Spinal Cord (2012) 50, 467–471

www.nature.com/sc

ORIGINAL ARTICLE

The protective effects of the lentivirus-mediated neuroglobin gene transfer on spinal cord injury in rabbits

X-W Chen[1,3], W-P Lin[2,3], J-H Lin[1], C-Y Wu[1], L-Q Zhang[1], Z-D Huang[1] and J-M Lai[1]

Study design: We introduced a lentiviral vector containing the *neuroglobin* (*Ngb*) gene into the injured spinal cords to evaluate the therapeutic potential of Ngb in a rabbit model of spinal cord injury (SCI).
Objectives: It is not clear whether Ngb has the neuroprotective role to SCI. The purpose of this study was to investigate the possible protective effects of the Ngb overexpression on traumatic SCI in rabbits.
Setting: Department of Orthopedic Surgery, The First Affiliated Hospital, Fujian Medical University, Fuzhou, People's Republic of China.
Methods: A lentiviral vector containing *Ngb* gene was constructed and injected at the SCI sites 24 h after SCI. The rabbits' motor functions were evaluated by the Basso–Beattie–Bresnahan rating scale. Quantitative real-time PCRs, western blots, malondialdehyde (MDA) tests and terminal deoxynucleotidyl transferase-mediated UTP end labeling assays were also performed.
Results: The Ngb expression in the LV-Ngb group increased significantly at days 7, 14 and 21. A more significant functional improvement was observed in the LV-Ngb group compared with the improvements in all other groups at days 14 and 21. The traumatic SCI seemed to lead to an increase in the levels of MDA and in the number of the apoptotic cells, which could be prevented by the LV-Ngb treatment.
Conclusion: This study demonstrated that the Ngb overexpression may have potential therapeutic benefits for both reducing secondary damages and improving the outcomes after traumatic SCI.
Spinal Cord (2012) **50,** 467–471; doi:10.1038/sc.2011.138; published online 22 November 2011

Keywords: spinal cord injury; neuroglobin; gene transfer; malondialdehyde; apoptosis

INTRODUCTION

Traumatic spinal cord injury (SCI) is one of the most devastating forms of trauma cases. It can cause severe functional impairment, paraplegia and tetraplegia. The pathophysiology of SCI is not fully known. But it has been suggested that there are primary and secondary injury mechanisms. Primary injury is inevitable. After the primary injury, the spinal cord will undergo a few sequential pathological changes, including hypoxia, reactive oxygen species (ROS) production, lipid peroxidation, ischemia, apoptosis and inflammation, and so on.[1] These secondary injury processes are the potential targets for therapeutic intervention. Although some therapeutic agents are being used to protect the injured spinal cords from those secondary pathological processes,[2] there is still no effective treatment for SCI.

Neuroglobin (Ngb) is a vertebrate globin that is expressed in the central and the peripheral nervous systems.[3] Several studies indicated that the Ngb overexpression can protect neurons from hypoxia and ischemia by enhancing either hypoxia sensing or hypoxia responding.[4] In addition, it is known that Ngb may provide an unidentified mean of apoptosis regulation in the neurons.[5] Ngb has demonstrated promising therapeutic effect on acute brain injury.[6] However, there is a paucity of literature concerning a role for Ngb in reducing the secondary damages in traumatic SCI.

Based on the previous studies, we hypothesized that Ngb might have a neuroprotective role in SCI in the rabbit. We introduced a lentiviral vector containing the *Ngb* gene into the injured spinal cords to evaluate the therapeutic potential of Ngb in a rabbit model of SCI.

MATERIALS AND METHODS

Production of the lentiviruses

The rabbit *Ngb* gene (Ref sequence: NM_001082133) was synthesized by Sangon (Shanghai, China) and confirmed by sequencing. The expression vector pGCFU containing *eGFP* gene was double cut with *AgeI*. An *Ngb* fragment was aligned to produce pGCFU-Ngb. A three-plasmid-envelope system was used to make the lentiviruses of pGCFU and pGCFU-Ngb in 293T cells. The quantitative real-time PCR (qRT-PCR) test of the eGFP expression revealed that the titers of Ngb and control lentiviruses ranged from 0.5 to 1.0×10^9 TU ml^{-1}. The Ngb lentivirus (LV-Ngb) contained *Ngb* gene and *eGFP* gene whereas the control lentivirus (LV-GFP) only contained *eGFP* gene.

The SCI model

Ninety-six New Zealand white male rabbits weighing 2.5–3.0 kg were used in this study. The animal experiments were performed in accordance with the guidelines in the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of Fujian Medical University.

The SCI model was established by epidural balloon compression in the rabbits, as described in detail previously.[7] Briefly, all rabbits received intramuscular injections of ketamine 50 mg kg^{-1} and xylazine 5 mg kg^{-1} anesthesia. Midline skin incisions were performed to expose the T9-T11 spinous processes. Self-retaining retractors were then used for retraction of the paraspinous

[1]Department of Orthopedic Surgery, The First Affiliated Hospital, Fujian Medical University, Fuzhou, PR China and [2]Department of Orthopedic Surgery, The Second Affiliated Hospital, Fujian Medical University, Quanzhou, PR China
[3]These authors contributed equally to this work.
Correspondence: Professor J-h Lin, Department of Orthopedic Surgery, The First Affiliated Hospital, Fujian Medical University, 20 Chazhong Road, Fuzhou 350005, PR China.
E-mail: jianhua0918@126.com
Received 30 June 2011; revised 3 October 2011; accepted 12 October 2011; published online 22 November 2011

muscles. Laminectomies were performed later at T10 level. Balloon angioplasty catheters (Medtronic-10851631, 2.0×20 mm, USA) filled with normal saline (NS) were inserted into the epidural spaces from the T10 level to the T9 level. The balloons were then inflated slowly until 2 atm pressure was achieved. After 5 min of the compression period, the balloons were deflated and removed. Then muscles and skins were sutured in separate layers. The SCI model in the present study was to form a partial spinal cord lesion and the severity of SCI was moderate.

Injections of LV-Ngb

The Basso–Beattie–Bresnahan (BBB) scores were obtained 24 h after SCI. The rabbits were randomly assigned to four groups (n=24 per group) including the Control, the NS, the LV-GFP and the LV-Ngb groups. Also BBB scores were performed on days 3, 7, 14 and 21 (n=24 per time point) after SCI in each group. The rabbits were anesthetized using the methods described previously. Then a T9 laminectomy was performed. In the Control group, no media or lentivirus was injected at the SCI sites. In the NS group, 15 μl NS was injected at each SCI site using microsyringes (Hamilton, Reno, NV, USA). In the LV-GFP group, 15 μl LV-GFP was injected at each SCI site. In the LV-Ngb group, 15 μl LV-Ngb was injected at each SCI site. After removal of the injectors, the muscles and skins were sutured in separate layers.

QRT-PCR analysis of Ngb

16 mm of the injured spinal cord (8 mm rostral to the center of injured spinal cord and 8 mm caudal to the center of injured spinal cord) was extracted and divided into four segments. One segment was used for qRT-PCR analysis, another segment for Western blot analysis, the third segment for malondialdehyde (MDA) tests and the last segment for Terminal deoxynucleotidyl transferase-mediated UTP nick end-labeling (TUNEL) assays.

The spinal cord segments were homogenized and total RNA was extracted with Trizol reagents (Invitrogen Life Technologies, Carlsbad, CA, USA) and quantified by ultraviolet spectroscopy. A reverse transcription was performed in a 20-μl reaction system with 4 μg of the total RNAs treated by RNase-free DNase I (TAKARA BIO, Inc., Otsu, Japan) according to the manufacturer's specifications. The PCR primers were purchased from TAKARA BIO, Inc. The primers used were as follows: the GAPDH-forward primer: 5′-CCACTTTGTG AAGCTCATTTCCT-3′; the GAPDH-reverse primer: 5′-TCGTCCTCCTCT GGTGCTCT-3′; the Ngb-forward primer: 5′-CTGGACCACATCAGGAAG GT-3′; the Ngb-reverse primer: 5′-CCCAGACACTTCTCCAGCAT-3′. The qRT-PCR was performed with the Thermal Cycler Dice Real Time System (TP800, TAKARA BIO, Inc.). The reaction was conducted in a 20-μl mixture containing 10 μl of SYBR Premix Ex Taq (TAKARA BIO, Inc.), 5 μM of each primer and 1 μl of the DNA extract. Each sample was processed in triplicate. The relative Ngb mRNA expression data were quantified using $2^{-\Delta\Delta CT}$ method, where CT was the cycle threshold.

Western blot analysis of Ngb

The protein homogenates of the spinal cord samples were prepared by rapid homogenization in lysis buffer and the lysates were separated by 10% SDS–polyacrylamide gel electrophoresis. The proteins were transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). After incubation with goat anti-Ngb (1:200; Santa Cruz Biotechnology, Paso Robles, CA, USA), the blot was washed with phosphate buffered saline and incubated with mouse anti-goat IgG conjugated with horseradish peroxidase (Santa Cruz Biotechnology). Immunoreactive complexes were visualized by enhanced chemiluminescene (ECL) and exposed to X-ray film. The protein signals were quantified by scanning densitometry using AlphaEaseFC (AlphaInnotech, San Leandro, CA, USA). The results from each experimental group were expressed as the relative integrated intensity compared with β-actin.

Neurologic evaluation

The hindlimb locomotor function was assessed at 1, 3, 7, 14 and 21 days after SCI using the BBB locomotor test developed by Basso *et al*.[7] The hindlimb movements during locomotion were quantified using a scale ranging from 0 to 21. The rabbits were observed for 5 min at each time point by two observers who were blinded to the experimental protocol.

MDA tests

The extent of the lipid peroxidation in the spinal cords was estimated by the MDA level, which was measured using the commercial assay kits produced by Jiancheng Bioengineering, Nanjing, China. The levels of MDA in the spinal cords were determined by the thiobarbituric acid method and expressed in nmol mg^{-1} proteins.

TUNEL assays

The spinal cord samples from each group were fixed in 4% PFA and embedded in wax. Sections were deparaffinized and hydrated using graded alcohols. Sections were then permeabilized with proteinase K (2 mg ml^{-1}, 1:100 in 10 mM Tris, pH 8) solution for 20 min at room temperature. The TUNEL assays were conducted using TUNEL detection kits according to the manufacturer's instruction (TdT-FragEL, Calbiochem, Bad Soden, Germany). In each section, eight high-power visual fields (×400) were selected to calculate the apoptotic index (AI=(the number of the TUNEL-positive cells)/(the total number of the nucleated cells)).

Statistical analysis

All data were presented as the mean ± s.d. A two-way ANOVA was performed to determine the differences among the groups for all measures followed by the least significant difference test. A P-value <0.05 would be considered significant. All statistical analyses were performed using the SPSS 16.0 (SPSS Inc., Chicago, IL, USA) statistical software package.

RESULTS

Overexpression of the Ngb

As shown in Figure 1, the expression of the *Ngb* gene at 3, 7, 14 and 21 days after SCI in the spinal cords dissected from the rabbits in all four groups were assessed by qRT-PCR. The level of the Ngb mRNA expression was not statistically significant among the level in the four groups 3 days after SCI. It then gradually decreased at days 7, 14 and 21, and there was no difference among the levels in the Control, the NS and the LV-GFP groups. However, the level of the Ngb mRNA expression in the LV-Ngb group was significantly higher at days 7, 14 and 21 compared with the levels in all other groups ($P<0.05$).

As shown in Figure 2, the Ngb protein expression was investigated by Western blot analysis. The level of the Ngb protein expression was not statistically significant among the level in the four groups 3 days after SCI. It then gradually decreased at days 7, 14 and 21, and there was no significant difference among the levels in the Control, the NS and the LV-GFP groups. However, the level of the Ngb protein expression in the LV-Ngb group increased more significantly at days 7, 14 and 21 compared with the levels in all other groups ($P<0.05$).

Neurological outcomes

The evaluations of the hindlimb locomotor functions at 1, 3, 7, 14 and 21 days after SCI were shown in Figure 3. All the rabbits became paraparetic and there was no significant difference among the four groups 1 day after SCI. Partial improvements were observed and there was no significant difference among the four groups 3 or 7 days after SCI. However, the neurological improvements were significantly greater in the LV-Ngb group compared with the improvements in all other groups at 14 and 21 days after SCI ($P<0.05$).

Changes of the MDA levels

The MDA levels in the spinal cord tissues were shown in Figure 4. The levels of MDA were not statistically significant among the levels in the four groups 3 days after SCI. The MDA levels reached a peak on the day 7 after SCI in the four groups. A more significant decrease of the MDA was observed in the LV-Ngb group compared with the levels in all other groups ($P<0.05$). At 14 and 21 days after SCI, the MDA levels decreased in all the groups, and MDA levels in the LV-Ngb

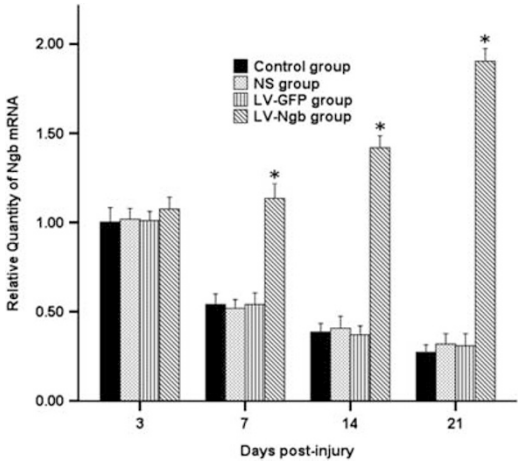

Figure 1 The levels of the Ngb mRNA expression in the injured spinal cords on days 3, 7, 14 and 21 after SCI in each group. The data were plotted as the mean ± s.d. $^{*}P<0.05$ versus the Control, the NS and the LV-GFP groups.

group were significantly lower compared with the levels in all other groups ($P<0.05$).

TUNEL labeling

The AIs were shown in Figure 5 and Figure 6. There was no difference among the AIs in the Control group, the NS group and the LV-GFP groups. A few TUNEL-positive cells were detected 3 days after SCI. There was no significant difference among the AIs in the four groups. The number of the apoptotic cells reached a peak at 7 days after SCI in the four groups. A more significant decrease in the AI was observed in the LV-Ngb group compared with the AIs in all other groups ($P<0.05$). Then the number of the TUNEL-positive cells decreased in all the four groups. The AI in the LV-Ngb group was significantly lower compared with the AIs in all other groups at days 14 and 21 ($P<0.05$).

DISCUSSION

It is important to develop a therapy that can reduce the evolution of the secondary damages in injured spinal cords. A number of recent studies have focused on the effects of gene therapies on secondary injury.[8] The data from the present study demonstrated a protective effect of the lentivirus-mediated *Ngb* gene transfer on traumatic SCI. To the best of our knowledge, the present study was the first to show the effects of the overexpression of Ngb on traumatic SCI.

Secondary injury in traumatic SCI is believed to be a result of a several destructive process such as excessive calcium release, hypoxia, ROS and lipid peroxidation, apoptosis, edema and inflammation, hemorrhage and ischemia, all of them can cause dysfunction and death in neuronal cells. Previous reports stated that one of the most important factors precipitating post-traumatic degeneration in the spinal cord is ROS-induced lipid peroxidation.[9] Furthermore, apoptosis is believed to have an important role in the pathogenesis of secondary injury.[10] Consequently, decreasing lipid peroxidation or reducing apoptosis may improve neurological recovery or facilitate nerve regeneration.

The gene transfer of specific genes for therapeutic purposes offers a valuable approach to the treatment of SCI.[8] Recombinant lentiviral vectors have been proven superior to other vectors in terms of gene transfer because they can not only provide long-term expression of the therapeutic gene, but also efficiently transduce non-dividing cells such as neurons.[11] Therefore, in the present study, we used a lentiviral vector to deliver Ngb to the injured spinal cords of the rabbits *in vivo*. As result, the overexpression of Ngb in the injured spinal cords was achieved successfully.

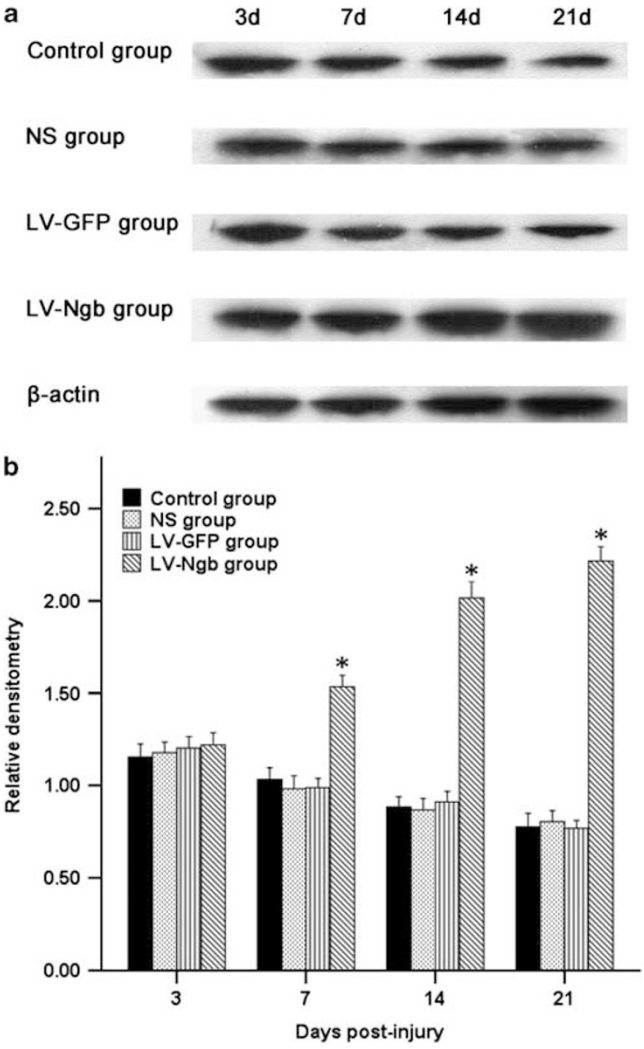

Figure 2 The detection of the Ngb protein in the spinal cords. (**a**) Ngb and β-actin were detected by Western blot in the spinal cord lysates obtained from the four groups at four different time points. The blots were representative examples from the six experiments per group per time point. (**b**) The quantitative mean ± s.d. data showed the level of the Ngb protein expression. The band density was normalized as the ratio of Ngb: β-actin ($n=6$). $^{*}P<0.05$ versus the Control, the NS and the LV-GFP groups.

The BBB open field scoring system is frequently used for the quantitative evaluation of the neurological status after SCI in animals. We demonstrated that the SCI had the ability to significantly reduce the BBB scores. However, the reduced scores could be significantly ameliorated by the Ngb overexpression. We also found that there was a progressive recovery over time in all the four groups. The functional recovery in the LV-Ngb group showed a more statistically significant improvement compared with the recoveries in all other groups. These results indicated that the lentivirus-mediated *Ngb* gene transfer effectively mitigated traumatic SCI-related neurodeficit.

Although we have demonstrated the neuroprotective effect of the Ngb overexpression, the neuroprotective mechanisms of the overexpression of Ngb have not yet been fully elucidated.[12] One possible

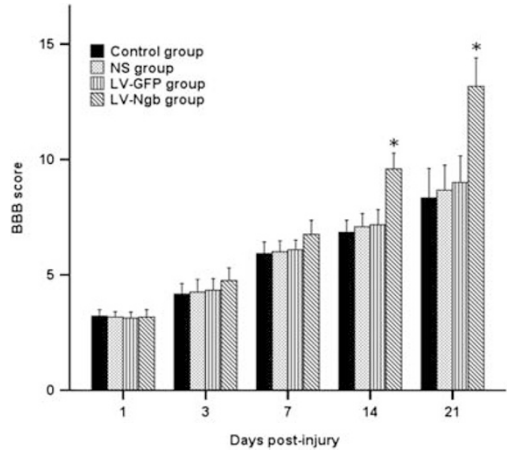

Figure 3 The hindlimb functional assessment with the BBB rating scale on days 1, 3, 7, 14 and 21 after SCI in each group. The data were plotted as the mean ± s.d. $^{*}P<0.05$ versus the Control, the NS and the LV-GFP groups.

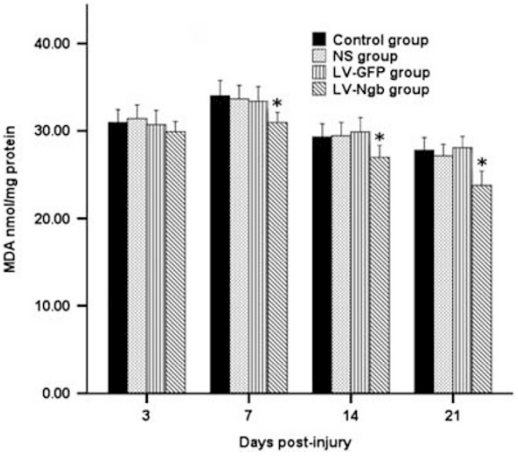

Figure 4 The MDA levels in the spinal cord tissues in the four groups at four different time points. The data were plotted as the mean ± s.d. $^{*}P<0.05$ versus the Control, the NS and the LV-GFP groups.

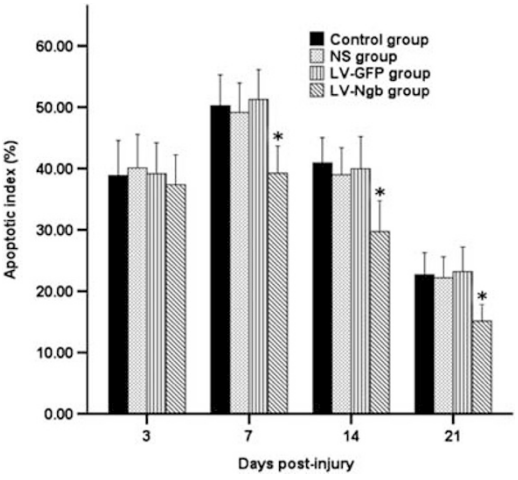

Figure 5 The AIs at four different time points in each group. The data were plotted as the mean ± s.d. $^{*}P<0.05$ versus the Control, the NS and the LV-GFP groups.

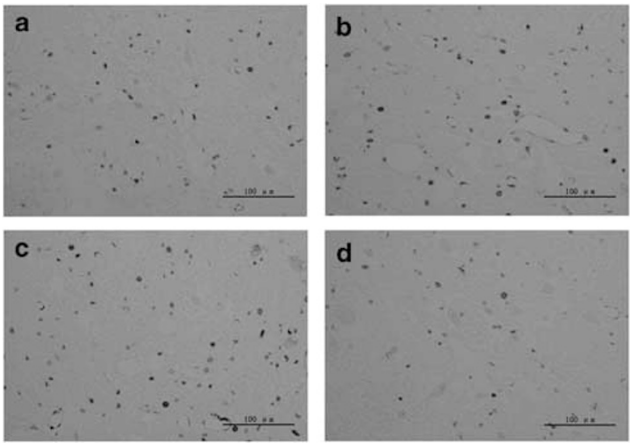

Figure 6 The apoptosis in the injured spinal cords on day 7 after SCI (magnification, ×400). (**a**) The Control group; (**b**) the NS group; (**c**) the LV-GFP group; (**d**) the LV-Ngb group. The TUNEL-positive cells were stained dark brown. The number of the positive cells was significantly lower in the LV-Ngb group compared with the numbers in the Control, the NS and the LV-GFP groups. Bar=100 μm (**a**, **b**, **c** and **d**). A full color version of this figure is available at the *Spinal Cord* journal online.

mechanism is that Ngb may function as a regulator of ROS.[13] It was believed that the ROS-induced lipid peroxidation was one of the most important precipitating components of neuronal degeneration after SCI.[9] MDA is widely used as one of the most important indicators for lipid peroxidation. The levels of MDA can significantly increase in animals exposed to traumatic SCI.[14] Previous experimental studies have also documented that the overexpression of Ngb was neuroprotective against direct oxidative stress.[15] In a recent study, the reduction of the MDA level in Ngb-Tg mouse brains suggested that Ngb can promote neuronal survival in part by reducing oxidative stress after focal cerebral ischemia.[6] The results presented in this study indicated that the MDA level increased significantly in the spinal cord tissues of the rabbits in the Control, the NS and the LV-GFP groups. However, the Ngb overexpression could significantly attenuate the increase of the MDA level because of its ability to reduce ROS. The protective effect of the Ngb overexpression in the SCI may also be caused by the reduction of the lipid peroxidation occurred in the neurons of the spinal cords.

Another possible mechanism is that Ngb may have the ability to protect neuronal cells from apoptosis.[16] Ngb proteins had been found in a high concentration (up to 100 μM) in the neurons and the retina. It was also closely associated with the mitochondria.[17] The induction of mitochondrial dysfunction can lead to the overproduction of superoxide anions, which is essential to promote enhanced apoptosis in neuronal cells.[18] Maintaining the mitochondrial function may be crucial for cell survival. Ngb may have the ability to reduce a small amount of the cytochrome *c* leaking from the damaged mitochondria, thereby inhibits the intrinsic pathway of apoptosis.[19] Apoptosis is an important mediator of the secondary damages after SCI.[10] In the present study, we examined the expression of the apoptotic cells in the traumatic spinal cords using the TUNEL detection kits. We found that the number of the apoptotic cells in the spinal cords significantly increased after SCI. The overexpression of Ngb also caused a more significant reduction in the expression of the apoptotic cells in the LV-Ngb group compared with the reductions in all other groups. This result suggested that the Ngb overexpression may have a role in reducing the secondary damages in traumatic SCI.

CONCLUSION

In summary, the present study shows that Ngb overexpression clearly had the ability to ameliorate oxidative stress and prevent apoptosis after the traumatic SCI. Our results indicated that the Ngb overexpression may have potential therapeutic benefits not only for reducing the secondary damages but also for improving the outcomes after traumatic SCI.

DATA ARCHIVING

There was no data to deposit.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We would like to thank Dr Yu Su for editorial assistance in preparing this paper. This study was supported by a grant from the Specialized Research Fund for the Doctoral Program of Higher Education of China (Grant No. 20060392003).

1 Nakahara S, Yone K, Sakou T, Wada S, Nagamine T, Niiyama T *et al.* Induction of apoptosis signal regulating kinase 1 (ASK1) after spinal cord injury in rats: possible involvement of ASK1-JNK and -p38 pathways in neuronal apoptosis. *J Neuropathol Exp Neurol* 1999; **58**: 442–450.
2 Blesch A, Lu P, Tuszynski MH. Neurotrophic factors, gene therapy, and neural stem cells for spinal cord repair. *Brain Res Bull* 2002; **57**: 833–838.
3 Burmester T, Weich B, Reinhardt S, Hankeln T. A vertebrate globin expressed in the brain. *Nature* 2000; **407**: 520–523.
4 Sun Y, Jin K, Mao XO, Zhu Y, Greenberg DA. Neuroglobin is up-regulated by and protects neurons from hypoxic-ischemic injury. *Proc Natl Acad Sci USA* 2001; **98**: 15306–15311.
5 Fago A, Mathews AJ, Brittain T. A role for neuroglobin: resetting the trigger level for apoptosis in neuronal and retinal cells. *IUBMB Life* 2008; **60**: 398–401.
6 Wang X, Liu J, Zhu H, Tejima E, Tsuji K, Murata Y *et al.* Effects of neuroglobin overexpression on acute brain injury and long-term outcomes after focal cerebral ischemia. *Stroke* 2008; **39**: 1869–1874.
7 Basso DM, Beattie MS, Bresnahan JC. A sensitive and reliable locomotor rating scale for open field testing in rats. *J Neurotrauma* 1995; **12**: 1–21.
8 Nakajima H, Uchida K, Yayama T, Kobayashi S, Guerrero AR, Furukawa S *et al*. Targeted retrograde gene delivery of brain-derived neurotrophic factor suppresses apoptosis of neurons and oligodendroglia after spinal cord injury in rats. *Spine (Phila Pa 1976)* 2010; **35**: 497–504.
9 Hall ED. Lipid antioxidants in acute central nervous system injury. *Ann Emerg Med* 1993; **22**: 1022–1027.
10 Merrill JE, Ignarro LJ, Sherman MP, Melinek J, Lane TE. Microglial cell cytotoxicity of oligodendrocytes is mediated through nitric oxide. *J Immunol* 1993; **151**: 2132–2141.
11 Lewis P, Hensel M, Emerman M. Human immunodeficiency virus infection of cells arrested in the cell cycle. *EMBO J* 1992; **11**: 3053–3058.
12 Brunori M, Vallone B. Neuroglobin, seven years after. *Cell Mol Life Sci* 2007; **64**: 1259–1268.
13 Brunori M, Giuffre A, Nienhaus K, Nienhaus GU, Scandurra FM, Vallone B. Neuroglobin, nitric oxide, and oxygen: functional pathways and conformational changes. *Proc Natl Acad Sci USA* 2005; **102**: 8483–8488.
14 Ates O, Cayli S, Altinoz E, Gurses I, Yucel N, Kocak A *et al*. Effects of resveratrol and methylprednisolone on biochemical, neurobehavioral and histopathological recovery after experimental spinal cord injury. *Acta Pharmacol Sin* 2006; **27**: 1317–1325.
15 Li RC, Morris MW, Lee SK, Pouranfar F, Wang Y, Gozal D. Neuroglobin protects PC12 cells against oxidative stress. *Brain Res* 2008; **1190**: 159–166.
16 Duong TT, Witting PK, Antao ST, Parry SN, Kennerson M, Lai B *et al*. Multiple protective activities of neuroglobin in cultured neuronal cells exposed to hypoxia re-oxygenation injury. *J Neurochem* 2009; **108**: 1143–1154.
17 Hankeln T, Ebner B, Fuchs C, Gerlach F, Haberkamp M, Laufs TL *et al.* Neuroglobin and cytoglobin in search of their role in the vertebrate globin family. *J Inorg Biochem* 2005; **99**: 110–119.
18 Weber T, Dalen H, Andera L, Negre-Salvayre A, Auge N, Sticha M *et al.* Mitochondria play a central role in apoptosis induced by alpha-tocopheryl succinate, an agent with antineoplastic activity: comparison with receptor-mediated pro-apoptotic signaling. *Biochemistry* 2003; **42**: 4277–4291.
19 Fago A, Mathews AJ, Dewilde S, Moens L, Brittain T. The reactions of neuroglobin with CO: evidence for two forms of the ferrous protein. *J Inorg Biochem* 2006; **100**: 1339–1343.