study, the right sciatic nerve of rats was lesioned with a waterjet. Waterjet dissection represents a surgical technique that combines highly precise parenchymal dissection with preservation of even small vessels, without thermal damage to the surrounding tissue.[25-30] The technique has been well evaluated in intracranial pathologic entities for many years.[28-30] The system has been approved by the regulatory authorities for surgical use in humans in Germany and the United States. The jet is pushed through a small nozzle of 120 μm in diameter that can be preset to various pressures. Sterile 0.9% isotonic saline is emitted as a separating medium with a volume flow of 1–55 mL/minute, resulting in a pressure of 1–80 bar. Experimentally, the waterjet device has been applied for dissection of adjacent structures in peripheral and cranial nerve surgery.[31,32] It has been shown that the sciatic nerves of rats can be preserved in their integrity, when waterjet pressures of ≤30 bar have been applied.[31] Functional damage will be observed at pressures >40 bar. With waterjet pressures of 50–80 bar, an incomplete nerve lesion will occur, with clinical and electrophysiological regeneration requiring ≤12 weeks. Depending on the pressure of the waterjet dissector, distinct and reproducible morphological characteristics will occur.[31] With these findings, in the present study, a waterjet pressure of 80 bar was used, resulting in a marked neurologic deficit but sustained macromorphologic nerve structure, such as is often observed with peripheral nerve stretch or contusion. In the case of nerve transection or nerve ligation, the trauma will be clearly identifiable visually. After inducement of a waterjet lesion, only indirect signs of marked nerve trauma, such as bleeding of superficial vessels or bubble formation at the epineurium, will be observed.[31] Thus, the presence of an immediate and adequate nerve lesion was confirmed by neurological and electrophysiological investigations conducted before and after inducement of the nerve lesion on a regular basis.

METHODS

All applicable international, national, and institutional guidelines for the care and use of animals were followed. All experiments were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee and the German State Committee of Laboratory Animal Research, and our report was prepared in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. The rats were housed 1–2 per cage with a 12-hour light/dark cycle and had free access to rat chow and water.

Study Design

In 48 adult male Sprague-Dawley rats (weight, 300–400 g), the right sciatic nerve was lesioned using the waterjet device and a pressure of 80 bar, with the rats under general anesthesia. In all the rats, the lesion was confirmed electrophysiologically with calculation of the motor nerve conduction velocity (mNCV) and clinically using pre- and postoperative walking track analysis and the sciatic functional index (SFI). The rats were allocated to 2 treatment groups, and each group was divided into 4 subgroups of 6 rats. Of the 48 rats, 24 were treated with recombinant human G-CSF (filgrastim [Neupogen] [Amgen GmbH, Munich, Germany]) administered intravenously into the tail vein at a dose of 60 μg/kg body weight (BW) directly after lesion inducement and on days 1, 3, and 5 after surgery. In 24 rats, 5% glucose (G5%) solution was administered intravenously as vehicle (control group).

On the final day of the observation periods, the rats were sacrificed for histological evaluation of spinal cord segments L4–L6. Immunohistochemical staining for the detection of choline acetyltransferase (ChAT), G-CSFR, the antiapoptotic protein Bcl-2, and the proapoptotic protein Bax was performed. Additionally, fluorescence double staining was used for confirmation of the co-expression of ChAT and Bcl-2, Bax, and G-CSFR. Owing to the pronounced contrast of the fluorescence dye with unmarked cell compartments, it was possible to verify co-expression of 2 different proteins within 1 compartment very clearly, even in the case of weak expression of a target. In an experimental ALS model of neonatal mice, Pitzer et al.[11] verified the co-expression of ChAT and G-CSFR using fluorescence staining in α-motoneurons. Thus, the goal of the present study

was to confirm the consistent protein expression of Bcl2, Bax, and G-CSFR, in addition to ChAT, as specific markers for α-motoneurons.

Surgical Procedure and Waterjet Lesion

The rats were anesthetized with isoflurane/oxygen inhalation, followed by intraperitoneal injection of xylazine hydrochloride at a dose of 5 mg/kg BW and ketamine hydrochloride at a dose of 80 mg/kg BW for general anesthesia. A posterolateral skin incision was performed parallel to the right femur, and the muscle fascia of the gluteus muscles was opened. The sciatic nerve was carefully exposed at the midthigh level with the aid of a wound expander. Under microscopic view, the nerve was mobilized with microforceps and microscissors from the surrounding muscle fascia until it was exposed from the sciatic notch exit to the division of the sciatic nerve motor branches. Waterjet dissection of the sciatic nerve was performed with the pressure set at 80 bar (Figure 1). For sciatic nerve dissection, the Erbejet 2 (Erbe Elektromedizin Co., Tuebingen, Germany) was used. To induce the nerve lesion, the waterjet was applied to the right sciatic nerve at a 90° angle and a cutting distance of 2 mm from the nozzle tip to the nerve surface. To obtain comparable results regarding the duration of the jet-induced lesion on the sciatic nerve, a computer-controlled linear device was used (Software Servomanager, version 6.4.1; Parker Automation [Erbe Elektromedizin Co., Tuebingen, Germany]). After dissection, the muscle fascia and skin were closed with 4-0 sutures.

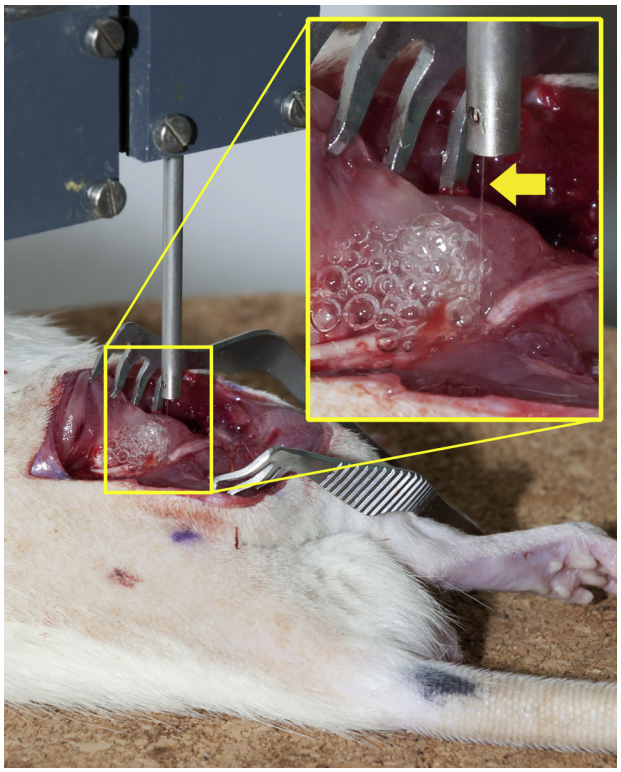

Figure 1. Waterjet lesion of a rat's right sciatic nerve with a pressure of 80 bar. For the sciatic nerve lesion, the jet was applied at a 90° angle (*arrow*).

Clinical and Electrophysiological Scoring of Neurologic Deficits

All the rats underwent walking track analysis pre- and postoperatively.[33] Hind paw prints were recorded using black ink. The number of steps per meter and possible limping after nerve lesion inducement were evaluated as previously characterized.[31] The factors for SFI were calculated as described by De Medinaceli et al.[33] An SFI of −25 or less was graded as a marked neurological deficit and an SFI of −75 or less graded as a severe neurological deficit. The pre- and postoperative electrophysiological measurements and follow-up measurements were performed on both sciatic nerves of each rat (EMS Biomedical, Surpass; Ambu Neuroline Subdermal Electrodes [Ambu GmbH, Bad Nauheim, Germany]). Owing to the small number of rats in each group, no statistical evaluation was performed.

Histologic and Immunohistochemical Examination

For histological analysis of the spinal α-motoneurons, the spinal cords were removed in total, fixed in 4% paraformaldehyde, and the spinal cord segments L4–L6 (for the sciatic nerve) were prepared.[34] The spinal cord sections were fixed in paraffin and cut into 5-µm-thick slices. For every spinal cord, 10 slices of 10 different sections (each composed of 100 µm) were analyzed. Nissl and hematoxylin and eosin staining and immunohistochemical staining for G-CSFR, Bcl-2, and Bax were performed.

All α-motoneurons in lamina 8 and 9 of the ventral horn that were clearly identifiable were counted on both sides. α-Motoneurons were defined as neurons ≥300 µm² in diameter, with an intact cell membrane, a clearly definable nuclear membrane with a nucleolus, a clearly definable cytoplasm, and positive expression for ChAT. The α-motoneurons were counted by 2 of us (J.-P.K. and A.H.) in a blinded fashion with encoded slices.

In every spinal cord section, α-motoneurons with positive expression for ChAT, G-CSFR, Bcl-2, and Bax were counted (Olympus XC30 [Olympus Soft Imaging Solutions, Hamburg, Germany]). The number of α-motoneurons on the lesion side was compared with those on the contralateral side in every rat.

To provide proof of a potential colocalization of anti- or pro-apoptotic antigens within the α-motoneurons, fluorescence double staining was performed for the α-motoneuron–specific marker ChAT and for G-CSFR, Bcl-2, and Bax. Fluorescent-microscopic images of stained and irradiating motoneurons were taken using 100-fold enlargement (UPlanFLN 100×/1.3 NA Oil Ph3 [Olympus, Hamburg, Germany]), with automatic setting of the exposure time without contrast enhancement and filters for ultraviolet, blue, and green light (Fraen Corp. Srl, Trivolzio, Italy).

Statistical Analysis

The data were statistically evaluated and plotted using Excel 2010 (Microsoft Corp., Redmond, Washington, USA). Statistical significance was calculated using the independent *t* test. In addition to the actual *P* values, the threshold indicating statistically significant differences is given in the figures. Data failing significance is marked as not significant. Before the *t* test calculations, the collected data were analyzed for outliers (values with 4 times the difference from the standard deviation of the mean value).

A Kolmogorov-Smirnov test for evaluation of normal distribution was performed.

RESULTS

Motor Function and Electrophysiological Measurements

A walking track analysis was performed in all rats before lesion inducement to allow the rats to become accustomed to the examination and to measure the motor function. Additionally, the mNCV was measured. Calculation of the SFI and mNCV confirmed intact motor function with regular toe spreading in all the rats preoperatively.

After nerve lesion, measurement of the SFI at day 1 after surgery showed, as expected, a motor deficit in all the rats. During the 14-day follow-up period, many rats showed recovery of the motor deficit and electrophysiological deterioration. On day 3 after surgery, 12 of 18 rats (67%) in of the G-CSF group and 14 of 18 rats (88%) in the control group had no or only a slight residual motor deficit (SFI, −25% to 0%). However, a marked conduction block was measured in the electrophysiological examination in 83% (15 of 18) of the rats in both groups. In the remaining rats, the conduction block was less severe. On day 14, intact motor function or a slight residual deficit was found in all the rats in the G-CSF group and in 5 of 6 rats (83%) in the control group. Electrophysiologically, the mNCV had recovered in 33% of the rats (2 of 6) in the control group and 50% of the rats (3 of 3) treated with G-CSF.

Histologic and Immunohistochemical Examination

ChAT Expression. Overall, counting of the α-motoneurons revealed a significantly lower number of ChAT-stained α-motoneurons on the lesion side compared with the contralateral intact side in the rats treated with the G5% solution. In contrast, the number of α-motoneurons in the G-CSF—treated rats was equal between the 2 sides. The median value of the counted α-motoneurons per slice that showed positivity for ChAT in the 10 regions of a single rat was 40 ± 1 on the left side and 31 ± 1 on the lesioned right side in the rats treated with G5% solution. The decrease in the number of α-motoneurons showed statistical significance ($P < 0.001$). Counting the α-motoneurons in the rats treated with G-CSF revealed only a slight difference in the total number of motoneurons, with 29 ± 1 on the left side and 30 ± 1 on the right side ($P = 0.79$; Figure 2). The results for the different subgroups, accordingly, showed a significantly decreased number of ChAT-positive α-motoneurons on the lesion side compared with the contralateral side on days 1–14 in the rats in the control group. The number of ChAT-positive α-motoneurons in the rats treated with G-CSF was equal (Table 1).

Bcl-2 and Bax Expression. Similar to the results with ChAT staining, the number of α-motoneurons on each slide showing positive expression for the antiapoptotic protein Bcl-2 was significantly lower on the lesion side than on the contralateral side in the control group ($P < 0.05$). In contrast, the number of α-motoneurons with Bcl-2 positivity did not differ significantly on the lesion side compared with the nonlesion side in the rats treated with G-CSF ($P = 0.15$). Accordingly, analysis of the subgroups revealed significantly lower numbers of Bcl-2—positive α-motoneurons on the lesion side after G5% treatment in the subgroups at days 4, 7, and 14, with exception of day 1 ($P = 0.07$). The number of Bcl-2—positive α-motoneurons of each side in the rats that received G-CSF was equal in all subgroups (Figure 3).

Regarding the proapoptotic protein Bax, counting of the cells showing a strong expression for Bax, with a significantly greater number of Bax-positive α-motoneurons per slice on the lesion side compared with the contralateral intact side in the control group ($P = 0.02$). The number of Bax-positive α-motoneurons of each side of the ventral horn in the rats treated with G-CSF did not show a statistically significant difference ($P = 0.90$).

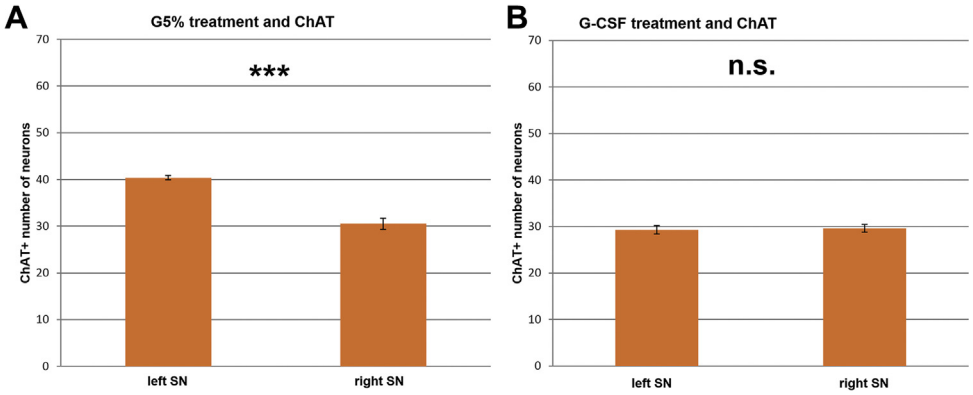

Figure 2. (**A**) Counting α-motoneurons showed positive expression for choline acetyltransferase (ChAT) in all rats ($n = 24$) treated with glucose 5% (G5%) solution (control group) and revealed a significantly decreased number of ChAT-positive α-motoneurons on the lesion side compared with the contralateral side (***$P < 0.001$). (**B**) A significant difference was observed in all subgroups and in every subgroup investigated on days 1, 4, 7, and 14. The number of ChAT-positive α-motoneurons in rats that received granulocyte-colony stimulating factor (G-CSF) was equal on both sides of the ventral horn (P = n.s.). SN, sciatic nerve.

Table 1. Results of Subgroups From Days 1 to 14 and Mean Difference in Counted α-Motoneurons

	G5%			G-CSF		
ChAT	**Left**	**Right**	***P* Value**	**Left**	**Right**	***P* Value**
Day 1	40 ± 2	30 ± 1	0.0004*	32 ± 2	31 ± 2	0.75†
Day 4	41 ± 3	32 ± 2	0.04‡	29 ± 2	27 ± 1	0.35†
Day 7	42 ± 2	32 ± 1	0.0023§	27 ± 1	30 ± 1	0.07†
Day 14	40 ± 2	27 ± 1	0.0015§	29 ± 1	30 ± 2	0.59†
Mean	40 ± 1	31 ± 1	0.00002*	29 ± 1	30 ± 1	0.79†

ChAT staining was performed for confirmation of α-motoneurons in the ventral horn of sciatic nerve spinal cord sections. After G-CSF treatment, no significant difference was observed between the lesion (right) and contralateral (left) side of the spinal cord section. In contrast, in the control group, the number of α-motoneurons was significantly decreased on the lesion side compared with the contralateral side.

ChAT, choline acetyltransferase; G5%, glucose 5%; G-CSF, granulocyte-colony stimulating growth factor; G-CSFR, granulocyte-colony stimulating factor receptor.

*$P \leq 0.001$.

†Not statistically significant.

‡$P \leq 0.05$.

§$P \leq 0.01$.

Accordingly, in all subgroups of rats treated with G5% solution, significantly greater numbers of Bax-positive α-motoneurons on the lesion side compared with the intact side were counted. In contrast, the number of α-motoneurons with positive Bax expression was equal on both sides in the rats that had received G-CSF (Figure 4).

G-CSFR Expression. In addition to the analysis of Bcl-2 and Bax, immunohistochemical staining for G-CSFR was performed. Only α-motoneurons with a strong expression for G-CSFR were counted. In summary, in accordance with ChAT expression and Bcl-2 analysis, counting the α-motoneurons in the control group revealed a significantly lower number of G-CSFR–stained α-motoneurons on the lesion side compared with the contralateral side of the ventral horn. In contrast, the number of α-motoneurons with strong G-CSFR positivity in the G-CSF–treated rats was equal. The median value of the counted α-motoneurons per slice showing strong positivity for G-CSFR in the 10 regions of a single rat was 46 ± 2 on the left (intact) side and 32 ± 1 on the lesion right side in the control group, with a statistically significant difference ($P < 0.001$). Counting the α-motoneurons in the rats treated with G-CSF revealed only a slight difference in the total number of α-motoneurons (43 ± 3 on the left side and 44 ± 2 on the lesion side; $P = 0.84$). Analysis of the subgroups showed a significantly decreased number of G-CSFR–positive α-motoneurons on the lesion side compared with the contralateral side on

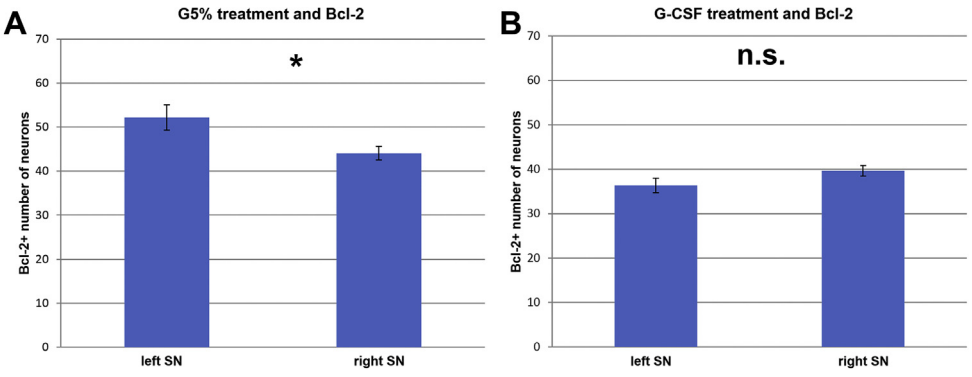

Figure 3. (**A**) According to the α-motoneurons showing positive expression of choline acetyltransferase, the number of α-motoneurons on both sides of the ventral horn showing positive expression for Bcl-2 was significantly decreased (*$P < 0.05$), with exception of day 1 ($P = 0.07$ [NS]). (**B**) The number of Bcl-2–positive α-motoneurons on each side of the ventral horn was equal in all subgroups of rats treated with granulocyte-colony stimulating factor (G-CSF) after lesion inducement (P = n.s.).

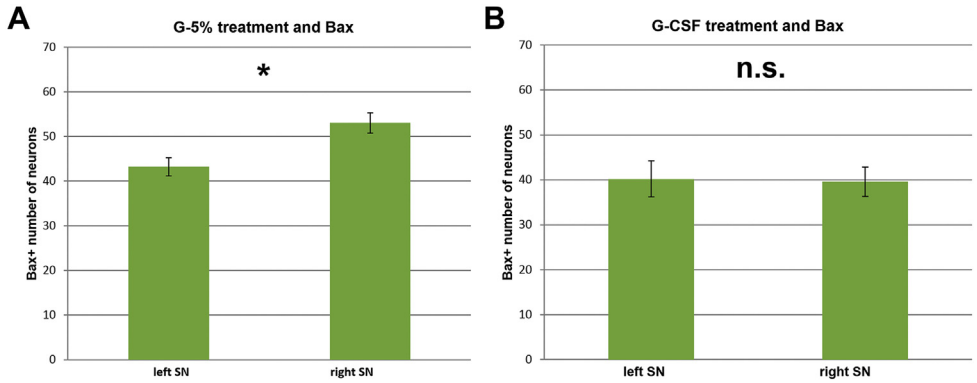

Figure 4. (**A**) Counting α-motoneurons showing a strong positive expression for Bax revealed a significantly greater number of α-motoneurons on the lesion side compared with the contralateral side in the control group (*$P < 0.05$). Accordingly, in every subgroup of rats treated with glucose 5% (G-5%) solution, significantly greater numbers of Bax-positive α-motoneurons were counted. (**B**) The number of Bax-positive α-motoneurons on both sides of the ventral horn was equal in all subgroups of rats treated with granulocyte-colony stimulating factor (G-CSF; P = n.s.).

days 1–14 in the control group. In contrast, the number of G-CSFR–positive α-motoneurons in the G-CSF–treated group did not differ significantly.

Fluorescence Double Staining of ChAT/Bcl-2, ChAT/Bax, and ChAT/G-CSFR

A qualitative analysis was performed for potential colocalization of different immunohistochemical markers within 1 cell. An α-motoneuron with positive expression for ChAT and Bcl-2 is shown in **Figure 5A**. The ChAT signal could be identified within the cell perikaryon, except for the nucleus. Bcl-2 staining revealed colocalization in the same region of the perikaryon, except for the nucleus. In the area surrounding the nucleus, the fluorescence staining of both markers was increased. Colocalization of ChAT and Bcl-2 within 1 α-motoneuron was revealed by overlaying both fluorescence images. Similar results were achieved with fluorescence double staining for ChAT/Bax and ChAT/G-CSFR (**Figure 5B, C**). Additionally, using fluorescence double staining for ChAT/G-CSFR colocalization, cell staining could be differentiated into α-motoneurons revealing weak and strong expression of G-CSFR.

DISCUSSION

Traumatic and iatrogenic lesions of the peripheral nerves often result in severe—and often persistent—neurologic deficit. Several therapeutic options have been intensely investigated. In addition to biomechanical approaches such as nerve graft transplantation techniques and neurophysiologic approaches, scientific research in the past years has focused on different systemic drugs.[35-39] Growth factors, including G-CSF, represent 1 group of drugs that have been examined for their possible benefits to treat nerve lesions.[40,41]

G-CSF is well known for its application in the treatment of neutropenia.[7] In past years, various experimental studies have shown the antiapoptotic, anti-inflammatory, and neuroregenerative properties of G-CSF in pathologic entities and lesions of the CNS. In the CNS, G-CSF and the G-CSFR have been found in the cortex layers II and V, hippocampus, subventricular zone, Purkinje cells, cerebellum, and brain stem. Additionally, G-CSF has been detected in the α-motoneurons of the ventral horn.[3,11,42]

In a study reported in 2010, Henriques et al.[42] showed that the application of G-CSF had a positive effect on the survival of spinal α-motoneurons and led to increased expression of G-CSFR in neonatal mice after complete sciatic nerve axotomy. Another experimental study reported that G-CSF promoted the survival of spinal α-motoneurons in an ALS model counteracting muscle atrophy.[11] However, to the best of our knowledge, the effect of G-CSF on neuroregeneration and neuroprotection after incomplete nerve lesion, which is often encountered in daily clinical practice, has not yet been closely investigated.

The present study investigated the potential regenerative effect of G-CSF after inducement of an incomplete traumatic lesion in the peripheral nervous system. Using waterjet dissection with a confined jet, a reproducible and incomplete lesion was set on the right sciatic nerve of rats, resulting in a marked neurological and electrophysiological deterioration such as is often seen in patients after nerve strain or partial nerve crush.[31] The nerve lesion was confirmed clinically and electrophysiologically. The possible influence of G-CSF on the survival of α-motoneurons in the related spinal cord section was analyzed in the initial period after the setting of the lesion.

Regarding the clinical and electrophysiological course after the nerve lesion, which had mainly been performed to confirm the presence of an adequate lesion, more rats receiving G-CSF after the traumatic lesion seemed to improve during follow-up. However, owing to the short follow-up period of 14 days, no significant differences were observed. In a study reported in 2010 by Tschan et al.,[31] the severity of the rats' sciatic nerve trauma after waterjet lesions of different pressures were analyzed. Different preset jet

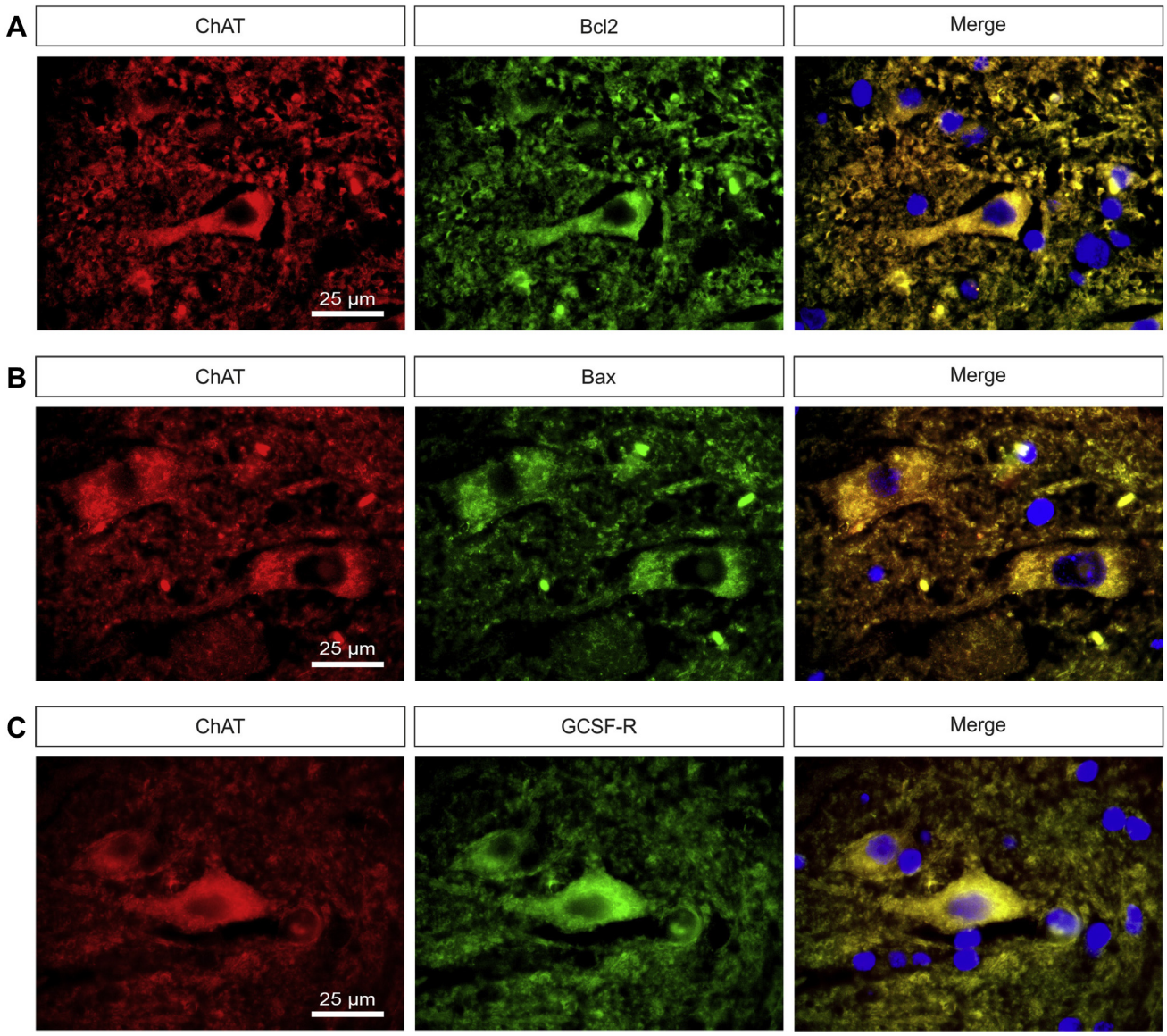

Figure 5. Fluorescence double staining of choline acetyltransferase (ChAT)/Bcl-2 revealed a positive colocalization of both antibodies. Positive expression was shown within the cell perikaryon, except for the nucleus, after ChAT and Bcl-2 staining. The nucleus was stained with DAPI dihydrochloride. After overlying the images, the fluorescence signal of both markers was increased in the area surrounding the nucleus (**A**). Similar colocalization was observed after ChAT/Bax and ChAT/granulocyte-colony stimulating factor receptor fluorescence double staining (**B, C**). Scale bar = 25 µm.

pressures led to distinct and reproducible histological lesions and to reproducible neurological impairment. A lesion of 80 bar, which was applied in the present study, resulted in a marked deficit, with significant signs of motor function impairment and significant improvement of motor function after 12 weeks.[31]

In a study reported by Pan et al.[43] in 2009, superior improvement of motor function after sciatic nerve lesion inducement and administration of G-CSF was detected after 7 days. However, other factors, such as the severity of the nerve lesion and the experimental setup, could have contributed to the different outcomes. In general, G-CSF has been proved to be associated with better neurological outcomes.[43]

The present study focused on the histological and immunohistochemical apoptotic and antiapoptotic signs of spinal motoneurons. Apoptosis usually occurs within the first days after a nerve injury.

ChAT staining revealed a significantly reduced number of α-motoneurons on the injured right side of the spinal cord compared with the contralateral left side. In the rats that had received G-CSF, no difference between the numbers of α-motoneurons on the 2 sides was detected. Antibody staining against G-CSFR obtained the same result. In the control group, the number of α-motoneurons and positivity for G-CSFR were significantly lower on the lesion side of the spinal cord sections. In contrast, the number of α-motoneurons with positivity for G-CSFR was not decreased significantly compared with the contralateral side in the G-CSF group. Thus, the survival of a larger number of spinal α-motoneurons might represent the basis for better nerve regeneration and improved motor function several weeks after the nerve lesion had occurred.

The results are in line with a study reported by Pitzer et al.[11] in 2008 investigating the survival of α-motoneurons in an experimental ALS model in mice. The application of G-CSF was associated with improved survival of the spinal α-motoneurons. Additionally, in an α-motoneuron cell culture, staurosporine-induced apoptosis was reduced.[11] Nishio et al.[44] reported that G-CSF was connected to a greater amount of α-motoneuron survival after spinal cord injury in mice.

Regarding the G-CSFR, a positive correlation between G-CSF application and G-CSFR expression in α-motoneurons was revealed in the present study. Tanaka et al.[45] reported in 2006 that the G-CSFR expression of α-motoneurons was reduced in an experimental ALS model. They concluded that downregulation of G-CSFR might play a part in the development of ALS, because a greater concentration of G-CSF was associated with neuroprotection.[45] Additionally, in the study by Henriques et al.,[42] sciatic nerve axotomy was connected to upregulation of G-CSFR expression in the spinal α-motoneurons.

Regarding antiapoptosis, in the present study, the number of Bcl-2—positive spinal motoneurons was, on average, 15% lower on the lesion side in rats that had not received G-CSF. In contrast, the number of Bcl-2—positive cells was equal on the lesion and non-lesion side in rats treated with G-CSF. In several experimental studies investigating cerebral ischemia in a rat model, G-CSF application was connected to an increased cytosol level of Bcl-2 and a decreased level of cytochrome C.[10,46,47] Furthermore, a high level of Bcl-2 has been shown to protect cerebral neurons from apoptosis and necrosis.[48-50]

Accordingly, antibody staining against the proapoptotic protein Bax displayed 20% more spinal motoneurons with positive staining on the damaged side without application of G-CSF. After G-CSF treatment, no difference in Bax positivity was observed. Concerning the expression of the proapoptotic protein Bax, G-CSF might reduce the transcription and expression of Bax, which induces apoptosis by binding at mitochondrial membranes, resulting in increased activation of proapoptotic caspases.[51-53] In rats, cerebral ischemia results in an increased level of Bax.[54] However, after G-CSF treatment, a low concentration of Bax was found.[10,47]

Fluorescence staining enables the identification and localization of distinct proteins within neurons. Moreover, in the case of low antigen expression or in small structures, the high contrast of the fluorescence markers to other tissue enables the visualization, in particular, if simultaneous expression of 2 structures is under examination. The technique was used by Pitzer et al.[11] in the experimental ALS study. A colocalization of ChAT and G-CSFR was detected. In the present study, fluorescence double staining of α-motoneurons also revealed positive coexpression of ChAT/G-CSFR. Additionally, double staining for ChAT/Bcl-2 and ChAT/Bax revealed colocalization of pro- and antiapoptotic factors within the α-motoneurons.

Study Limitations

One limitation of the present study was the variability in the total numbers of α-motoneurons in the different groups and different rats. The analysis of ChAT and Bcl-2 showed a smaller total number of positive α-motoneurons (without a statistically significant difference between both sides) in rats receiving G-CSF compared with the control group. However, the total numbers of α-motoneurons with positive Bax and G-CSFR expression were in equal in both treatment groups, except that the lesion side in the control groups had a significantly lower number of α-motoneurons. Overall, the number of α-motoneurons from a single side of the spinal cord varied from 27 ± 1 to 58 ± 5. These differences could have resulted in a certain susceptibility to bias. However, despite numerous experimental studies that have investigated rat spinal motoneurons, only rare data regarding the total number are available and often the data have varied according to the rat strain, age, and gender. The number of α-motoneurons in the present study is in line with the numbers reported in a study by Mierzejewska-Krzyzowska et al.[55] in 2014. Nevertheless, despite the differences in the counted motoneurons in the single rats, the rats in the control group show significantly fewer α-motoneurons on the lesion side compared with the contralateral side, and these differences were not detected in the rats that had received G-CSF.

CONCLUSION

The administration of G-CSF after peripheral nerve trauma revealed strong antiapoptotic effects on the spinal cord α-motoneurons of the sciatic nerve section and are line with the results from other studies. Thus, in addition to the positive effect of G-CSF on neuroregeneration in the treatment of central lesions, neuroregenerative effects mediated by G-CSF after peripheral nerve lesions are likely. Although no statistically significant functional benefit could be shown in the first 2 weeks, long-term beneficial effects might exist regarding peripheral nerve regeneration after a traumatic lesion. We believe that further investigation of nerve regeneration with G-CSF treatment is worth elucidating. Additional studies investigating the neuroprotective and neuroregenerative mechanisms after peripheral nerve lesions with a special emphasis on G-CSF application in terms of the dose, duration, and form of administration are needed.

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