Adenovirus-mediated delivery of GDNF ameliorates corticospinal neuronal atrophy and motor function deficits in rats with spinal cord injury

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The aim of the present study was to assess the effect of glial cell line-derived neurotrophic factor (GDNF) delivery mediated by a recombinant adenovirus (AdCMV*gdnf* or AdCMV*lacZ*) on the functional recovery and central neuronal atrophy in adult rats with spinal cord injury. Bilateral electrolytic lesions were made in the corticospinal tracts at the T10 vertebral level in a rat model. AdCMV*gdnf* or AdCMV*lacZ* was injected at the lesion site immediately after operation. After 2–3 weeks the neurological score and the inclined plane angle were significantly higher and the soma size of corticospinal motoneurons was larger in the AdCMV*gdnf* group compared with the AdCMV*lacZ* group. These results demonstrated that adenovirus-mediated delivery of GDNF could prevent the retrograde atrophy of corticospinal motoneurons and improve the motor function in rats with spinal cord injury. *NeuroReport* 15:425–429 © 2004 Lippincott Williams & Wilkins.

Key words: Adenovirus; Corticospinal motoneuron; GDNF; Gene therapy; Spinal cord injury

INTRODUCTION

Motoneuron diseases have long been recognized as a largely unresolved challenge to clinicians and scientists. Generally speaking, it is accepted that corticospinal motoneurons (CSMN) are responsible for controlling voluntary muscle activities in the trunk and limbs. Their damage following traumatic brain and spinal cord injuries or by neurodegenerative disorders are usually followed by paralysis at sublesional levels due to loss of descending control of motor functions. Many studies have revealed the trophic effects of glial cell line-derived neurotrophic factor (GDNF) on motoneurons under both physiological and pathophysiological conditions [1], making it a good candidate for the treatment of motoneuron disease. However, few studies have explored the influences of GDNF on central motoneurons [2,3].

In the present study, we examined the neurotrophic effects of recombinant adenovirus-mediated delivery of GDNF on injured corticospinal motoneurons and possible motor function improvement in a rat model of spinal cord injury (SCI).

MATERIALS AND METHODS

Experimental animals: Eighty-one adult female Wistar rats (220–250 g) were used in this study. All experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23) and approved by our University.

Recombinant adenovirus construction: The adenovirus was developed by homologous recombination *in vivo*, as reported previously [4]. The E1/E3-deleted recombinant AdCMV*gdnf* adenovirus was propagated in 293 cells and purified by $CsCl_2$ density gradient centrifugation. Virus titers were determined by plaque assays. The same strategy was applied for the recombinant adenovirus containing the *lacZ* gene (AdCMV*lacZ*).

Surgery and lesion generation: Rats were anaesthetized with chloral hydrate (300 mg/kg, *i.p.*) and positioned in a rat stereotaxic frame. A laminectomy was performed at the T10 vertebra level. A 0.5 mm stainless steel electrode was insulated to within 0.1 mm of its tip and inserted at a 30° angle left to the vertical axis and 0.3 mm to the right of the dorsal midline of the cord, as viewed rostro-caudally. It was then lowered 0.86 mm into the cord. In the electrolytic lesion group, a current of 1.0 mA, 210 s was applied through a SEM-20 electric stimulator, while no current was applied in the sham-operation group. The pathological confirmation of the lesion was made as reported in our previous study [5].

Injection of adenovirus: After surgery 3–4 µl of 10^8 pfu/ml AdCMV*lacZ* or AdCMV*gdnf* at 10^6, 10^7 or 10^8 pfu/ml was injected immediately into the cord rostral to the lesion site at a rate of 0.5 µl/min. Each group contained 6–8 animals.

Behavioral assessment of motor deficits: At 1, 2, and 3 weeks after surgery, behavioral assessments were made under single-blind conditions. First, a modified Tarlov score reflecting a semi-quantitative assessment of hind limb function was used [6]: 0 = total paraplegia of hind limbs; 1 = no spontaneous movements but responds to hind limb pinch (just detectable movement); 2 = movement at all limb joints but no walk or weight bearing; 3 = able to walk and support weight on a 1.8 cm wide ledge with abnormal gait; 4 = walks on a ledge. This neuroscore was tested independently in the right and left hind limbs. Values of minimum, at 25% position (25% percentile), the median, and 75% position (75% percentile) and the maximum were used for statistical analysis. Second, the inclined plane angle test was applied to assess the animal's ability to maintain its position on a corrugated rubber board [7]. The maximum angle at which an animal could support its weight for 5 s was the capacity angle.

The retrograde labeling of CSMN: Fast Blue (FB, Sigma) was used for retrograde tracing of CST in 12 rats. Following surgery, 1 µl of FB in (2% in 0.2% DMSO) was injected into the lesion site or into the same point of the lumbar enlargement in sham-operated rats. Three weeks thereafter, the anaesthetized rats were perfused with 4% paraformaldehyde (PFA), post-fixed in PFA overnight and equilibrated in 20% sucrose for 24 h. The forebrains, including the sensory motor cortex, were cut into 20 µm cryostat coronal sections. One-in-five serial sections were mounted onto gelatin-subbed slides, and observed under a fluorescence microscope (Leica) in a 360 nm excitation light. Ten neurons with clear nuclei from each of 10 slide sections for each rat were randomly selected and examined, and 300 neurons were examined in total in each group ($n = 3$ animals, 300 neurons). The scan area of the neuronal somata was measured and recorded using an image analysis system (Imaging Research Inc.).

X-Gal staining: To demonstrate adenovirus-mediated expression of the *lacZ* transgene *in vivo*, three rats were sacrificed 10 days after injection of AdCMV*lacZ*. The segment of the spinal cord including the injection site was dissected and serial transverse cryostat sections were cut and processed for X-Gal staining [8].

Quantitative RT-PCR analysis of relative GDNF expression in vivo: A coupled RT-PCR/HPLC method [9,10] was used. Rats were sacrificed by decapitation 3 weeks after surgery. A segment of the spinal cord around the lesion site was removed and total RNA was isolated. For GDNF RT-PCR, the first strand of cDNA was amplified for 40 cycles with the following primers: 5′-CTGTCTGCCTGGTGT-TGCGC-3′ (sense) and 5′-CTGGTGAACTTTTCAGTCTTTT-GA-3′ (antisense). One amplification cycle consisted of 45 s denaturation at 94°C, 1 min annealing at 63°C and 1.5 min extension at 72°C. GAPDH was used as the internal standard, and there were 25 amplification cycles. The GDNF and GAPDH PCR products were separated and quantified by a reverse-phase HPLC system (column: TSKDNA-NPR 4.6 mm, i.d. 75 mm, 2.5 µm, UV/Vis detector). The peak area ratio of GDNF *vs* GAPDH was used to represent the relative expression of GDNF in tissue.

Data analysis: The Tarlov neuroscores were analyzed with non-parameter tests (Mann-Whitney or Kruskal-Wallis test). All other data were expressed as mean ± s.e. and analyzed by one-way ANOVA followed by Newman-Keul's *post-hoc* test. $p \leq 0.05$ was considered statistically significant.

RESULTS

The motor performance of adult rats with electrolytic lesions: Results are shown in Fig. 1. Before surgery there was no difference in the Tarlov scores and inclined plane angles between the electrolytic lesion group and the sham-operation group, whereas at 1, 2, and 3 weeks after surgery, a significant decrease ($p < 0.05$ or 0.01) was observed. These results indicated that sham operation had no effect on motor function, whereas the electrolytic lesion caused a decline in motor function, which lasted at least 3 weeks.

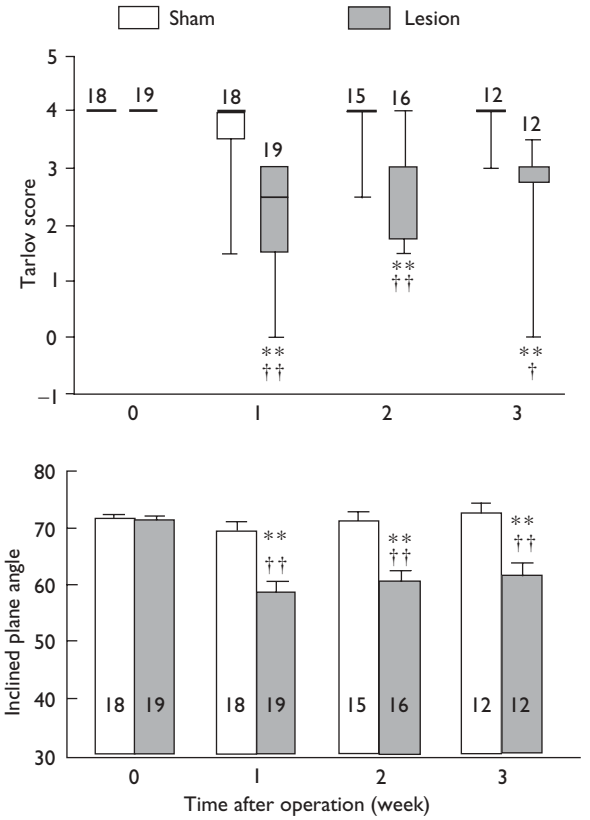

Fig. 1. Behavioral evaluation of rats before and 1, 2, 3 weeks after the operation. (**a**) Tarlov score. The box and whiskers represent the median (box waists), 25% and 75% percentile (outer limits of boxes), minimum and maximum (whiskers) values, respectively. (**b**) Inclined plane angle. Bars represent mean and vertical lines represent s.e. The numbers above the bars (in a) or within the columns (in b) represent the number of rats in each group. $^{**}p < 0.01$ compared with the sham-operation group, $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ compared with pre-operation tested by Mann-Whitney test for Tarlov score or one-way ANOVA followed by Newman-Keuls *post-hoc* test for inclined plane angle.

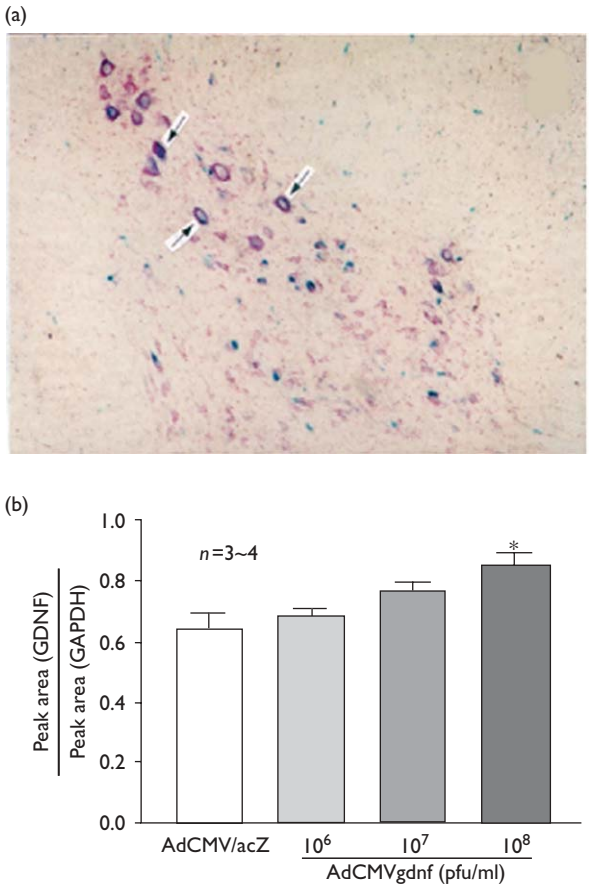

Fig. 2. Recombinant adenovirus mediated delivery of *lacZ* or *gdnf* expression in the spinal cord 3 weeks after SCI and injection of adenovirus. (**a**) The expression of reporter gene *lacZ* after AdCMV*lacZ* injection. Arrows point to the β-galactosidase(+) cells. Magnification: × 200. (**b**) The expression of GDNF as measured by RT-PCR/HPLC. Bars represent mean and vertical lines represent s.e. $*p < 0.05$ compared with the AdCMV*lacZ* group tested by ANOVA followed by Newman-Keuls *post-hoc* test.

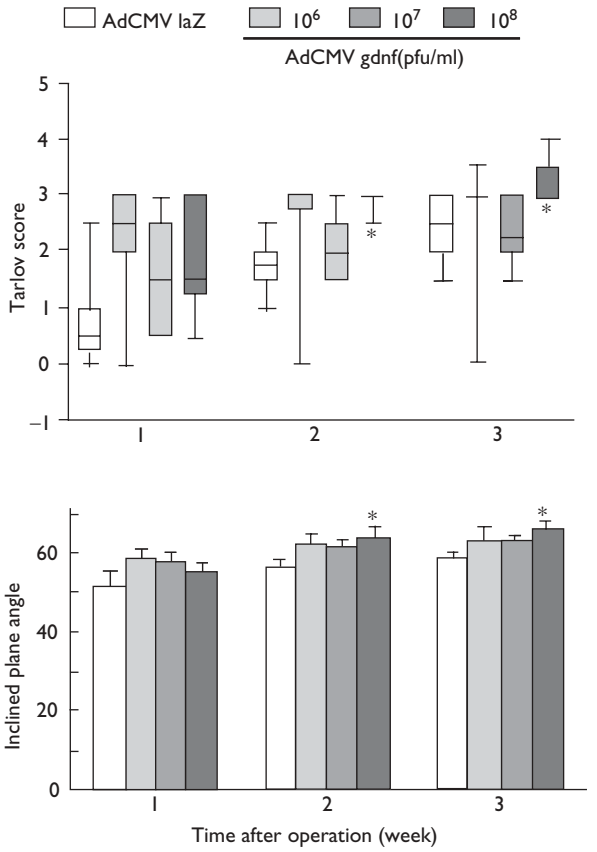

Fig. 3. Motor performance at different time points after SCI and injection of adenovirus. (**a**) Tarlov score, (**b**) inclined plane angle. $*p < 0.05$ compared with AdCMV*lacZ* group tested by Kruskal-Wallis test for Tarlov score or ANOVA followed by Newman-Keuls *post-hoc* test for the inclined plane angle. $n = 6–8$ in each group. The illustration of boxes and whiskers was the same as in Fig. 1.

Adenovirus-encoded gene expression in vivo: The distribution of β-galactosidase, the product of the reporter gene *LacZ*, in the spinal cord after AdCMV*lacZ* injection was shown by X-Gal staining (Fig. 2a). Bluestaining was located in the grey and white matter. GDNF expression in the spinal cord measured with a combination of RT-PCR and HPLC 3 weeks after SCI and injection of adenovirus is shown in Fig. 2b. In the group receiving 10^8 pfu/ml AdCMV*gdnf*, GDNF expression was signiicantly higher than that in the AdCMV*lacZ* group. GDNF expression increased with increasing virus titres, although no significant change was found in 10^6pfu/ml and 10^7 pfu/ml AdCMV*gdnf* groups.

Functional recovery after AdCMVgdnf injection in rats: The motor performance assessed weekly for 3 weeks after SCI and injection of adenovirus is shown in Fig. 3. From 2 to 3 weeks, the neuroscore and the inclined plane angle increased significantly following injection of 10^8 pfu/ml of AdCMV*gdnf*, suggesting that AdCMV*gdnf* accelerated motor function recovery.

Quantitative analysis of CSMN soma size: Quantitative analysis of the CSMN soma size in the sham-operation, SCI, SCI with AdCMV*lacZ* and SCI with AdCMV*gdnf* groups was performed 3 weeks after surgery. The results are shown in Fig. 4. CSMN soma in the electrolytic lesion groups were much smaller than in the sham-operation group ($p < 0.01$). In the group injected with 10^8 pfu/ml AdCMV*gdnf*, CSMN soma size was significantly larger than that in SCI only or AdCMV*lacZ* group.

DISCUSSION

In the present study, we followed Mathers' approach [11] to prepare the electrolytic lesion model of spinal cord injury with a slight modification in the parameters of electric stimulation and in the coordinates for inserting the electrode. The tip of the electrode was located along the midline of bilateral CST in dorsal funiculus, so when an appropriate current was applied, the CST in the contiguous segment was destroyed by electrolysis. CST injury was confirmed by histological evaluation and an HRP retrograde tracing test [5]. CSMN soma size decrease revealed by FB retrograde labeling confirmed this CST lesion. Motor performance, revealed by a decrease in the Tarlov scores and inclined plane angles, indicated that this model was appropriate. No other tract was found to be involved in our experiment.

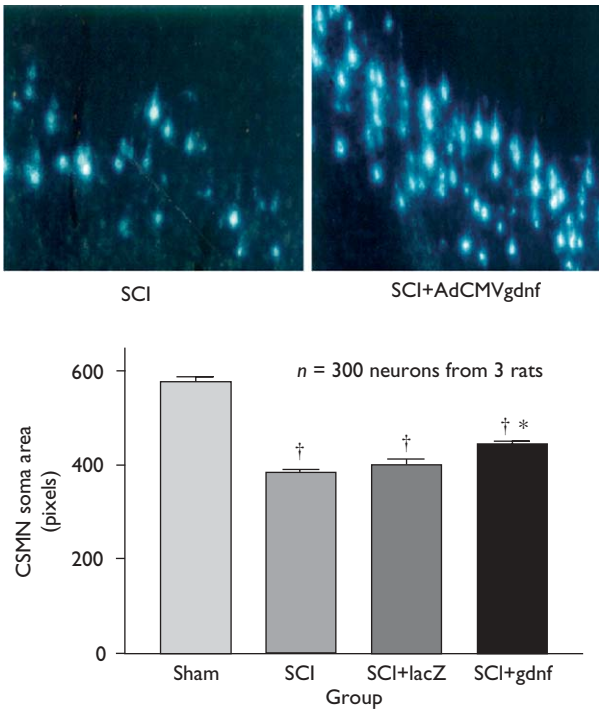

Fig. 4. Effect of AdCMV*gdnf* on the soma area of the corticospinal motoneurons (CSMNs) after spinal cord injury (SCI). (**a**) CSMNs labeled by fast blue (FB) obtained from SCI or SCI + AdCMV*gdnf* treated rat. Magnification: × 200. (**b**) The soma area of CSMN in rats with sham-operation or 3 weeks after SCI and injection of adenovirus. A total of 300 neuronal soma from 3 rats were measured in each group. Bars represent mean and vertical lines represent s.e. $^{\dagger}p < 0.05$ compared with sham-operation group, $^{*}p < 0.05$ compared with the SCI only group tested by ANOVA followed by Newman-Keuls *post-hoc* test.

GDNF, a distant member of the transforming growth factor-β family, was firstly cloned from a B49 glial cell line [12]. It was originally identified as a trophic factor specific for dopaminergic neurons, while further investigation found it to be a potent trophic factor for motoneurons [1]. GDNF mRNA was found in the immediate vicinity of motoneurons during the period of cell death during development. GDNF enhanced the survival of cultured motoneurons and rescues facial motoneurons from axotomy-induced degeneration. It promoted the survival of corticospinal motor neurons *in vitro* [3], and could increase survival of axotomized CSMN after internal capsule lesion [2]. Like other neurotrophic factors, GDNF is a protein that does not cross the blood–brain barrier, therefore treatment of CNS neurons would require intracerebraventricular injection. Gene therapy could provide an alternative method for neurotrophic factor therapy. We reported here that adenovirus-mediated GDNF could reverse the retrograde atrophy of CSMN. Furthermore our data demonstrated that it could improve the functional outcome after SCI in adult rats.

In the present experiment, adenovirus was proven to mediate successful expression of target genes (*lacZ* gene revealed by X-gal staining and GDNF mRNA detected by quantitative RT-PCR combined with HPLC). Our pilot study showed that injection with recombinant adenovirus did not cause significant cell death or degeneration throughout the observation time of 3 weeks (data not shown). The activity of the expressed GDNF was not assayed in the present experiment, but our previous studies showed that the AdCMV*gdnf*-expressed GDNF protein had survival effects on cultured dopaminergic neurons [4] or protective effects on glutamine-injured motoneurons [13].

The neuronal tracer FB was used in this study to identify the cell bodies of the CST. FB can be taken up by either nerve terminals or axon stumps, and then retrogradely transported to the cell bodies. FB was injected into the lumbar enlargement (in the sham-operation group) or into the lesion site (in electrolytic-lesion group). Under both conditions, FB entered the CST, and was then transported along the axon, finally reaching the CSMN soma, which lies in the motor cortex. The retrograde atrophy, similar to the axotomy-induced atrophy of red nucleus neurons after SCI reported by Bregman *et al.* [14], was observed in our experiment. The reason for this might be that CNS neurons required target-derived neurotrophic support. In our experiment, it was obvious that AdCMV*gdnf* (actually the expressed functionally active GDNF protein) prevented the CSMN soma from atrophy and improved motor performance.

The mechanism underlying the functional improvement by AdCMV*gdnf* is not yet clear. It seems that recovery of function does not require regenerative growth of large numbers of axons over long distances in a point-to-point topographical manner for a complete re-establishment of suprasegmental control. As a matter of fact, regrowth over relatively short distances had major functional consequences [15]. It is possible that injection of AdCMV*gdnf* causes sprouting and collateral formation of the injured or uninjured axons, which led to recovery of function. This could be supported to some extent by the prevention of CSMN atrophy by AdCMV*gdnf* seen in our experiment, as the maintenance of cell morphology and axon outgrowth might be closely related [14]. Direct evidence of the regenerative fibers is the object of further investigation.

In conclusion, the present study demonstrated that recombinant adenovirus-mediated delivery of GDNF could significantly improve motor function following spinal cord injury in adult rat, accompanied by rescue of central motoneurons from retrograde atrophy. Further experiments are required to dissect the underlying mechanism in more detail.

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