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Short Communication

Adenoviral interneuronal transportation after retrograde gene transfer in mice

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Abstract

Although retrograde gene transfer from infected muscles to neurons by viral vectors has been known for years, it is still unknown whether interneuronal gene transportation of viral vectors occurs after retrograde gene transfer. To determine this, we injected adenoviral vectors carrying *eGFP* gene with or without a neural tracer into the right gastrocnemius muscles of mice. After 7 days, some spinal motor neurons were detected with green fluorescence but without the signal of neural tracer. In addition, nerves with green fluorescence could be noted in the right lumbosacral paraspinal muscles of viral-injected mice. The green fluorescence in the right lumbosacral paraspinal muscles might have resulted from retrograde gene transportation from the viral-injected gastrocnemius muscles to the spinal neurons, followed by interneuronal transfer and anterograde expression of eGFP in the axons belonging to neurons innervating the paraspinal muscles. This phenomenon of interneuronal transportation raises the possibility that we could treat motoneuron diseases by injection of viral vectors containing therapeutic genes into a few muscles resulting in widespread beneficial effects.

Theme: Motor systems and sensorimotor integration
Topic: Spinal cord and brainstem

Keywords: Recombinant adenovirus; Motor neuron; Spinal cord; Gene therapy

Degenerative motor neuron diseases, such as amyotrophic lateral sclerosis and spinal muscular atrophy, cause limb paralysis and respiratory failure. Some neurotrophic factors have been found to be candidates as gene therapy for these diseases due to their ability to rescue degenerative motor neurons [1]. Since intramuscular injection of adenoviral vectors results in retrograde gene transport from skeletal muscles to motor neurons, so it can be used as a gene delivery method for the treatment of motor neuron diseases [2,3]. The possibility of interneuronal gene transportation after recombinant adenoviral retrograde gene transfer from injected muscles to neurons is still undetermined. Previous study has shown that retrograde transportation of adenoviral vectors was limited to neurons having axon terminals at the virus injected sites and did not suggest the possibility of interneuronal transportation [5]. In this study, we try to confirm the retrograde transportation capabilities of recombinant adenoviruses and provide evidences of adenoviral interneuronal transportation after retrograde transfer.

The *eGFP* gene was subcloned into an adenovirus shuttle vector under the control of a human phosphoglycerate kinase gene promoter. Recombinant adenovirus carrying the *eGFP* gene (Ad*eGFP*) was generated by homologous

Abbreviations: eGFP, enhanced green fluorescence protein; AdeGFP, adenoviral vector carrying enhanced green fluorescence protein gene; CHAT, choline acetyltransferase; GFAP, glial fibrillary acidic protein

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recombination and amplified in 293 cells as previously described [4]. Large quantities of viral vectors were purified by double CsCl ultracentrifugation. After dialysis, purified Ad*eGFP* was stored in 10 mM Tris–HCl (pH 7.4), 1 mM $MgCl_2$, and 10% (vol/vol) glycerol at −70 °C until used. Virus titer was determined by a plaque formation assay on the 293 cell monolayer [4]. To verify that cells infected with Ad*eGFP* could efficiently express eGFP, we infected mouse neuroblastoma cells (Neuro 2A, ATCC CCL-131) with Ad*eGFP* and then monitored eGFP expression with a fluorescence microscope IX70 (Olympus Optical Co., Japan) fitted with a SPOT cooled color digital camera and SPOT software v2.2 (Diagnostic Instruments, Inc., USA). Two days after Ad*eGFP* (6×10^8 PFU) infection, nearly all of the Neuro 2A cells (1×10^6 cells) expressed green fluorescence. In addition to their cell bodies having green fluorescence, most of the Neuro 2A cells exhibited green fluorescence in their neurite-like processes (Fig. 1A). We could still detect green fluorescence 7 days after viral infection.

Twelve-day-old FVB/NJ mice were briefly anesthetized with chloral hydrate (0.5 mg/g, i.p.). We injected 10 μl of phosphate-buffered saline (PBS) or Ad*eGFP* (6×10^8 PFU/μl) into the right gastrocnemius muscles of these mice, using a 30 G × 1/2 needle and a 100 μl microsyringe (ITO, Fuji, Japan). Some mice also received the neural tracer, Diamidino Yellow (DY, 1 μl at 3% in 0.9% saline; Sigma, St. Louis, MO), in the same muscles at the same time. The mice were killed within 4 to 9 days after virus injection. Gastrocnemius muscles, triceps muscles, paraspinal muscles, sciatic nerves, the spinal cord around the thoracolumbar enlargement, dorsal root ganglia from the lumbar regions, and the liver were removed, fixed in 4% paraformaldehyde at room temperature for 3 h, placed in 15% sucrose for 3 h, then in 30% sucrose at 4 °C overnight, and then rapidly frozen in liquid nitrogen-cooled isopentane. These tissues were subjected to serial cryostat sectioning at 7–15 μm thickness. Samples were examined by fluorescence microscopy. Through the testing course, mice were supplied with sterile water ad libitum and rodent pellets and were under the care of the animal facility of the Institute of Molecular Biology, Academia Sinica. All procedures were approved by the Academia Sinica Animal Care and Use Committee, Master Protocol #RMiRbIMBLH2001132.

From 4 to 7 days after muscular inoculation of Ad*eGFP*, we detected strong green fluorescence in the injected gastrocnemius muscles. The green fluorescence dramatically decreased after day 9 post-inoculation (data not shown). Thus, we analyzed the green fluorescence at day 7 after viral inoculation. In addition to the injected right gastrocnemius muscles, the right sciatic nerves of the injected mice showed linear green structures in the nerve bundles (Fig. 1B). In the right lateral aspect of anterior horn regions of the spinal cord (L4–6), we detected some neuron-like cells exhibiting green fluorescence (Fig. 1C). Under hematoxylin and eosin stain, these cells had pyramid-shape cell bodies, large nuclei, prominent nucleoli, and Nissle bodies without adjacent gliosis (Fig. 1D). After immunostaining for NeuN (neuron marker), choline acetyltransferase (CHAT, motoneuron marker) and glial fibrillary acidic protein (GFAP, glial cell marker), we confirmed that the cells with green fluorescence were motor neurons (Fig. 1E). By comparing the numbers of green fluorescent neurons and DY-labeled neurons from the mice with both virus and DY injection, the estimated retrograde efficacy of our adenoviral vector was about 15% (Fig. 1F). RT-PCR analysis for *eGFP* confirmed the *eGFP* gene expression in the lumbar spinal cord of Ad*eGFP*-injected mice (data not shown). We did not detect any green fluorescence in the left gastrocnemius muscles, the left sciatic nerves, or the left aspect of lumbar spinal cord. Therefore, we had reproduced the retrograde transport of recombinant adenoviruses reported previously [2,3]. In addition, some cells and nerve fibers in the right lumbar dorsal root ganglia expressed green fluorescence (Fig. 1G). We did not detect any green fluorescence in the left dorsal root ganglia. Green reticular fibers were also noted in the right dorsal horn area from the lumbar to the lower thoracic spinal cord (Fig. 1H). These results indicated that Ad*eGFP* was also retrogradely transported from the injected muscles to the sensory neurons. Thus, by means of intramuscular injection of therapeutic recombinant adenoviruses, we may be able to treat not only the motor neuron diseases but also the sensory neuronopathy.

In the mice with both viral vectors and the DY injected into the gastrocnemius muscles, some spinal neurons (2%) located in the lateral or medial aspects of anterior horn regions showed green fluorescence without expression of a tracer signal (Fig. 2A). Since immunostaining for CHAT confirmed these neurons to be motor neurons (Fig. 2B), adenoviral interneuronal transportation was thus suspected. To test the phenomenon of adenoviral interneuronal transportation, we investigated the lumbosacral paraspinal muscles in the mice with Ad*eGFP* inoculation into the right gastrocnemius muscle. We could detect green fibrous structures in the right lumbosacral paraspinal muscles (Fig. 2C) in 50% of Ad*eGFP* inoculated mice prior to muscle sectioning. After muscle sectioning, nerves with green fluorescence were found throughout the connective tissue of the right lumbosacral paraspinal muscles (Fig. 2D), while muscle fibers or vascular structures did not express green fluorescence. Neuronal transportation pathways have been identified from muscle afferents innervating hind-limb muscles to motor neurons innervating trunk muscles [9]. Since the gastrocnemius muscles and the dorsal lumbosacral paraspinal muscles belong to the same myotome, we speculate that the green fluorescence seen in nerves embedded in paraspinal muscles may result from adenoviral retrograde transportation from the injected gastrocnemius muscles to spinal sensory and motor neurons, followed by interneuronal transfer from sensory and/or motor neurons to the motor neurons innervating the paraspinal muscles, and expression of eGFP in the spinal motor axons embedded in