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Expression of brain-derived neurotrophic factor in the central nervous system of mice using a poliovirus-based vector

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Brain-derived neurotrophic factor (BDNF) is a promising candidate for the gene therapy of neurological disease. To deliver BDNF to neurons of the central nervous system (CNS), a nucleotide sequence encoding the mature peptide of BDNF was inserted into the genome of poliovirus, a neurotropic virus that is known to replicate mainly in motor neurons of the spinal cord of the CNS. Thus, the recombinant poliovirus constructed was replication-competent. The expression of BDNF in cultured cells infected with the recombinant poliovirus was evident when the cells were analyzed using an immunofluorescence assay and Western blotting. When the recombinant viruses were injected intramuscularly into transgenic mice that carry the human poliovirus receptor gene, the antigens of poliovirus and BDNF were detected in the motor neurons of the spinal cord at 3 days postinfection, and had disappeared by 7 days postinfection. This study suggests that poliovirus can be used as a virus vector for the delivery of neurotrophic factors to the motor neurons of the central nervous system and may provide a new approach for the treatment of motor neuron diseases. *Journal of NeuroVirology* (2002) **8,** 14–23.

Keywords: transgenic mouse; intracerebral inoculation; intramuscular inoculation; immunostaining

Introduction

In mammals, the neurotrophin family consists of nerve growth factor, brain-derived neurotrophic factor (BDNF), neurotrophin-3, and neurotrophin-4 (Leibrock *et al*, 1989; Hohn *et al*, 1990; Maisonpierre *et al*, 1990; Berkemeier *et al*, 1991; Ip *et al*, 1992; Lewin and Barde, 1996). Neurotrophin genes are candidate therapeutic genes for neurodegenerative diseases, because they regulate neuronal development and promote the survival of peripheral sensory neurons, sympathetic neurons, and various types of central nervous system (CNS) neurons in cell culture systems and animal injury models (Eide *et al*, 1993; Korsching, 1993). Much effort has been made to develop virus vectors that could deliver neurotrophins into the CNS. Adenovirus vectors have been used as tools of gene therapy for neurodegenerative diseases (Barkats *et al*, 1998; Di Polo *et al*, 1998; Haase *et al*, 1998). Adeno-associated virus and herpes simplex virus vectors have also been explored for gene therapy in neurodegenerative disease (Battleman *et al*, 1993; Goodman *et al*, 1996; Klein *et al*, 1998; Goins *et al*, 1999; Mandel *et al*, 1999). In addition, transplants of fibroblasts modified by retrovirus-mediated

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expression of BDNF have also shown promising effects on the promoting the regeneration of severed neurons (Liu *et al*, 1999).

Because neurotrophic factors influence various types of CNS neurons, the broad delivery of neurotrophic factors to the CNS may affect multiple nontarget populations. Thus, for gene therapy of a particular type of neurological disease it would be more desirable to use vectors that display specificity to the neurons of interests. Poliovirus is known to have a distinct neuron tropism in the CNS, especially to the motor neurons in the anterior horn of the spinal cord. In addition, intramuscularly inoculated poliovirus can be transported to the cell body of projection neurons. Therefore, a poliovirus-based vector may offer promising applications for the specific targeting of selected neuronal populations such as the motor neurons in the spinal cord, which are not easily accessible by noninvasive approaches.

The genome of poliovirus is a single-stranded RNA molecule of positive polarity, composed of approximately 7,500 nucleotides. A unique open reading frame (ORF) encodes a large polyprotein precursor that is processed by two viral proteinases, $3C^{pro}$ and $2A^{pro}$. The major proteinase, $3C^{pro}$, recognizes and cleaves at specific amino acid sequences (AXXQ/G) within the exposed polyprotein domains. Many studies have been performed to engineer poliovirus for the delivery of foreign proteins (Burke *et al*, 1988; Choi *et al*, 1991; Alexander *et al*, 1994; Andino *et al*, 1994; Ansardi *et al*, 1994; Lu *et al*, 1995; Porter *et al*, 1995). These are classified into two strategies.

One of these two strategies is to generate replication-competent recombinant viruses in which foreign ORFs are directly fused to the ORF of the poliovirus polyprotein. In this case, foreign peptides are designed to be separated from poliovirus polyprotein by artificial cleavage sites of $3C^{pro}$ or $2A^{pro}$ (Andino *et al*, 1994; Mattion *et al*, 1994; Yim *et al*, 1996; Mandl *et al*, 1998; Crotty *et al*, 1999). Another strategy using poliovirus as an expression vector is to replace the coding sequence for the capsid protein region (P1) with a foreign ORF. Cleavage of the foreign polyprotein from the P2-P3 regions is then carried out by the viral proteinase $2A^{pro}$ at an endogenous cleavage site (Percy *et al*, 1992; Ansardi *et al*, 1994; Morrow *et al*, 1994; Porter *et al*, 1995). In the latter case, progeny virions cannot be generated because no viral capsid protein is produced in the infected or transfected cells.

By using the poliovirus replicon strategy (the latter case), foreign proteins were expressed in motor neurons of the spinal cord in transgenic (Tg) mice carrying the gene for the human poliovirus receptor (PVR; CD155) (Bledsoe *et al*, 2000a, 2000b). We report here on the construction of a replication-competent poliovirus recombinant that is manipulated to express a neurotrophin BDNF and the characterization of its nature in the cultured cell lines and CNS neurons of PVR-Tg mice, mice transgenic for the human PVR gene.

Results

Construction and replication characteristics of recombinant polioviruses expressing BDNF As the first step in construction of a replication-competent poliovirus vector to deliver a neurotrophin to the CNS, a nucleotide sequence encoding the mature peptide of BDNF was inserted at the 5′ terminus of the ORF in poliovirus type 1 Mahoney strain (PV1/Mahoney) (Figure 1). This recombinant cDNA, designated pMah/BDNF, yielded a replication-competent virus after transfection of African green monkey kidney (AGMK) cells with the *in vitro* synthesized RNA from pMah/BDNF. The virus (Mah/BDNF) harvested displayed smaller plaques and replicated more slowly than the parental

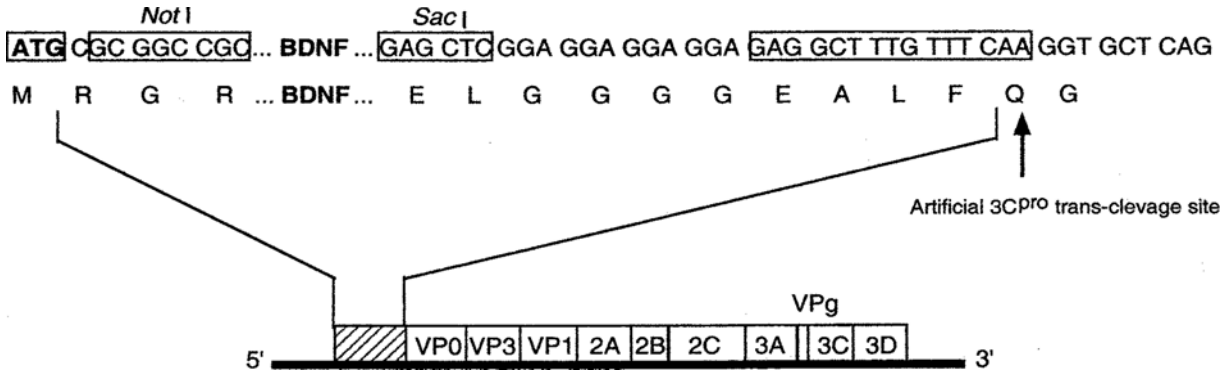

Figure 1 Genome structure of recombinant poliovirus. Schematic diagram of recombinant poliovirus expressing BDNF is shown. The virus genome is indicated by the bold line, and the organization is within the corresponding boxes. VPg at the 5′ end and poly A at the 3′ end are omitted in this figure. A linker sequence carrying restriction enzyme sites for *Sac*I and *Not*I, and a nucleotide sequence for an artificial $3C^{pro}$ cleavage site, were inserted at the 5′ end of the poliovirus ORF. The sequence encoding the mature peptide of BDNF was inserted into the linker. The start codon for the poliovirus polyprotein, the restriction cleavage sites, and a sequence for the artificial $3C^{pro}$ cleavage site are squared.

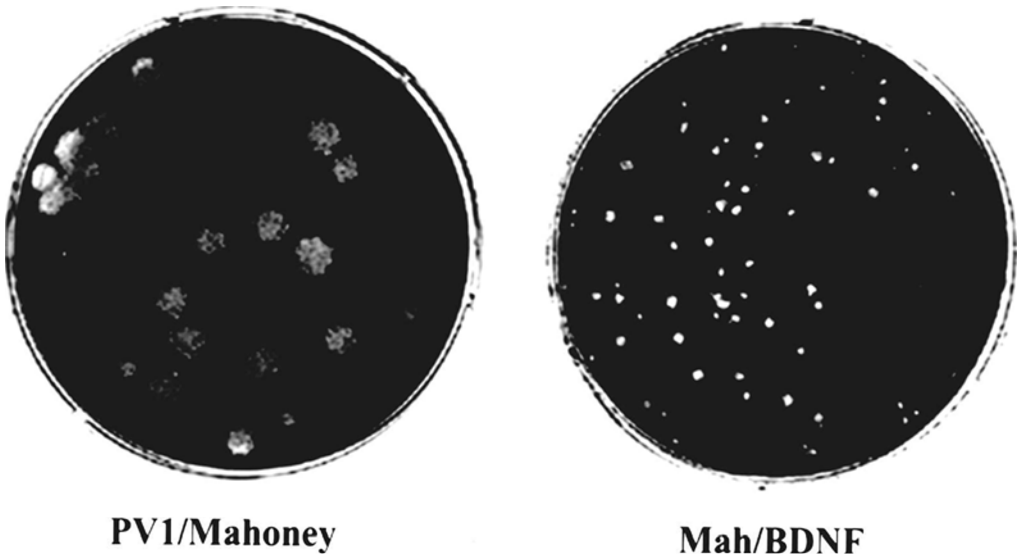

Figure 2 Plaque phenotypes. AGMK cells grown overnight in 60-mm-diameter dishes were infected with PV1/Mahoney or Mah/BDNF. Cells in the dishes were incubated for 72 h at 37°C before being fixed and stained with 1% crystal violet.

PV1/Mahoney in AGMK cells (Figures 2 and 3). The titer of the Mah/BDNF reached only 10% of the wild-type virus at 9 h postinfection (Figure 3).

Because poliovirus, like all other RNA viruses, shows a high rate of genetic variation (Mueller and Wimmer, 1998), foreign sequences, which are dispensable for poliovirus replication, might be deleted rapidly after a few passages in cultured cells. To examine the genetic stability of the Mah/BDNF in cultured cells, the virus recovered from the cells transfected with its RNA was passaged for three more times in AGMK cells. Then, 29 plaques were picked up and the total RNA isolated. RT-PCR was employed to amplify the genome region containing the inserted sequence encoding the mature peptide of BDNF. The bands of intact length (1068 nt) were detected in all the 29 plaque-purified viruses (Figure 4), suggesting that the foreign sequence was stably maintained in the majority of recombinant virus population for up to three passages in the cultured cells. However, the further passage of Mah/BDNF caused appearance of virus population that displayed slightly larger plaques, although the plaque size was much smaller than that of the parental PV1/Mahoney (Figure 5). Based on these observations, all further experiments presented in this report were performed using virus stocks obtained after three passages in AGMK cells.

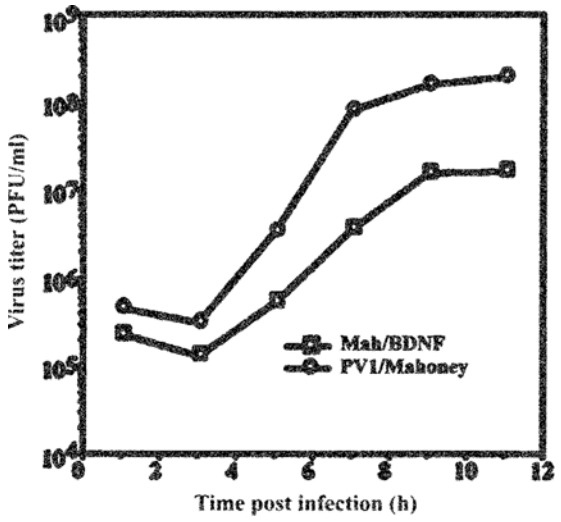

Figure 3 One-step growth curve of polioviruses in cultured cells. AGMK cells grown overnight in 60-mm-diameter dishes at 37°C were infected at an m.o.i. of 10 with PV1/Mahoney or Mah/BDNF obtained after three successive passages in AGMK cells. The cultures were collected at the indicated times. Virus titers were determined by plaque assay.

Expression of BDNF in cultured cells To examine the expression of BDNF by the poliovirus-based vector in cultured cells, AGMK cells were infected with PV1/Mahoney or Mah/BDNF at a multiplicity of infection (m.o.i.) of 10, and then double stained with antibodies against PV1/Mahoney or BDNF. Almost all the cells infected with Mah/BDNF expressed both antigens of PV1/Mahoney and BDNF at 7 h postinfection (Figure 6A–C), suggesting that no significant deletions occurred on the foreign sequence of the recombinant virus genome after three passages of the virus. No expression of BDNF was detected in cells infected with PV1/Mahoney (Figure 6D–F) or mock-infected cells (Figure 6G–I). To further examine the expression of BDNF by Mah/BDNF in cultured cells, Western blot analysis was performed on AGMK cells infected with PV1/Mahoney or Mah/BDNF. Cytoplasmic extracts were prepared at 7 h postinfection and analyzed by immunoblotting as described in Materials and methods. Antibodies to BDNF detected a band with a molecular mass of about 15.2 kDa only in the extracts from Mah/BDNF-infected cells, and not

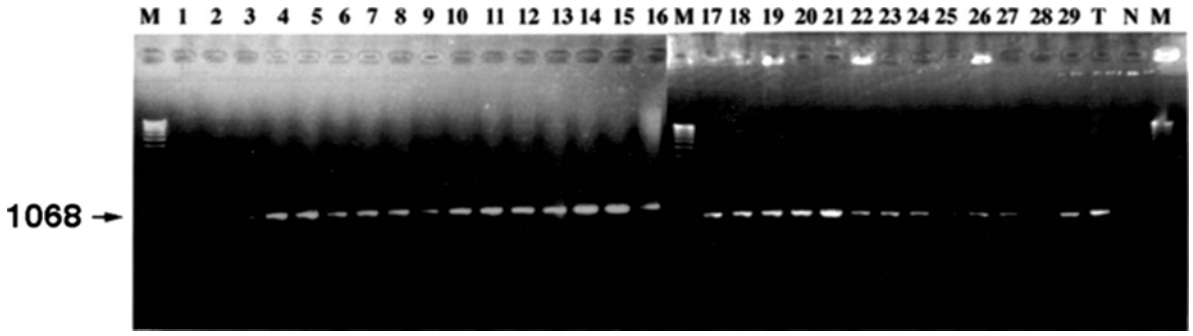

Figure 4 Analysis of the genetic stability of Mah/BDNF in cultured cell. AGMK cells grown in 24-well plates were infected with individual Mah/BDNF isolates obtained after three successive passages in AGMK cells. Total RNAs prepared from the infected cells were subjected to RT-PCR with oligonucleotides 04 and 07 as primers to amplify the genome region that contained the inserted sequence encoding the mature peptide of BDNF. M, DNA molecular weight marker 1KB. T, RNA *in vitro* transcribed from pMah/BDNF. N, RNAs from mock-infected cells as a negative control. Products were analyzed by electrophoresis on a 1% agarose gel. Position of expected RT-PCR product is indicated by an arrow with the length in nucleotides given.

in those from PV1/Mahoney- or mock-infected cells (Figure 7). The 15.2-kDa polypeptide corresponds to the expected molecular mass of BDNF. In addition to the 15.2-kDa band, a 78-kDa band was also detected only in the extracts from Mah/BDNF-infected cells, although the intensity of the 78-kDa band was approximately 10% that of the 15.2-kDa band (data not shown). The 78-kDa band may correspond to unprocessed polyprotein that consists of BDNF (15 kDa), VP0 (37 kDa), and VP3 (26 kDa).

Expression of exogenous BDNF in the central nervous system of transgenic mice Poliovirus is a neurotropic virus, which replicates mainly in the motor neurons of the CNS. Based on this characteristic, we tested whether Mah/BDNF delivers BDNF to the motor neurons of the CNS. Twenty PVR-Tg mice were infected with PV1/Mahoney or Mah/BDNF via an intracerebral inoculation route, and were then observed for up to 4 weeks. All the mice inoculated with 10^6 plaque-forming units (PFU) of PV1/Mahoney died within 7 days, but no mice inoculated with the same amount of Mah/BDNF prepared by three passages developed any paralysis during the observation period. Both exogenous BDNF antigens and PV1/Mahoney antigens were detected in a portion of the neurons from the brains, but no Mah/BDNF-mediated expression of the BDNF was found in the spinal cords (data not shown), of the mice intracerebrally inoculated with Mah/BDNF.

To explore an indirect way of delivering BDNF to the CNS, we injected 10^5 PFU of Mah/BDNF into the quadriceps muscle of PVR-Tg mice, and examined the expression of BDNF and PV1/Mahoney antigens in the lumber spinal cords and brain stems of the mice. Strong expression of the poliovirus antigens was observed in the ventral horn of the lumber cords and some in the dorsal horn from the mice 3 days after the intramuscular injection with Mah/BDNF (Figure 8C). Poliovirus antigens were also detected in the brain stem structures, for example, medial reticular formation, inferior vestibular nucleus, and spinal trigeminal nucleus (Figure 8A). Expression of exogenous BDNF was also detected in motor neurons of the lumber cords (Figure 8D) and in brain-stem neurons (Figure 8B) of these mice. However, both the antigens disappeared in the spinal cords 7 days after the intramuscular inoculation with Mah/BDNF (data not shown). Endogenous BDNF in the spinal cord was detected only in the superficial laminae of the dorsal horn (data not shown). Its presence in the ventral

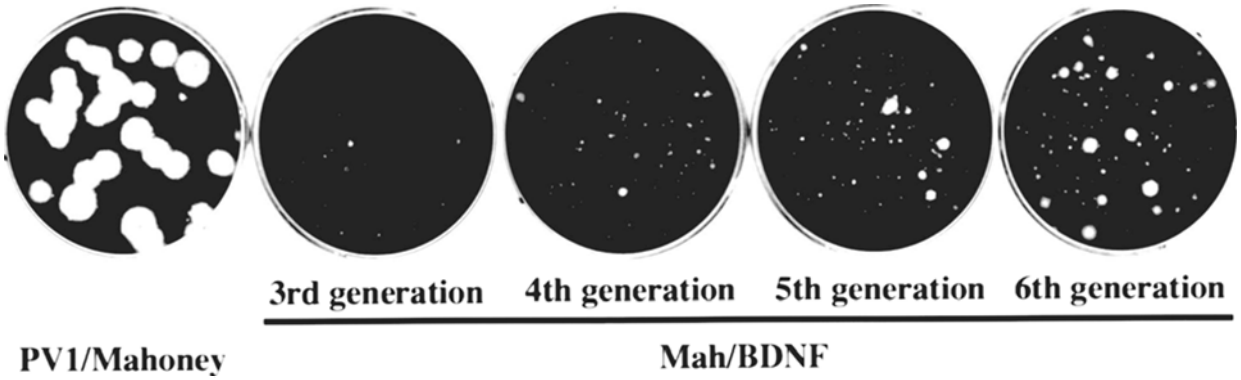

Figure 5 Alteration of plaque phenotypes during passages of Mah/BDNF. PV1/Mahoney, Mah/BDNF preparations of 3rd, 4th, 5th, and 6th generations were used for plaque formation. Other conditions were the same as indicated in the legend to Figure 2.

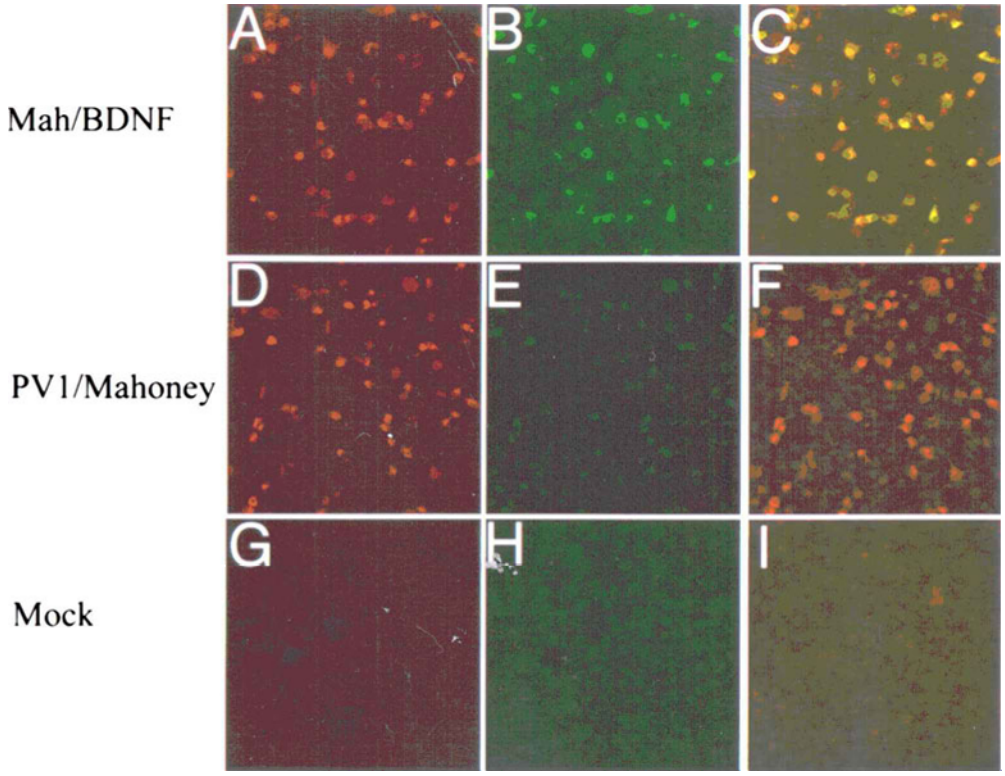

Figure 6 BDNF expression in cultured cells infected with Mah/BDNF. Monolayers of AGMK cells were infected at an m.o.i. of 10 with Mah/BDNF (**A–C**), PV1/Mahoney (**D–F**), or mock-infected with PBS (**G–I**). Cells were fixed and stained as described in Materials and methods. The preparations were visualized with a confocal laser-scanning microscope. PV1/Mahoney antigens (**A**, **D**, and **G**) and BDNF antigens (**B**, **E**, and **H**) are merged in pictures (**C**, **F**, and **I**, respectively).

horn neurons was too sparse to detect by the method used. Motoneuronal cell bodies in the normal and PV1/Mahoney-infected Tg mice were mostly negative in their immunostaining for BDNF.

Replication efficiency of Mah/BDNF in the CNS of PVR-Tg mice We observed that no mouse intracerebrally infected with 10^6 PFU of Mah/BDNF showed any paralysis even 4 weeks after the infection, whereas the LD_{50} of PV1/Mahoney was less than 10^3 PFU. Because efficient replication of poliovirus in the brain of mice appears to be necessary for the virus to cause paralysis (Jia *et al*, 1999), it is possible that Mah/BDNF has an inefficient replicating capacity in the brain as well as in cultured cells (Figures 2 and 3). To test the possibility, we measured virus titers in the brains of PVR-Tg mice infected with 10^4 PFU of PV1/Mahoney or Mah/BDNF (Figure 9). The titer of PV1/Mahoney increased up to 10^8 PFU per brain until 72 h postinfection. However, the titers of Mah/BDNF in the brains increased only to 10^5 PFU per brain at 24 h postinfection, and this level was maintained until 72 h postinfection. This poor replication capacity of Mah/BDNF in the brains of PVR-Tg mice may be a determinant for the decreased neurovirulence of the virus, and may also explain why BDNF antigens were not detected in the spinal cords of mice intracerebrally injected with Mah/BDNF.

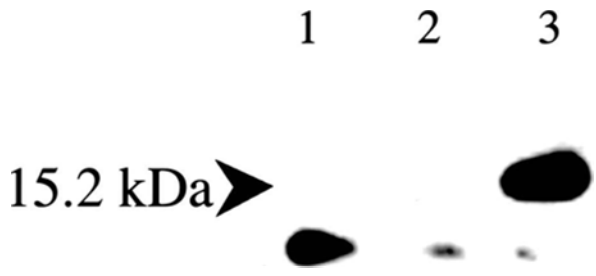

Figure 7 Western blot analysis of BDNF. Cytoplasmic extracts from AGMK cells infected with PV1/Mahoney (lane 2), Mah/BDNF (lane 3), or mock-infected with PBS (lane 1) were analyzed by Western blotting using antibodies against BDNF. Specific bands detected by antibodies against BDNF are indicated by the arrowheads. Four μg of total protein was loaded on each lane.

Discussion

Previous studies have shown that poliovirus in the CNS primarily localizes to motor neurons in the ventral horn of the spinal cord (Bodian, 1949; Ren *et al*, 1990; Koike *et al*, 1991), and that intramuscularly inoculated polioviruses are transported through the axon and cause paralysis in PVR-Tg mice (Ren and Racaniello, 1992; Gromeier and Wimmer, 1998; Ohka *et al*, 1998). Therefore, poliovirus is a promising candidate vector for the gene therapy of motor

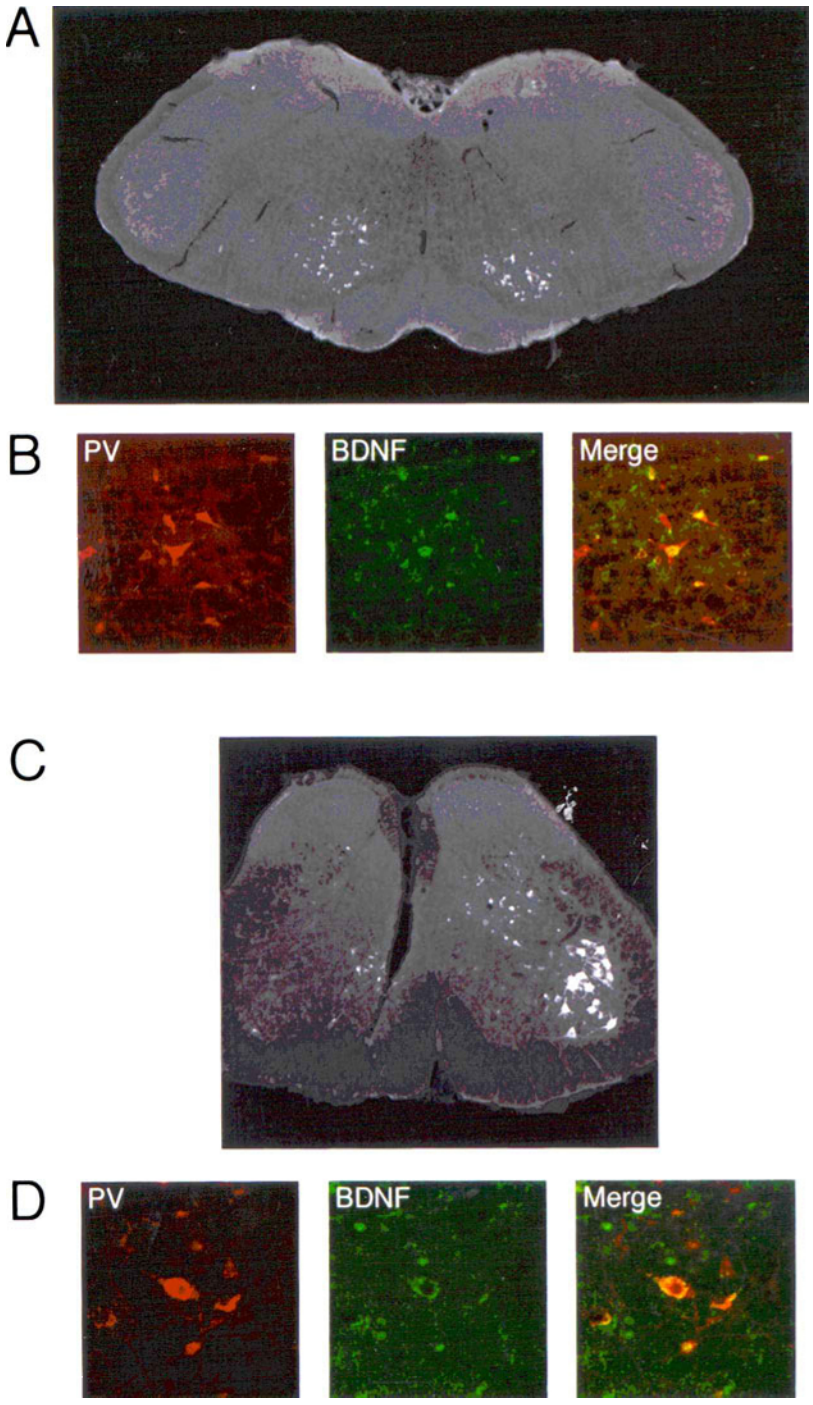

Figure 8 Expression of exogenous BDNF by Mah/BDNF in the CNS of PVR-Tg mice. Three days after the intramuscular inoculation of Mah/BDNF in PVR-Tg mice, poliovirus immunoreactive neurons were detected in the brain stem (**A**) and spinal cord (**C**). Many of poliovirus antigen-positive neurons also expressed BDNF immunoreactivity in their cell bodies, for example, in the brain stem reticular formation (**B**) and spinal lumber cord (**D**).

neuron-related diseases or injuries. BDNF has been reported to prevent motor neuron death in cultures of embryonic motor neurons (Henderson *et al*, 1993; Becker *et al*, 1998). In the case of nerve injury, exogenous BDNF attenuates degeneration and biochemical changes in the affected neurons (Chiu *et al*, 1994; Yan *et al*, 1994). In this report, as a first step toward developing a neurotropic virus vector, we chose poliovirus to express BDNF in the CNS of PVR-Tg mice. PV1/Mahoney, rather than the vaccine virus Sabin 1 strain, was chosen to be the vector to express BDNF because the available strain of PVR-Tg mice does not support the replication of Sabin 1 strain well.

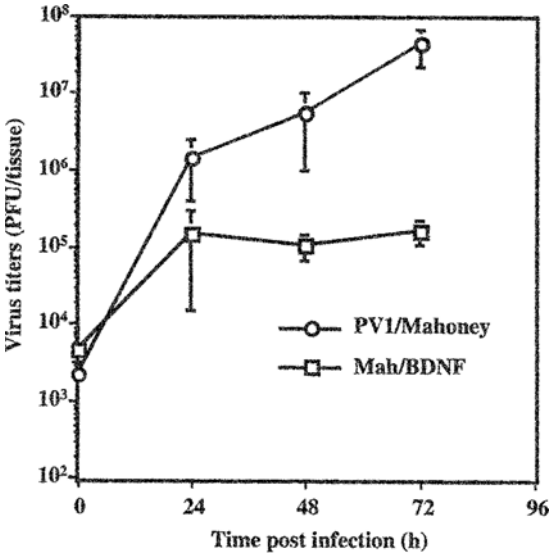

Figure 9 Viral replication in the brains of PVR-Tg mice. PVR-Tg mice were intracerebrally inoculated with 10^4 PFU of PV1/Mahoney (open circle) or Mah/BDNF (open square). Mice were anesthetized and dissected at the indicated times. Virus titers were measured by plaque assays. The values are the means of virus titers obtained from triplicate independent infection experiments. The extent of scattering values is indicated by the vertical lines.

Viral replications of recombinant polioviruses with long foreign nucleotide sequences are severely impaired, and the inserts of more than 700-nt long are usually not stably maintained during passages (Mueller and Wimmer, 1998). However, recombinant polioviruses carrying inserts less than 400-nt long are generally much more stable (Mattion *et al*, 1994, 1995; Tang *et al*, 1997; Yim *et al*, 1996). In this study, BDNF sequence of 423-nt long in the Mah/BDNF genome appeared to be stably maintained until three passages of the virus (Figure 4). Three time-cell passages is enough to prepare virus stocks in practice. However, variants showing larger plaque phenotypes generated during further passages (Figure 5). This may result in difficulties in practical use of poliovirus-based vectors.

When Mah/BDNF was used to infect PVR-Tg mice via an intracerebral inoculation route, none of the mice showed any paralysis. The replication efficiency of Mah/BDNF in the brain was greatly reduced as compared with that of PV1/Mahoney (Figure 9). Furthermore, in PVR-Tg mice intramuscularly inoculated with Mah/BDNF, antigens of BDNF were detected in the motor neurons at 3 days postinfection (Figure 8) but not at 7 days postinfection (data not shown). The poliovirus antigens also disappeared at 7 days postinfection. These results suggest that the foreign sequence encoding BDNF unexpectedly reduced the replication efficiency and therefore lowered the neurovirulence of PV1/Mahoney, although 1 of 12 or 2 of 6 mice developed paralysis after the intramuscular inoculation with 1 of 12; 2×10^4 or 2 of 6; 4×10^5 PFU of Mah/BDNF, respectively (data not shown). Thus, it is clearly necessary to reduce neurovirulence of Mah/BDNF for practical use. By contrast, the observation of short-lived Mah/BDNF in the CNS may be indicative of a relatively safe character of the recombinant. The expression of exogenous BDNF itself may have an ability to reduce poliovirus replication in the CNS, although BDNF showed no effect on poliovirus replication in AGMK cells (data not shown).

Infectious poliovirus replicons, in which the nucleotide sequence encoding the capsid protein (P1) of poliovirus was replaced by the foreign sequence, have been reported to express foreign proteins in the motor neurons of the spinal cords from mice transgenic for the human PVR (Bledsoe *et al*, 2000a, 2000b). The poliovirus replicons do not generate infectious virus particles in the infected cells because no capsid protein is produced, resulting in expression of the foreign gene only in the first target cells. This transient expression system may be safer than that employing replication-competent poliovirus vectors. On the other hand, expression of foreign genes by the former system must be less efficient than the replication-competent one. In any event, both strategies for the expression of foreign genes take advantage of the natural tropism of poliovirus which targets neurons in the CNS, and therefore holds promise for continued development of poliovirus-based vectors for neural gene delivery.

Materials and methods

Cells, viruses, and mice Monolayers of AGMK cells were grown in Dulbecco modified Eagle's medium supplemented with 5% newborn calf serum. They were used for transfections, virus preparations, and plaque assays.

PV1/Mahoney and the recombinant virus Mah/BDNF were recovered from AGMK cells transfected with the corresponding infectious RNA transcripts. The infection of AGMK cells with the recovered virus was regarded as the first passage. Mah/BDNF was amplified for three more times by passaging in AGMK cells at an m.o.i. of 10. The virus titers were determined by plaque assays in AGMK cells. The third generation of Mah/BDNF was used for most experiments.

The transgenic mouse line expressing PVR (CD155) (PVR-Tg mice), ICR-PVRTg21 (Koike *et al*, 1991), at the age of 6 to 10 weeks, was used as an animal model for the infection experiments. All mice used were maintained under specific-pathogen-free conditions.

DNA manipulation All cloning enzymes and reaction buffers were purchased from New England Biolabs or Takara Ltd. Co. (Japan), and used as recommended by the manufacturers.

A poliovirus 5′-proximal portion-based vector was constructed by inserting a synthetic linker which contained the unique *Not*I and *Sac*I sites, coding

sequences of a four-glycine tract and a cleavage recognition site for poliovirus proteinase $3C^{pro}$, at nucleotide position (nt) 746 of pOM, an infectious cDNA clone of PV1/Mahoney (Shiroki *et al*, 1995). This was done by means of PCR using primers 01–02 and 03–04 to amplify the portions of pOM corresponding to nt 1 to 746 and 746 to 1201. The primers 02 and 03 introduced the synthetic linker into the nt 746 of pOM. The two PCR fragments were used as templates for further nucleotide amplification with primers 01 and 04. The 1212-bp *Eco*RI and *Nru*I (nt 1172) fragment was then inserted into the *Eco*RI-*Nru*I sites of the plasmid pBR322 to yield pBR-PV5′(N^+S^+).

A 423-bp nucleotide that included the sequence encoding the mature peptide of rat BDNF (nt 439–819) (Maisonpierre *et al*, 1991) flanked by the *Not*I and *Sac*I restriction sites was amplified from the plasmid pBS-BDNF (kindly provided by EG Jones from University of California at Davis) by PCR using primers 05–06 and then inserted into the *Not*I-*Sac*I sites of pBR-PV5′(N^+S^+) to yield pBR-PV5′BDNF. The AatII fragment of pOM was replaced by the corresponding fragment of pBR-PV5′BDNF to yield pMah/BDNF. The nucleotide sequences of the PCR products and the inserted foreign sequences in the recombinant infectious cDNAs were determined to confirm that no additional mutations occurred and that the inserted sequences were not deleted.

RNA transfection RNA transcripts were synthesized from P*vu*I-linearized infectious cDNAs. AGMK cells in 60-mm-diameter dishes were transfected with 1 to 5 μg of RNA using a DEAE-dextran method (Jia *et al*, 1999). The viruses recovered from the cells transfected with pMah/BDNF were designated Mah/BDNF.

One step growth curve Monolayers of AGMK cells grown in 60-mm-diameter dishes were infected with viruses at an m.o.i. of 10, and incubated at 37°C. Infected cells were collected at the indicated times and lysed by three rounds of freezing and thawing. The cell debris was spun down with a low-speed centrifugation and virus titers (PFU/ml) determined by plaque assays.

RT-PCR AGMK cells were infected at an m.o.i. of 10 with Mah/BDNF obtained after three successive passages in AGMK cells. Total RNAs from the infected cells were prepared by ISOGEN (Nippongene, Fukuyama, Japan)/chloroform extraction 7 h postinfection and precipitated with ethanol according to the instructions of the manufacturer. Reverse transcription (RT) was performed with Superscript™ II RT (Gibco) for 60 min at 37°C using primer 04. After the reaction was completed, the enzyme was inactivated by a 2-min incubation at 95°C. An aliquot (2 μl) of the product was used as a template for the PCR. PCR was performed by using Ex Taq polymerase (Takara, Japan) with primers 07 and 04 to amplify the region of the poliovirus genome that contained the inserted sequence encoding the mature peptide of BDNF.

Immunofluorescence and Western blot analysis Immunofluorescence and Western blot analysis were performed to detect the expression of BDNF in cultured cells infected with Mah/BDNF. For the immunofluorescence assay, AGMK cells grown on 8-well culture slides were infected with PV1/Mahoney or the recombinant Mah/BDNF at an m.o.i. of 10, and then incubated for 7 h at 37°C. The infected cells were washed once with phosphate-buffered saline (PBS) (10 mM phosphate buffer, pH 7.0, 137 mM NaCl, and 2.6 mM KCl), and fixed with 2% paraformaldehyde for 10 min at room temperature. The fixed cells were double stained with a mouse monoclonal antibody against PV1/Mahoney and rabbit polyclonal antibodies against BDNF (Santa Cruz, Biotechnology). They were then subsequently stained with Texas Red-conjugated goat anti-mouse IgG and FITC-conjugated goat anti-rabbit IgG. The preparations were visualized with a confocal laser-scanning microscope (BioRad).

For Western blot analysis, AGMK cells grown in 60-mm-diameter dishes (2×10^6 cells/dish) were infected with PV1/Mahoney or Mah/BDNF at an m.o.i. of 10 and then incubated for 7 h at 37°C. The infected cells were harvested, and lysed in chilled buffer H (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM MgCl, 1 mM DTT, 1% Triton X-100, and 0.1 mM phenymethylsulfonyl fluoride) (Andino *et al*, 1993). The nuclei were removed by a low speed centrifugation. Approximately 4 μg of proteins from the total lysates were subjected to electrophoresis on a 12% polyacrylamide gel containing 0.1% SDS and then analyzed by immunoblotting. The blots were incubated with rabbit polyclonal antibody against BDNF (Santa Cruz, Biotechnology). Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins and enhanced-chemiluminescence detection kits were purchased from Amersham. The immunoblotting was performed according to the recommendations of the manufacturer.

Infection of PVR-Tg mice with Mah/BDNF and preparation of tissues for immunohistochemistry PVR-Tg mice were anesthetized by intraperitoneal injection with 0.3 to 0.4 ml of ketamine (10 mg/ml) and xylazine (0.2 mg/ml) in PBS. The anesthetized mice were injected with virus suspensions using an intracerebral (10^6 PFU/30 μl/mouse) or intramuscular (10^5 PFU/100 μl/mouse) inoculation route. For the intramuscular inoculation, the virus suspension was injected into quadriceps. The mice were observed daily for up to 4 weeks. At 2, 3, 5, and 7 days postinfection, the mice were anesthetized, perfused with cold 2.5% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.4), and dissected. The tissues were postfixed in the same fixative, and then equilibrated in 0.1 M PB with 30% sucrose. Brains and spinal cords were sectioned at 30-μm thickness using a cryostat and floated in cold 0.1 M PB.

Immunohistochemistry All the reactions were carried out at room temperature, unless otherwise specified. Nonspecific reactions were blocked with

10% normal goat serum. Sections were incubated first with primary rabbit antiserum against PV1/Mahoney overnight at 4°C. After washing in PB, a mixture of Alexa 594-conjugated anti-rabbit IgG (produced in goat) and anti-human BDNF (produced in chicken) antibodies were applied to the sections overnight at 4°C, followed by incubation in 10% normal rabbit serum for 1 h to block excess amount of anti-rabbit IgG. To localize BDNF immunoreactivity, biotinylated anti-chicken IgY (produced in rabbit), avidin-conjugated peroxidase and thyramid solution were used according to the manufacturer's instruction (TSA method, NEN). Between the incubation steps, the sections were extensively washed. Histological examination was carried out with a laser scanning microscope (LSM510, Zeiss).

Virus recovery from the brains of PVR-Tg mice
Mice were intracerebrally infected with 10^4 PFU of viruses and anesthetized as described previuosly. The brains were removed from the mice at the indicated times and homogenized in PBS to prepare 10% tissue emulsions. The emulsions were centrifuged at low speed to remove tissue debris, and the supernatant containing viruses subjected to plaque assay.

Oligonucleotide primers

01 5′CTGAGAATTCGTAATACGACTCACTATAGG TTAAAACAGCTCTGGGG 3′
02 5′TCCTCCTCCTCCGAGCTCTAGGCGGCCGCG CATTATGATACAATTGTC 3′
03 5′CTAGAGCTCGGAGGAGGAGGAGAGGCTTT GTTTCAAGGTGCTCAGGTTTCA 3′
04 5′ATCAGGCAACTTCCACCACC 3′
05 5′AATAGCGGCCGCAACATGTCTATGAGGGTT CG 3′
06 5′CAGGAGCTCTCTTCCCCTTTTAATGG 3′
07 5′TGGCTGCTTATGGTGACAATC 3′

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