Adenovirus-mediated GDNF protects cultured motoneurons from glutamate injury

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Received 9 July 2001; accepted 31 July 2001

The protective effects of adenovirus-mediated glia cell line-derived neurotrophic factor (GDNF) gene transaction was investigated on cultured motoneurons. First, the dose– and time–response relationship of glutamate neurotoxicity was determined on spinal motoneuron cultures. Then, the effect of the *gdnf* recombinant adenovirus (AdCMV*gdnf*) was tested in this cellular model. AdCMV*gdnf* at 20 MOI (multiplicity of infection) was found to significantly reduce the cell loss of motoneurons, as compared to AdCMV*gdnf* at 20 MOI, the recombinant adenovirus containing the marker gene *lacZ*. Furthermore, the adenovirus was proved to mediate erogenous gene expression using X-Gal staining and a semi-quantitative RT-PCR method. These results suggested a therapeutic potential of adenovirus vector-mediated *gdnf* gene therapy in human motoneuron diseases. *NeuroReport* 12:3073–3076 © 2001 Lippincott Williams & Wilkins.

Key words: Adenovirus; Excitatory amino acid; Gene therapy; Glia cell line-derived neurotrophic factor; Glutamate; Motoneuron; Spinal cord

INTRODUCTION

It has now been well established by many studies that glial cell line-derived neurotrophic factor (GDNF) is a potent and specific trophic factor for motoneurons [1-3], making it a potential therapeutic molecule for motoneuron diseases. However, most of these studies are involved with introduction of recombinant GDNF protein in microgram quantities into the CNS, which brings about such problems as high expense and side effects. Gene therapy, which can deliver neurotrophic factors continuously to a focal brain area through erogenous gene expression, is an effective tool to solve these problems.

The neurotoxicity derived from abnormalities in the uptake or metabolism of glutamate and related excitatory amino acid has contributes to neuronal loss in many motoneuron diseases [4,5]. For example, it has been suggested that motoneuron degeneration may be mediated by an endogenous excitotoxin identified in the cerebrospinal fluid of patients with amyotrophic lateral sclerosis, and this neurotoxicity could be blocked by AMPA receptor antagonists [6]. On the other hand, MK801, an NMDA receptor antagonist, has been shown to prevent motoneuron degeneration after traumatic injury of the spinal cord [7]. In the present study, we set up a cellular model of glutamate neurotoxicity on cultured spinal motoneurons, and then tested the protection of motoneuron by adenovirus-mediated *gdnf* expression in this system.

MATERIALS AND METHODS

Recombinant adenovirus: By homologous recombination *in vivo*, the *E. coli lacZ* transgene or the *gdnf* gene driven by a human cytomegalovirus immediate-early gene promotor was inserted into genome of the replication-defective adenovirus. The recombinant adenoviruses (AdCMV*lacZ* and AdCMV*gdnf*) were propagated in human 293 cell line and purified by double cerium chloride gradient ultracentrifugation. Titers were determined by the 293 plaque assay.

Cell culture: Fetal rats at 14 days gestation were used. Spinal cords were dissected under sterile conditions, stripped of meninges and dorsal root ganglia. With a razor blade, each side of the cords was cut longitudinally along the mediolateral line into ventral and dorsal halves. The ventral halves, which include the ventral horn, were then cut into small pieces and incubated in 0.1% trypsin at 37°C for 20 min. Fetal calf serum (1 ml) was added and cell dissociation was completed by trituration through narrow bore pipettes. A final suspension was plated on 24-well plates pre-coated with poly-lysine at a density of 3×10^5 cells/per well. The culture medium was Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal calf serum (10%) and horse serum (10%). Cultures were maintained at 37°C in 5% CO_2/95% air and saturating humidity.

Evaluation of motoneuron viability: The viability of cultured motoneurons after treatment with glutamate and adenovirus was assessed by counting the number of survival motoneurons over the entire well. Cultures were

fixed in 10% formalin, then acetylcholinesterase (AChE) histochemical staining was performed using Karnovsky-Roots' method [8]. AChE positive neurons, combined with morphological characteristics of cultured motoneurons (a large cell body, a prominent neuritic arborization, and generally a single long axon-like neurite) can be identified as motoneurons [9,10]. To reduce the differences between experiments, cell counts in different groups were expressed as the percentage of average counts in untreated control cultures.

Estimation of adenovirus-mediated gene expression: To show adenovirus-mediated *lacZ* expression, AdCMV*lacZ* was added to the cultures. Forty-eight hours later the cultures were fixed and processed for X-Gal staining [11]. To show *gdnf* transgene expression after the addition of AdCMV*gdnf* to the culture system, we used a coupled RT-PCR/HPLC method [12,13]. Seventy-two hours after treatment of adenovirus, total RNA was isolated from the primary cultures. First strand cDNA was synthesized using an oligo-dT primer and MMLV reverse transcriptase (BRL/Gibco). For *gdnf* RT-PCR, the first strand cDNA was then amplified with 40 cycles using the following primers: sense: 5′-CTGTCTGCCTGGTGTTGCGC-3′, antisense: 5′-CTGGTGAACTTTTCAGTCTTTTGA-3′. One amplication cycle consisted of 45 s denaturation at 94°C, 60 s, annealing at 63°C, and 90 s extension at 72°C; For GAPDH (as the internal standard) RT-PCR, the primers were: sense: 5′-TCCCTCAAGATTGTCAGCAA-3′, antisense: 5′-AGATC CACAACGGATACATT-3′. Twenty-five cycles and annealing temperature of 57°C were used, other conditions, were the same as *gdnf* amplication. The *gdnf* and GAPDH PCR product were separated and quantified by a reverse-phase HPLC system (column: TSKDNA-NPR 4.6 mm i.d., 75 mm length, 2.5 μm, UV/Vis detector). The peak area ratio of *gdnf* versus GAPDH was used to represent the relative expression of *gdnf* in cultures.

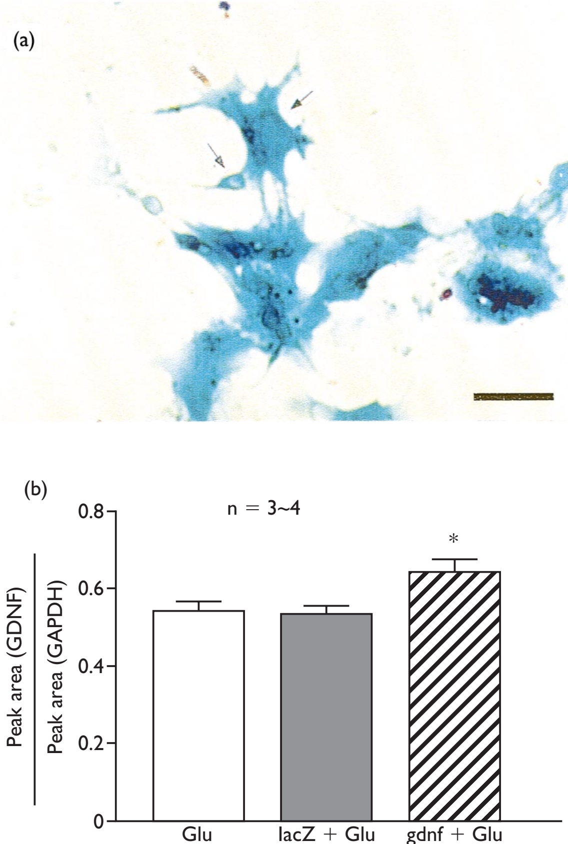

Fig. 1. (**a**) X-Gal staining of ventral spinal cord cultures, 48 h after the addition of AdCMV*lacZ*. Arrows point to the β-Gal(+) cells. Bar = 50 μm. (**b**) The expression of *gdnf* as measured by semi-quantification RT-PCR in ventral spinal cord cultures. Bars represent mean and vertical line s.e. * $p < 0.05$, compared with the glutamate group, tested by ANOVA followed by the Newman–Keul's *post-hoc* test.

RESULTS

Evaluation of adenovirus-mediated gene expression: Figure 1a shows that AdCMV*lacZ* could infect almost all the cells in the culture system and mediated *lacZ* gene expression in them, shown as blue staining with X-Gal. To investigate *gdnf* gene expression, we used a semi-quantitative RT-PCR method.

After 24 h *in vitro*, 20 MOI of AdCMV*lacZ* and AdCMV *gdnf* were added to the culture. Total RNA was extracted 72 h later and a relative amount of *gdnf* mRNA was determined by coupled RT-PCR/HPLC analysis. It was found that addition of AdCMV*gdnf* brought about a significant increase of *gdnf* expression, whereas there was no change was found in AdCMV*lacZ* group (Fig. 1b. These results confirmed that AdCMV*gdnf* could mediate erogenous *gdnf* gene expression, and then exert trophic effect on motoneurons.

Cellular model of glutamate-induced neurotoxicity on motoneurons: Motoneurons viability *vs* various glutamate concentration and incubation time was determined using percentage motoneuron counts. At 24 h after plating, medium was changed by serum-free DMEM, containing 0.02, 0.1, 0.5, 2.5, 12.5 mmol/L (final concentration) glutamate, respectively. Cultures were fixed 48 h later, and surviving motoneurons were counted. As shown in Fig. 2a, average counts in the control group were taken as 100%, glutamate toxicity on cultured motoneurons decreased with increasing extracellular concentrations of the amino acid. Glutamate at 0.5 mmol/l induced a 40.6 ± 4.6% cell loss ($p < 0.01$) compared with the non-treated group. Figure 2b shows the time–response relationship for glutamate. At 24 h after plating, medium was changed by serum-free DMEM containing 0.5 mmol/L (final concentration) glutamate. Over different incubation times, cultures were fixed and counted. Counts before the addition of glutamate was 100%. The neurotoxicity increased with the incubation time, and 48 h exposure to glutamate induced a 23.1 ± 5.0% cell loss, compared to the non-treated group cultured for the same period of time. The data were analyzed by two-way ANOVA, which showed that the number of the survived neurons decreased significantly as time increased from 0 to 48 h ($p < 0.01$, $F = 7.39$, $df = 5$). On the other hand, the number of surviving neurons in the glutamate group was much lower than that in the control group ($p < 0.01$, $F = 11.04$, $df = 1$). According to such a dose– and time–effect relationship, we chose 0.5 mmol/L glutamate,

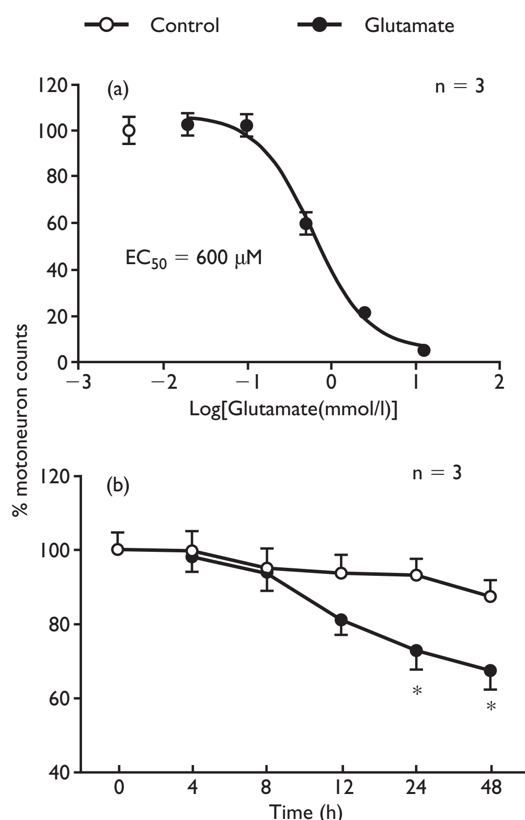

Fig. 2. (**a**) The dose–response relationship for glutamate neurotoxicity on cultured motoneurons as assessed by motoneuron counts. (**b**) The time–response relationship for glutamate (final concentration is 0.5 mmol/L) neurotoxicity on cultured motoneurons as assessed by motoneuron counts. Symbols represent mean and vertical line s.e. $^{*}p < 0.01$, compared with the control, tested by two-way ANOVA, followed by Bonferroni post-tests. (Interaction F = 2.04, df = 5, $p > 0.05$).

48 h incubation as adequate parameters of excitotoxic injury in later studies.

***Trophic effects of AdCMV*gdnf:** The protective effect of AdCMV*gdnf* was tested on glutamate-injured motoneurons. After 24 h *in vitro* motoneurons were cultured with serum-free DMEM and were infected with 5, 10, 20, 40, 80 MOI AdCMV*gdnf* for 1 h. Twenty-four hours later 0.5 mmol/L glutamate was added to the medium, and after another 48 h cultures were fixed and counted. As shown in Fig. 3, between 5 and 20 MOI, AdCMV*gdnf* has a tendency to decrease glutamate neurotoxicity dose-dependently. At MOI of 20, AdCMV*gdnf* significantly prevented glutamate-induced cell loss ($p < 0.01$). However, when higher concentrations were used (40 or 80 MOI), motoneuron counts decreased further, rather trhan increasing. In order to explore the mechanism of AdCMV*gdnf*, we compared AdCMV*gdnf* with AdCMV*lacZ* and *gdnf* protein at 20 MOI, 20 MOI and 10 μg/l, respectively. Glutamate exposure and motoneuron counts were performed as above. Figure 4 shows that both AdCMV*gdnf* and *gdnf* protein significantly attenuated the glutamate-mediated injury, led to an increase in the numbers of survival motoneurons, whereas AdCMV*lacZ* did not improve survival. This indicates that

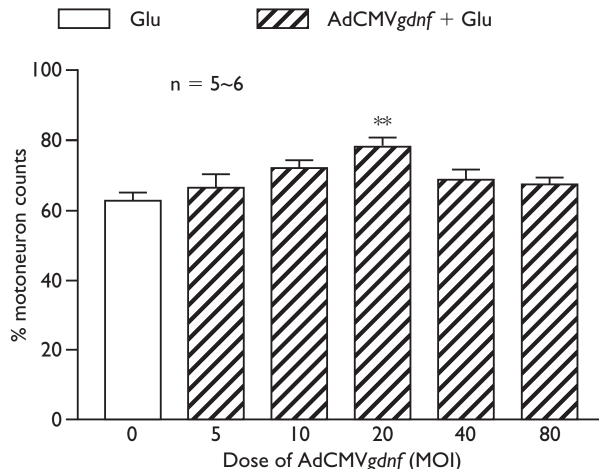

Fig. 3. The dose-effect relationship of AdCMV*gdnf* on glutamate-injured motoneurons. Bars represent mean and vertical line s.e. $^{**}p < 0.01$, compared with the glutamate group, tested by ANOVA followed by the Newman–Keul's *post-hoc* test.

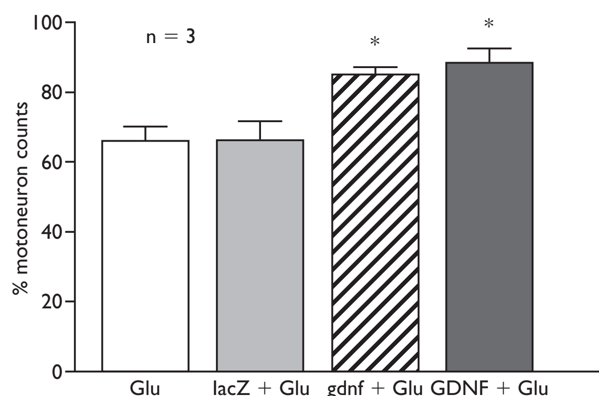

Fig. 4. The effect of adenovirus or *gdnf* on glutamate-injured motoneurons. Bars represent mean and vertical line s.e. $^{*}p < 0.05$, compared with the glutamate group, tested by ANOVA followed by the Newman–Keul's *post-hoc* test.

the rescue of motoneurons by AdCMV*gdnf* may be mediated by the expressed *gdnf* protein, not by the Ad vector in the culture system.

DISCUSSION

Spinal motoneurons appear to be particularly vulnerable to excitotoxic insults [14,15]. Moreover, growing evidence suggested that excitatory amino acid-induced neurotoxicity probably participates in the pathogenesis of motoneuron degeneration in a variety of acute and chronic neurological disorders [4,5]. It is therefore worthwhile exploring either the mechanisms of the excitatory neurotoxicity on motoneurons or effective treatment for such injuries. The susceptibility of motoneurons to excitotoxins varies with age, different culture system, indicators used to reflect injury, and so on [16–18]. Here we established the dose– and time–effect relationship of glutamate on cultured motoneurons using counts of motoneuron survival, and

then investigated the neuropretective effects of *gdnf* gene therapy in this model.

Concerning gene transfer techniques, a selection of *in vivo* and *ex vivo,* viral and non-viral, methods are available. In this study, gene transfer to the CNS was carried out with recombinant adenoviral vectors. Several advantages of adenovirus transduction made it to be a powerful method in research of gene therapy for neurological diseases. First, there is little cell specifity of transduction by a replication-deficient adenovirus. When injected into the CNS parenchyma, Ad vectors have been shown to transduce multiple cell types, including neurons, astrocytes, oligodendrocytes and microglia [19–22]. Moreover, neurons appeared to be the most successfully transduced [19-22]. Second, transgene expression following CNS gene transfer is relatively long-term [23] and, finally, recombinant adenoviral vectors are relatively simple to generate. In our experiment, the *in vitro* expression of AdCMV*lacZ* and AdCMV*gdnf* were proved by X-Gal staining and semi-quantitative detection of *gdnf* mRNA in the cultures.

The adenovirus used in this experiment was constructed in our laboratory through homologous recombination *in vivo* [24]. Its validity was identified by morphology, PCR, RT-PCR and restriction enzyme analysis. Using the immunoprecipitation method high level *gdnf* protein was found to be expressed in 293 cells and released in the supernatant of the culture medium. These results confirmed that *gdnf* could be produced and secreted by cells infected with AdCMV*gdnf*.

This experiment demonstrated, for the first time, the protective effect of AdCMV*gdnf* on glutamate-injured motoneurons. This effect was in a dose-dependent manner. A possible explanation for this phenomenon was that when MOI was between 5 and 20, the toxicity of adenovirus itself was not evident. As the viral dose increased, the infection efficiency increased, the amount of *gdnf* expression and secretion also increased, and so produced a better trophic effect. But when MOI reached 40 or 80, the toxic effect of the adenovirus appeared, so the motoneuron counts decreased.

When AdCMV*lacZ* (negative control), AdCMV*gdnf* or *gdnf* protein (positive control) was added to the culture system respectively, it was found that both AdCMV*gdnf* and *gdnf* protein prevented the motoneuron loss caused by glutamate exposure, while AdCMV*lacZ* had no such an effect. Because AdCMV*gdnf* was proved to mediate *gdnf* expression in the culture, we postulate that the effect of AdCMV*gdnf* was realized through the exogenous *gdnf* gene expression.

CONCLUSION

In this experiment, we reported the protective effects of recombinant adenovirus-mediated *gdnf* on motoneurons. The cultured motoneurons were injured by the excitatory amino acid, glutamate, as a model. The adenovirus-mediated gene expression was accessed with X-Gal staining and a semi-quantitative RT-PCR method on the cultured neurons. Our results suggested that the adenovirus could effectively express erogenous gene, and *gdnf* could protect the injured cultured motoneurons.

REFERENCES

1. Henderson CE, Phillips HS, Pollock RA *et al*. *Science* **266**, 1062–1064 (1994).
2. Zurn AD, Baetge EE, Hammang JP *et al*. *Neuroreport* **6**, 113–118 (1995).
3. Junger H and Varon S. *Brain Res* **762**, 56–60 (1997).
4. Faden A and Simon RP. *Ann Neurol* **23**, 623–626 (1988).
5. Plaitakis A, Constantakakis E and Smith J. *Ann Neurol* **24**, 446–449 (1988).
6. Couratier P, Hugon J, Sindou P *et al*. *Lancet* **341**, 265–268 (1993).
7. Mentis GZ, Greensmith L and Vrbova G. *Neuroscience* **54**, 283–285 (1993).
8. Karnovsky MJ. *J Histochem Cytochem* **12**, 219 (1964).
9. Satoh K, Armstrong DM and Fibiger HC. *Brain Res Bull* **11**, 693–720 (1983).
10. Carriedo SG, Yin HZ and Weiss JH. *J Neurosci* **16**, 4069–4079 (1996).
11. Sanes JR, Rubenstein JL and Nicolas JF. *EMBO J* **5**, 3133–3142 (1986).
12. Hayward-Lester A, Oefner PJ and Doris PA. *Biotechniques* **20**, 250–257 (1996).
13. Odin E, Larsson L, Arain M *et al*. *Tumour Biol* **19**, 167–175 (1998).
14. Faden AI, Lemke M, Simon RP and Noble LJ. *J Neurotrauma* **5**, 33–45 (1988).
15. Faden AI and Simon RP. *Ann Neurol* **23**, 623–626 (1988).
16. Estevez AG, Stutzmann JM and Barbeito L. *Eur J Pharmacol* **280**, 47–53 (1995).
17. Regan RF and Choi DW. *Neuroscience* **43**, 585–591 (1991).
18. Meldrum B and Garthwaite J. *Trends Pharmacol Sci* **11**, 3 79–3 87 (1990).
19. Akli S, Caillaud C, Vigne E *et al*. *Nature Genet* **3**, 224–228 (1993).
20. Davidson BL, Allen ED, Kozarsky KF *et al*. *Nature Genet* **3**, 219–223 (1993).
21. Horellou P, Vigne E, Castel MN *et al*. *Neuroreport* **6**, 49–53 (1995).
22. Lisovoski F, Cadusseau J, Akli S *et al*. *Neuroreport* **5**, 1069–1072 (1994).
23. Peltekian E, Parrish E, Bouchard C *et al*. *J Neurosci Methods* **71**, 77–84 (1997).
24. Xu GH, Lin Y, Wan Y *et al*. *Chin J Biochem Mol Biol* **15**, 42–47 (1999).

Acknowledgements: The authors would like to thank Dr Xiaoming Xu and Louisa Wirthlin for proof reading. This work was supported by the grants to You Wan from the National Natural Science Foundation of China (39600150, 39770241) and the National Basic Research Program of China (G1999054000).