Adenovirus-mediated GDNF protects cultured motoneurons from glutamate injury

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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 (AdCMVgdnf) was tested in this cellular model. AdCMVgdnf at 20 MOI (multiplicity of infection) was found to significantly reduce the cell loss of motoneurons, as compared to AdCMVgdnf 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 promoter was inserted into genome of the replication-defective adenovirus. The recombinant adenoviruses (AdCMVlacZ and AdCMVgdnf) were propagated in human 293 cell line and purified by double cesium 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/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% CO2/95% 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 a percentage of average counts in untreated control cultures.

Estimation of adenovirus-mediated gene expression: To show adenovirus-mediated lacZ expression, AdCMVlacZ 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 AdCMVgdnf 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'-CTGTCGGCATCAGCTTCTCTTTGA-3', antisense: 5'-CTGGTGAACCTTTCAGTTTTGAG-3'. One amplification 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'-TCCCTCAAAGATTGTCAGCAA-3', antisense: 5'-AGATCCACAGCCGATACATT-3'. Twenty-five cycles and annealing temperature of 57°C were used, other conditions, were the same as gdnf amplification. 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.

RESULTS
Evaluation of adenovirus-mediated gene expression: Figure 1a shows that AdCMVlacZ 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 AdCMVlacZ 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 AdCMVgdnf brought about a significant increase of gdnf expression, whereas there was no change was found in AdCMVlacZ group (Fig. 1b). These results confirmed that AdCMVgdnf could mediate ergogenous 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 24h 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 glutamate group, tested by ANOVA followed by the Newman–Keul’s post-hoc test. At 24 h after plating, medium was changed by serum-free DMEM containing 0.5 mmol/L (final concentration) glutamate. Other 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,
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48 h incubation as adequate parameters of excitotoxic injury in later studies.

Trophic effects of AdCMVgdnf: The protective effect of AdCMVgdnf 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 AdCMVgdnf 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, AdCMVgdnf has a tendency to decrease glutamate neurotoxicity dose-dependently. At MOI of 20, AdCMVgdnf significantly prevented glutamate-induced cell loss ($p < 0.01$). However, when higher concentrations were used (40 or 80 MOI), motoneuron counts decreased further, rather than increasing. In order to explore the mechanism of AdCMVgdnf, we compared AdCMVgdnf with AdCMVlacZ and gdnf protein at 20 MOI, 20 MOI and 10 mg/L, respectively. Glutamate exposure and motoneuron counts were performed as above. Figure 4 shows that both AdCMVgdnf and gdnf protein significantly attenuated the glutamate-mediated injury, led to an increase in the numbers of survival motoneurons, whereas AdCMVlacZ did not improve survival. This indicates that the rescue of motoneurons by AdCMVgdnf 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

![Fig. 2](image-url)  
(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, $p > 0.05$).

![Fig. 3](image-url)  
The dose-effect relationship of AdCMVgdnf 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.

![Fig. 4](image-url)  
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.
then investigated the neuroprotective 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 specificity 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 AdCMVlacZ and AdCMVgdnf 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 AdCMVgdnf.

This experiment demonstrated, for the first time, the protective effect of AdCMVgdnf 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 AdCMVLacZ (negative control), AdCMVgdnf or gdnf protein (positive control) was added to the culture system respectively, it was found that both AdCMVgdnf and gdnf protein prevented the motoneuron loss caused by glutamate exposure, while AdCMVLacZ had no such an effect. Because AdCMVgdnf was proved to mediate gdnf expression in the culture, we postulate that the effect of AdCMVgdnf 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 endogenous gene, and gdnf could protect the injured cultured motoneurons.

REFERENCES