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Successful Transduction of Mammalian Astrocytes and Oligodendrocytes by "Pseudotyped" Baculovirus Vector *in Vitro* and *in Vivo*

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ABSTRACT

Baculovirus vectors can efficiently transduce human hepatoma cells and primary hepatocytes in culture. We report the potential use of baculovirus as a vector for gene delivery into cells of the mammalian central nervous system. We generated a "pseudotyped" baculovirus encoding the bacterial β-galactosidase (β-Gal) gene (LacZ), under the control of the cytomegalovirus promoter, and the vesicular stomatitis virus G protein gene, under the control of the polyhedrin promoter. This virus was used to infect primary cultures of rat glial cells. Three days after infection, these cells were immunostained for β-Gal, glial fibrillary acidic protein (for astrocytes), or galactocerebroside (for oligodendrocytes) to identify the infected cell types. Positive β-Gal immunofluorescence was observed in 10.4% of glial fibrillary acidic protein-positive cells and 35.6% of galactocerebroside-positive cells at a multiplicity of infection of 50. When the virus was injected into adult mouse striatum, β-Gal-positive cells were demonstrated, and no cytological or histological evidence of cell damage, inflammation, or cell infiltration was observed after infection. These findings suggest that baculovirus-mediated gene transfer can be used for gene therapy against nervous system diseases, especially demyelinating disorders, affecting mainly oligodendrocytes. (Jikeikai Med J 2006 ; 53 : 55-62)

Key words : baculovirus, gene therapy, pseudotype, astrocytes, oligodendrocytes

INTRODUCTION

The baculovirus vector has been widely used to obtain high levels of expression of foreign genes under the control of the strong baculoviral promoter (polyhedrin promoter) in insect cells[1]. Although its host specificity had been thought to be restricted to insect cells, the recombinant baculovirus was recently shown to be capable of transferring and expressing foreign genes in mammalian cells, such as hepatocytes[2,3] and nonhepatic cell lines[4]. For the original baculovirus vector the foreign gene is cloned next to the viral polyhedrin promoter, whereas for the recombinant baculovirus the gene is fused to a mammalian pro-

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moter, such as cytomegalovirus immediate early promoter and inserted. Efficient gene transfer has been reported, and no viral DNA replication has been observed in mammalian cells. These results suggest that this virus system would be safe for humans.

Sarkis et al. have reported that the baculovirus vector can be used to transfer a marker gene to neurons and astrocytes *in vitro* and *in vivo*[5]. They have reported efficient gene transfer into astrocytes and neurons, but not into oligodendrocytes. Oligodendrocytes are the cells mainly affected in several neurological diseases, such as demyelinating disorders; therefore, for gene therapy in these diseases, investigating ways to transfer genes into oligodendrocytes is important. Barsoum et al. have reported that pseudotype baculovirus, expressing vesiculostomatitis virus G (VSVG) protein in its envelope, transduces mammalian cells much more efficiently than does nonpseudotype baculovirus[6]. In this study, we examined the ability of a pseudotype baculovirus vector to transfer a marker gene in central nervous system (CNS) cells, including oligodendrocytes.

MATERIAL AND METHODS

1. *Construction of pseudotype transfer plasmids*

The pseudotype baculovirus transfer vector, pCZPG (Fig. 1), was generated by inserting expression cassettes encoding the VSVG protein and bacterial β-galactosidase (β-Gal) gene (LacZ) into the standard baculovirus transfer vector BacPak9 (Clontech Laboratories, Inc., Palo Alto, CA, USA)[6]. First, the VSVG gene complementary DNA was excised from the plasmid pLGRNL[7]. This fragment was inserted into the *Bam*HI site of BacPak9, in a direct orientation with respect to the polyhedrin promoter, to create VSVG/BP9. Next, the LacZ gene preceded by the cytomegalovirus promoter and followed by the SV40 poly(A) signal was inserted into VSVG/BP9 such that the LacZ cassette was downstream from the VSVG gene and the direction of transcription was convergent[6].

2. *Pseudotype baculovirus production*

Cells of the insect cell line Sf9 were grown in TC-100 medium (GIBCO/Invitrogen, Carlsbad, CA, USA) with 0.26% Bacto tryptose phosphate broth (Difco, Detroit, MI, USA), 100 μg/ml kanamycin, and 10% fetal bovine serum (GIBCO/Invitrogen). Recombinant pseudotype baculovirus, CZPG, was generated by homologous recombination, as described previously[4,9]. Briefly, pCZPG and the *Bsu* 36I-digested baculovirus genomic DNA were cotransfected into Sf9 cells by lipofectin (GIBCO/Invitrogen). Two days later, the culture medium was harvested and used to infect Sf9 cells in a standard plaque assay. The plaques were isolated and purified by a second round of plaque isolation. After the presence of the predicted recombinant DNA restriction digestion pattern was determined, selected plaques were expanded twice in tissue culture flasks and then on a large scale in 100-ml spinner cultures to obtain a significant volume of virus preparation. The viral titer was determined with a plaque assay[10]. To purify the virus, conditioned media of Sf9 cells infected with the virus were harvested 3 days after infection, and cell debris was removed by centrifugation at 6,000 g for 15

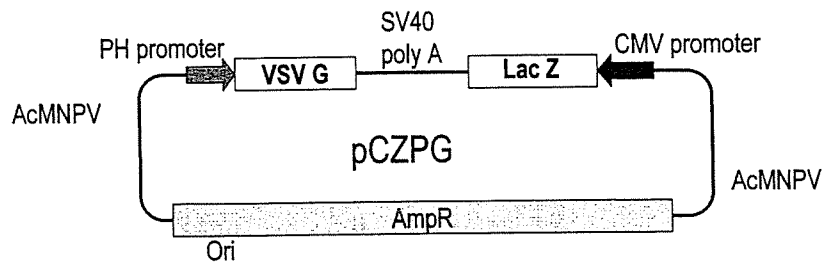

Fig. 1. Structure of pseudotype baculovirus transfer plasmid (pCZPG). AcNMPV, Baculovirus sequences; PH, polyhedrin promoter; AmpR, ampicillin-registance gene; Ori, replication origin; VSVG, Vesicular stomataitis virus G protein gene.

minutes at 4°C. The virus was pelleted by ultracentrifugation at 80,000 g (RPS27-2 rotor, Hitachi, Tokyo) for 90 minutes and resuspended in 1 ml of phosphate buffered saline (PBS), loaded on 10% to 60% (w/v) sucrose gradients, and ultracentrifuged at 77,600 g (P-40ST rotor, Hitachi) for 90 minutes. The virus band was collected and resuspended in PBS and ultracentrifuged at 77,600 for 90 minutes. The virus pellet was resuspended in PBS, and infectious titers were determined with a plaque assay[4].

3. Mixed glial cell culture

Cultures of rat astrocytes and oligodendrocytes were established with the enzyme digestion-Percoll (Pharmacia, Uppsala, Sweden) density gradient method[11-13]. The brains of 10-week-old male Wistar rats were minced and incubated in 0.25% trypsin and 20 μg/ml DNase in calcium- and magnesium-free Hanks balanced salt solution (HBSS) for 40 minutes at 37°C. Dissociated cells were passed through a 100-μm nylon mesh. Isolated cells suspended in HBSS were mixed with Percoll, and a gradient was formed by centrifugation for 25 minutes at 15,000 g in a high-speed refrigerated centrifuge with a fixed-angle rotor (Hitachi). The final concentration of Percoll was 30% in HBSS. An astrocyte- and oligodendrocyte-enriched fraction, bound by an upper myelin layer and a lower erythrocyte layer, was collected and diluted in three volumes of HBSS, before being harvesting by low-speed centrifugation for 10 minutes. The cells were washed twice in HBSS, suspended in feeding medium (1×10^5 cells/ml), and seeded on polysine-coated 9-mm-diameter round coverslips (Aclar, Honeywell/AlliedSignal, Pottsville, PA, USA). The feeding medium consisted of 5% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin in Isocove's modified Dulbecco's minimum essential medium. The cultures were maintained in 5% CO_2 at 37°C for 2 weeks, with the culture medium being changed every 4 days.

4. Infection of glial cell cultures with CZPG

Living cells of mixed rat glial cultures on coverslips after 2 weeks in vitro were infected with a 10-μl solution of purified CZPG at a multiplicity of infection of 50 for 1 hour and were incubated in feeding medium for 3 days in 5% CO_2 at 37°C. To detect β-Gal expression in infected cells, immunofluorescence was performed with a rabbit antibody to β-Gal. Cell types were identified with immunofluorescence using antibodies to glial fibrillary acidic protein (GFAP) and galactocerebroside (GC), specific markers for astrocytes and oligodendrocytes, respectively. For double immunofluorescence staining for β-Gal and GFAP, cells on coverslips were fixed in 1% paraformaldehyde in PBS for 10 minutes at 4°C and in methanol for 10 minutes at −20°C and incubated at room temperature for 1 hour with a mixture of a rabbit antibody to β-Gal (Eppendorf-5 Prime, Boulder, CO, USA) and rat monoclonal antibody (hybridoma soup) to GFAP[14] (kindly provided by Dr. Seung U. Kim, University of British Columbia) at final dilutions of 1 : 50 and 1 : 2, respectively. This incubation was followed by incubation at room temperature for 1 hour with a mixture of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Cappel, MP Biomedicals, Aurora, OH, USA) and rhodamine-conjugated goat anti-rat IgG (Cappel) at a final dilution of 1 : 50. For double immunofluorescence staining for β-Gal and GC, living cells on coverslips were incubated with mouse monoclonal antibody (hybridoma soup) to GC[15] (kindly provided by Dr. Seung U. Kim) at a dilution of 1 : 2 for 30 minutes at room temperature. After being washed with Isocove's modified Dulbecco's minimum essential medium, the cells were incubated with rhodamine-conjugated goat anti-mouse IgG (Cappel) at a final dilution of 1 : 50 for 30 minutes at room temperature. The cells were fixed with 1% paraformaldehyde in PBS at 4°C for 10 minutes and cold methanol at −20°C for 10 minutes. After being washed in PBS, the cells were incubated with rabbit antibody to β-Gal at a dilution of 1 : 50 for 1 hour at room temperature. After being washed, the cells were incubated with FITC-conjugated goat anti-rabbit IgG at a final dilution of 1 : 50 for 45 minutes at room temperature. After being washed, coverslips were mounted on glass slides with 20% glycerol/10% polyvinylalcohol in 0.1 M Tris-HCl buffer, pH 8.0. Cells were then examined under a universal microscope (Olympus Opti-

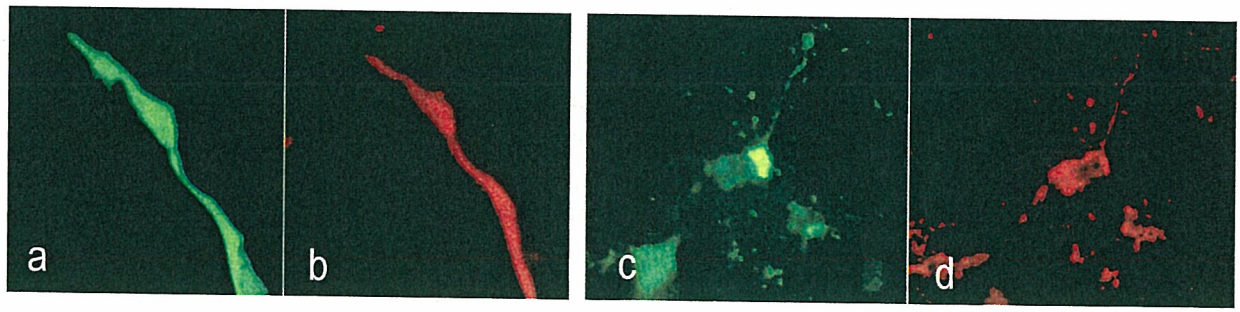

Fig. 2. β-Gal expression in rat primary culture astrocytes and oligodendrocytes *in vitro*. a, b: Astrocytes infected with CZPG *in vitro* for 3 days. β-Gal-FITC (a) and GFAP- rhodamine (b) double immunofluorescence. c, d: Oligodendrocytes infected with CZPG *in vitro* for 3 days. β-Gal-FITC (c) and GC-rhodamine (d) double immunofluorescence.

cal Co., Tokyo) equipped with fluorescein and rhodamine optics.

5. Infection of mouse brains with CZPG

Intracerebral injection of purified CZPG was carried out as follows: 6- to 8-week-old normal female C57BL/6 mice ($n=12$) were anesthetized with intraperitoneal injection of 40 mg/kg of pentobarbital sodium. Five microliters of purified CZPG (1×10^9 pfu/ml) was injected slowly into the left striatum (AP: to 1 mm; ML: 2 mm; DV: 3.6 mm to the bregma) or the ventricle (AP: to 1 mm; ML: 2 mm; DV: 2.6 mm to the bregma) through a syringe (Hamilton Co., Reno, NV, USA). Three days after the operation, the mice were deeply anesthetized through inhalation of diethyl ether and intracardially perfused with 4% paraformaldehyde in PBS. The brains were removed, postfixed in the same fixative for 24 hours, and cryoprotected in 30% sucrose in PBS, after which 20-μm-thick coronal sections were cut with a cryostat. Gene transfer into the CNS was identified with β-Gal staining. These sections were washed four times in PBS and stained in a solution of 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, and 2 mM $MgCl_2$ in PBS[16]. Cell types were identified with immunofluorescence using antibodies to GFAP and 3′,5′-cyclic nucleotide phosphodiesterase (CNP) for astrocytes and oligodendrocytes, respectively. The sections were washed in PBS and incubated in 0.1% Triton X-100 in PBS (PBST) for 30 minutes and in 3% normal goat serum in PBST for 1 hour at room temperature. For double immunofluorescence staining for β-Gal and GFAP, the sections were incubated at 4°C overnight with a mixture of rabbit antibody to β-Gal and rat monoclonal antibody to GFAP at final dilutions of 1 : 50 and 1 : 2, respectively. This incubation was followed by incubation at room temperature for 1 hour with a mixture of FITC-conjugated goat anti-rabbit IgG and rhodamine-conjugated goat anti-rat IgG at a final dilution of 1 : 50. For double immunofluorescence staining for β-Gal and CNP, the sections were incubated at 4°C for 12 hours with a mixture of rabbit antibody to β-Gal and mouse monoclonal antibody to CNP at final dilutions of 1 : 50 and 1 : 100, respectively. This incubation was followed by incubation at room temperature for 1 hour with a mixture of FITC-conjugated anti-rabbit IgG and rhodamine-conjugated anti-mouse IgG at a final dilution of 1 : 50.

RESULTS

1. Transduction of cultured glial cells by CZPG

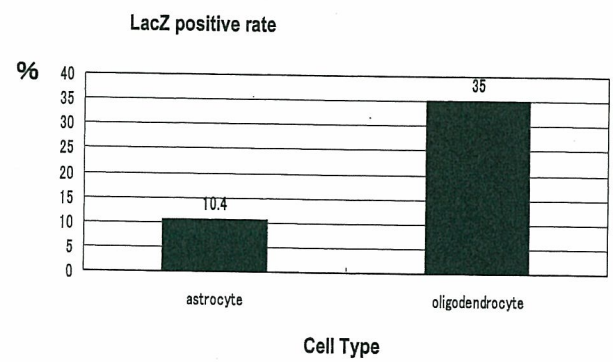

Fig. 3. Efficiency of LacZ gene transfer into rat primary culture *in vitro*.

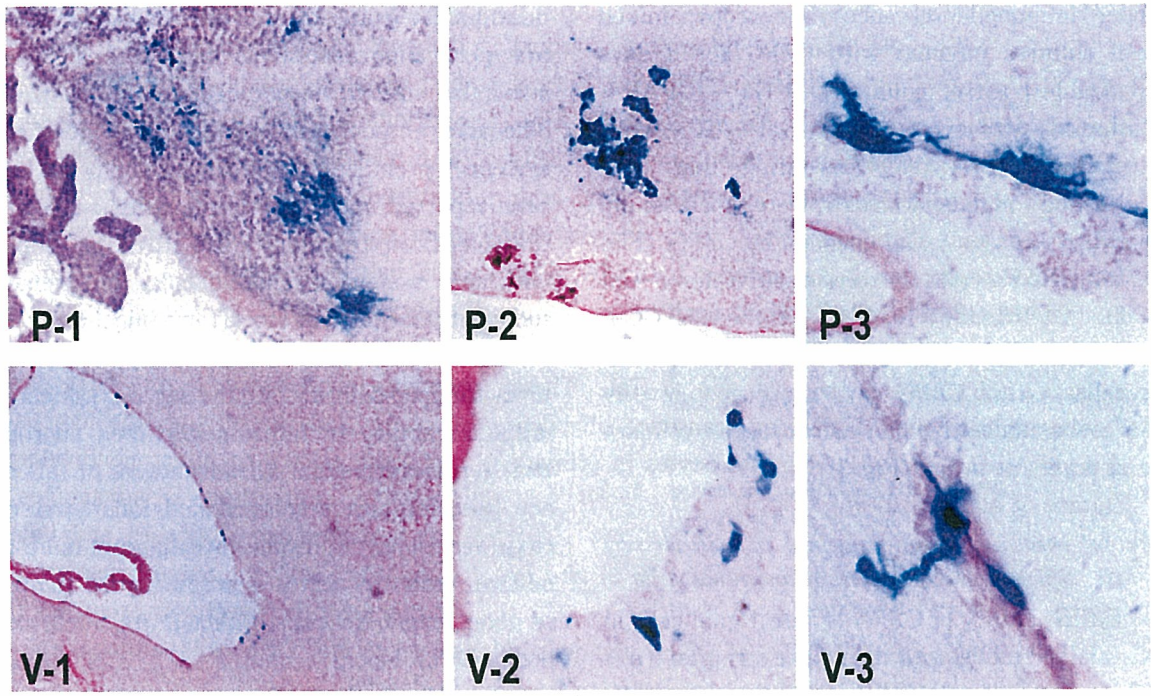

Fig. 4. β-Gal expression in normal mouse brain *in vivo*. 3 days after injection of CZPG into normal mouse (C57BL/6) striatum, gene transfer into CNS *in vivo* was identified by β-Gal staining.
P1-P3 ; the slides of the mouse brain injected in the parenchyma. Lac Z positive cells were seen in near the choroid plexus (P-1), striatum (P-2), and corpus callosum (P-3).
V1-V3 ; the slides of the mouse brain injected in the ventricle. Lac Z positive cells were seen in the ependimal cell (V-1, 2) and partially migrated into the parenchyma (V-3).

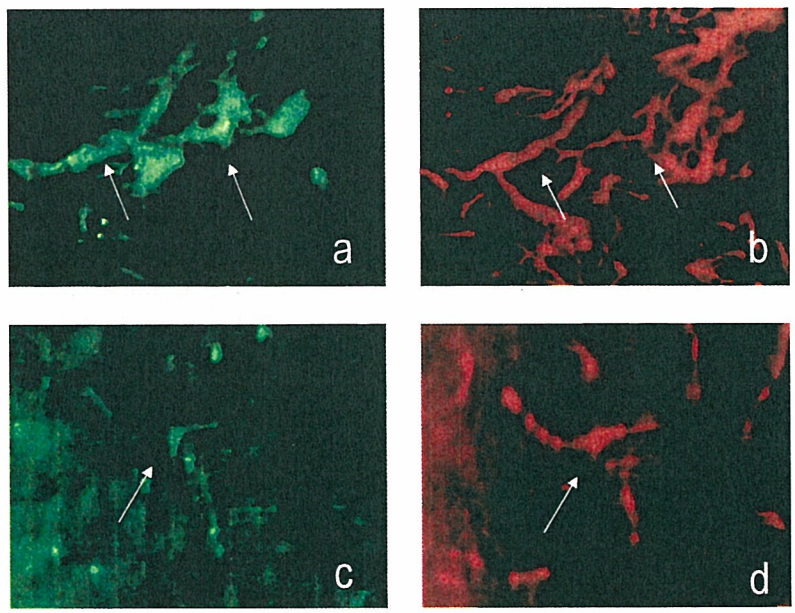

Fig. 5. The slides of the Immunohistochemistry of the mouse brain injected in the parenchyma, double-positive cells, i.e. β-Gal+/GFAP+ (an infected astrocytes ; a, b) and β-Gal+/CNP+ (infected oligodendrocyte : c, d) cells were demonstrated by double immunofluorescence microscopy, indicating both astrocytes and oligodendrocytes can also be transduced *in vivo* by CZPG.

Under immunofluorescence microscopy, mixed glial cell cultures infected with CZPG for 3 days showed double-positive cells, i.e., β-Gal+/GFAP+ cells (infected astrocyte) and β-Gal+/GC+ cells (infected oligodendrocytes), indicating that both astrocytes and oligodendrocytes were successfully transduced to express β-Gal encoded by CZPG (Fig. 2). Of 192 GFAP+cells, 20 were positive for β-Gal (transduction efficiency; 10.4%). Of the 202 GC+ cells, 72 (35%) were positive for β-Gal. These results indicate that CZPG can transduce oligodendrocytes more efficiently than astrocytes in primary culture of adult rat brain (Fig. 3).

2. Efficient transduction of neuronal cells in vivo by CZPG

To test the ability of CZPG to infect glial cells in vivo, we injected CZPG into the mouse striatum ($n=12$). Three days after injection, β-Gal positive cells were demonstrated in the parenchyma (Fig. 4 P1, P2, P3) and periventricular zone (Fig. 4 V1, V2, V3). We detected transduction mainly for parenchymal cells (P1, P2, P3) and ependymal cells (V1, V2), partially migrated into the parenchyma (V3). Next, we tried immunohistochemistry *in vivo*, double-positive cells, i.e., β-Gal+/GFAP+ cells (infected astrocytes) and β-Gal+/CNP+ cells (infected oligodendrocytes) were demonstrated by double immunofluorescence microscopy, indicating that both astrocytes and oligodendrocytes can also be transduced *in vivo* by CZPG (Fig. 5). We observed no cytological damage *in vitro* and no histological evidence of cell damage, inflammation, or cell infiltration after infection with CZPG.

DISCUSSION

Gene therapy for neurological disorders requires an efficient and stable gene delivery system for the CNS. As for gene transfer systems into the CNS cells, adenovirus, adeno-associated virus (AAV), herpesvirus, and lentivirus vectors have been studied[17]. Although adenovirus vector has been reported to be a useful gene transfer system for the nervous system[18-20], its expression is transient because the vector does not integrate into the host genome. Moreover, first-generation adenovirus vectors cause severe tissue inflammation when inoculated in brain tissue[21]. The adeno-associated virus-mediated gene transfer system is promising because no cellular toxicity has been reported and long-term expression by integration has been reported[20,22,23]. However, a problem with this system is the difficulty of preparing a sufficiently large virus stock for clinical use. Herpes simplex virus-based vectors have particular advantages for gene delivery into the nervous system including their ability to infect nondividing neurons and long-term expression. Disadvantages of this system are host immune responses and inflammatory and toxic reactions[24,25]. Some investigators have reported that lentivirus vector is promising for the transfer of genes into CNS cells, although its safety remains unclear[26,27].

To seek an alternative approach to overcome the limitations of current vector systems for transducing CNS cells, we tested a baculovirusmediated gene transfer system in rodent CNS cells in vivo and in vitro. The generation of recombinant baculovirus is relatively less time-consuming, and expansion of the virus stock is relatively easy. The titer of the virus stock after purification is high and comparable to that of adenovirus. Large DNA (up to 15 kb) can be inserted into the transfer vector. Moreover, baculovirus cannot replicate in mammalian cells, and no cell toxicity has been observed[28]. In fact, there was no evidence of tissue inflammation or cell damage in the present study.

The first attempt to transfer genes to mammalian cells with the baculovirus vector was reported by Hofman et al. in 1995. They successfully transferred a gene to mammalian hepatocytes. However, they failed to transfer genes to neural cell lines, such as mouse neuroblastoma Neuro-2a and human astrocytoma SW 1088[2]. Recently, Sarkis et al. have reported successful transduction of nervous system cells (both neurons and astrocytes) *in vitro* and *in vivo* using nonpseudotype baculovirus[5]. In the present study, we used a pseudotype vector. By pseudotyping, the VSVG protein is expressed in the viral envelope and mediates the escape of the recom-

binant pseudotype baculovirus from the intracellular vesicles. The efficiency of the escape may be the rate-limiting step for transduction[6]. We also tried a nonpseudotype baculoviral vector and detected efficient transduction *in vitro*, especially in the liver cell line Hep G2, but detected no transduction *in vivo* (data not shown). The baculovirus vector CZPG prepared in this study could transduce both astrocytes and oligodendrocytes. This ability implies a potential advantage of pseudotype baculovirus vectors for use in CNS gene therapy, because oligodendrocytes are the cells principally affected in many neurological diseases, such as globoid cell leukodystrophy (Krabbe's disease), metachromatic leukodystrophy, and adrenoleukodystrophy. We are now generating a baculovirus vector expressing galactocerebrosidase, which is lacking in Krabbe's disease, to investigate its therapeutic effects in a mouse model of Krabbe's disease. Our failure to find cytological or histological evidence of cell damage, inflammation, or cell infiltration after infection of CZPG *in vitro* and *in vivo* point is also important for gene transfer into CNS and other organ systems *in vivo*.

There are several unanswered questions regarding our baculovirus vector system; i.e., how long the expression persists and how efficiently it infects oligodendrocytes *in vivo*. For the treatment of Krabbe's disease, highly efficient transduction of oligodendrocytes and persistent expression of transferred genes will be required. Additional experiments are underway to answer these questions.

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Research Report

Adenoviral gene transfer of hepatocyte growth factor prevents death of injured adult motoneurons after peripheral nerve avulsion

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ABSTRACT

Hepatocyte growth factor (HGF) exhibits strong neurotrophic activities on motoneurons both in vitro and in vivo. We examined survival-promoting effects of an adenoviral vector encoding human HGF (AxCAhHGF) on injured adult rat motoneurons after peripheral nerve avulsion. The production of HGF in COS1 cells infected with AxCAhHGF and its bioactivity were confirmed by ELISA, Western blot and Madin-Darby canine kidney (MDCK) cell scatter assay. The facial nerve or the seventh cervical segment (C7) ventral and dorsal roots of 3-month-old Fischer 344 male rats were then avulsed and removed from the stylomastoid or vertebral foramen, respectively, and AxCAhHGF, AxCALacZ (adenovirus encoding β-galactosidase gene) or phosphate-buffered saline (PBS) was inoculated in the lesioned foramen. Treatment with AxCAhHGF after avulsion significantly prevented the loss of injured facial and C7 ventral motoneurons as compared to AxCALacZ or PBS treatment and ameliorated choline acetyltransferase immunoreactivity in these neurons. These results indicate that HGF may prevent the degeneration of motoneurons in adult humans with motoneuron injury and motor neuron diseases.

1. Introduction

Hepatocyte growth factor (HGF) was initially identified and purified as a potent mitogen of primary cultured hepatocytes (Nakamura et al., 1984, 1989). HGF is a heterodimeric protein composed of α and β chains and induces proliferation, migration, differentiation of target cells as well as organogenesis and neovascularization (Funakoshi and Nakamura, 2003). In the nervous system, HGF exhibits strong neurotrophic activities for motoneurons both in vitro and in vivo (Caton et al., 2000; Ebens et al., 1996; Funakoshi and Nakamura, 2003; Honda et al., 1995; Koyama et al., 2003, Maina and Klein, 1999; Naeem et al., 2002; Novak et al., 2000; Okura et al., 1999; Sun et al., 2002; Wong et al., 1997; Yamamoto et al., 1997). There have

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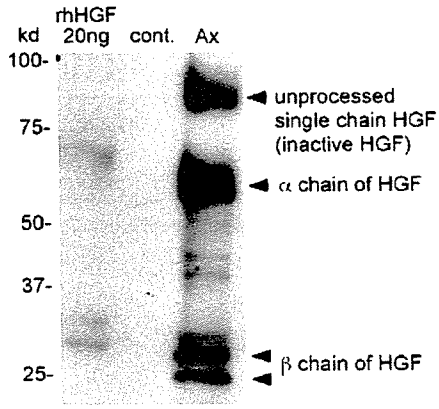

Fig. 1 – Western blot analysis of conditioned media (CMs) obtained from COS1 cells uninfected (cont.) or infected (Ax) with AxCAhHGF. The CMs harvested at 3 days after infection were concentrated by heparin beads, electrophoresed, blotted and immunolabeled for HGF as described in the text.

been no reports, however, concerning the neurotrophic effects of HGF on adult motoneuron death after proximal nerve injury. In animal models of adult motoneuron injury, avulsion of cranial and spinal nerves causes marked motoneuron degeneration in adult rats (Koliatsos et al., 1994; Moran and Graeber, 2004; Ruan et al., 1995; Sakamoto et al., 2000, 2003a, 2003b; Søreide, 1981; Watabe et al., 2000, 2005; Wu, 1993), so that these animal models can be useful for therapeutic evaluation of neurotrophic factors or neuroprotective molecules against adult motoneuron death (Ikeda et al., 2003; Sakamoto et al., 2000, 2003a, 2003b; Watabe et al., 2000, 2005). We have recently shown that adenoviral gene transfer of glial-cell-line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), transforming growth factor-β2 (TGFβ2) and growth inhibitory factor (GIF)/metallothionein-III (MT-III) prevented the death of adult rat facial and spinal motoneurons after facial nerve and cervical spinal root avulsion (Sakamoto et al., 2000, 2003a, 2003b; Watabe et al., 2000). In the present study, we investigated whether HGF protects injured motoneurons after facial nerve or spinal root avulsion by using a recombinant adenoviral vector encoding human HGF.

2. Results

2.1. *Bioassay of recombinant human HGF*

In this study, we constructed a recombinant adenoviral vector encoding human HGF (AxCAhHGF). To test the ability of AxCAhHGF to induce human HGF expression in vitro, COS1 cells were infected with AxCAhHGF and the conditioned media (CMs) were harvested at 3 days postinfection. The levels of human HGF in uninfected and infected CMs analyzed by enzyme-linked immunosorbent assay (ELISA) were 1.9± 0.4 ng/ml and 2004.8±160 ng/ml, respectively (n=3). Western blot analysis of the CM harvested at 3 days postinfection showed immunoreactive bands of α-chain, β-chain and pro-HGF (inactive, unprocessed single chain precursor form) (Fig. 1). The CM obtained from uninfected COS1 cells did not show any immunoreactive bands. The Madin-Darby canine kidney (MDCK) cell scatter assay showed definite bioactivitiy of AxCAhHGF-infected COS1 CM; i.e., the activity of 1:500-diluted CM containing 4 ng/ml HGF as measured by ELISA corresponded to that of 2 ng/ml recombinant human HGF (rhHGF) that induced scattering of MDCK cells (Fig. 2).

2.2. *Adenoviral-vector-mediated HGF gene expression in facial nuclei*

We then examined the expression of adenovirus-mediated HGF in injured motoneurons after avulsion. We have pre-

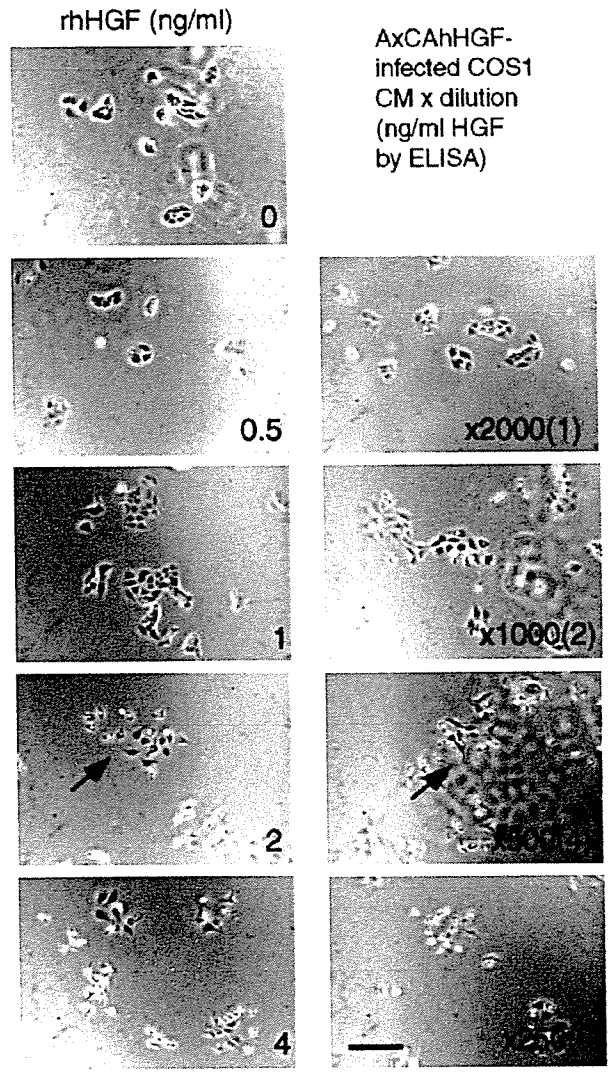

Fig. 2 – Madin-Darby canine kidney (MDCK) cell scatter assay for HGF bioactivity. MDCK cells were cultured in the presence or absence of AxCAhHGF-infected COS1 CM or rhHGF as described in the text. The activity of 1:500-diluted CM containing 4 ng/ml human HGF as measured by ELISA corresponds to that of 2 ng/ml recombinant human HGF that induced scattering of MDCK cells (arrows). Scale bar=50 μm.

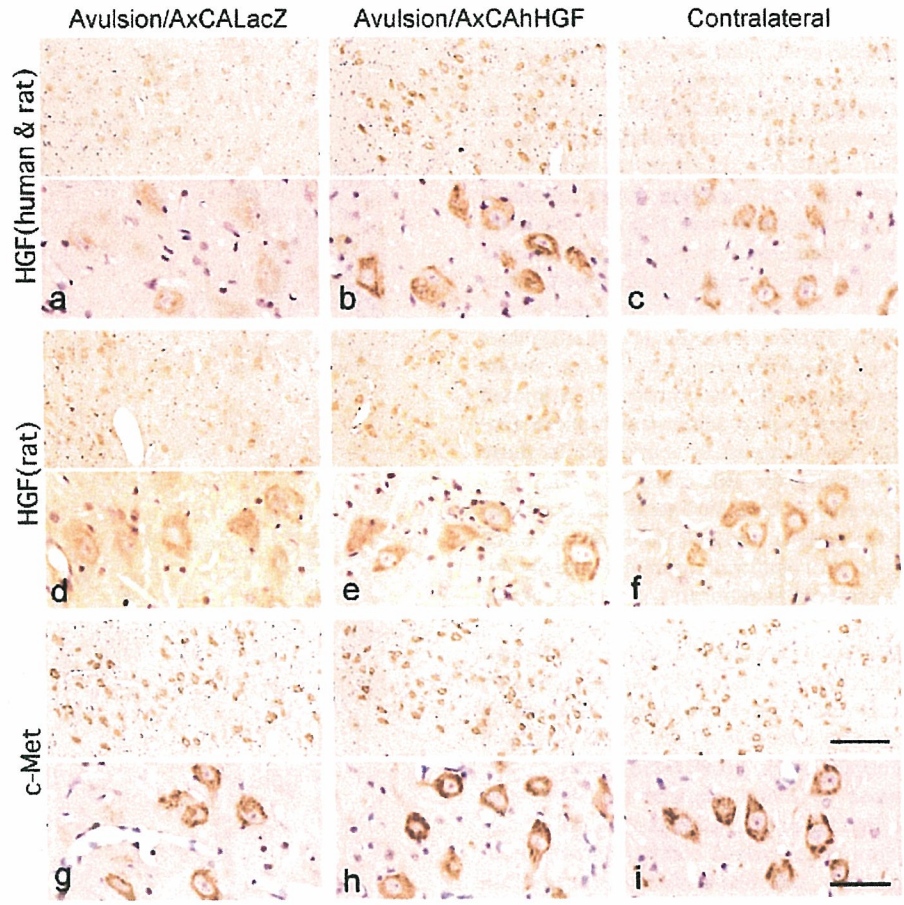

Fig. 3 – Low (top) and high (bottom)-magnified photomicrographs of immunohistochemistry of facial motoneurons at the ipsilateral (a, b, d, e, g, h) and contralateral (c, f, i) sides 7 days after facial nerve avulsion and the treatment with AxCALacZ (a, d, g) or AxCAhHGF (b, c, e, f, h, i) using antibodies against human and rat HGF (a–c), rat HGF (b–f) and c-Met (g–i). Counterstained with hematoxylin. Injured facial motoneurons after avulsion and AxCAhHGF treatment are more intensely immunolabeled by anti-human and rat HGF antibody (b) compared with injured motoneurons with AxCALacZ treatment (a) or contralateral intact motoneurons (c). Immunoreactivity of injured motoneurons treated with AxCALacZ (d) or AxCAhHGF (e) is comparable to that of contralateral intact motoneurons (f) when anti-rat HGF antibody was used. Immunoreactivity for c-Met is consistently demonstrated in both injured and contralateral motoneurons (g–i). Scale bars=200 μm (top), 50 μm (bottom).

viously demonstrated that injured motoneurons and their axons were labeled with X-Gal after facial or seventh cervical segment (C7) avulsion and inoculation of an adenovirus encoding bacterial β-galactosidase gene as a reporter (AxCA-LacZ) into lesioned stylomastoid or vertebral foramen, respectively (Sakamoto et al., 2000; Watabe et al., 2000). This indicates the diffusion of the virus through the facial canal or intervertebral foramen, its adsorption to injured axons, retrograde transport of the virus via intramedullary facial or spinal nerve tracts to soma of the motoneurons and

Table 1 – HGF protein levels in brain stem tissue containing facial nuclei after facial nerve avulsion and treatment with adenoviral vectors

Treatment (n=animal number)	Human HGF (ng/g)		Rat HGF (ng/g)	
	Ipsilateral	Contralateral	Ipsilateral	Contralateral
AxCALacZ (n=3)	u.d.	u.d.	20.3±3.8	17.2±2.3
AxCAhHGF (n=3)	61.8±36.1	13.1±11.4	21.2±3.1	22.8±2.8

Seven days after facial nerve avulsion and the treatment with AxCALacZ or AxCAhHGF, the brain stem tissues containing facial nuclei (10–14 mg wet weight) were examined by human- and rat-specific HGF ELISA. u.d. = under the detection limit (<2.4 ng/g tissue).

successful induction of the virus-induced foreign gene in these neurons (Sakamoto et al., 2000, 2003a,b; Watabe et al., 2000). In the present study, 1 week after avulsion and treatment with AxCAhHGF, injured facial motoneurons were more intensely immunolabeled by an antibody that recognizes both human and rat HGF (Fig. 3b), compared with injured motoneurons treated with AxCALacZ (Fig. 3a) or uninjured motoneurons on the contralateral side (Fig. 3c). Immunoreactivity of injured motoneurons treated with AxCAhHGF (Fig. 3e), AxCALacZ (Fig. 3d) or phosphate-buffered saline (PBS) (not shown) was comparable to that of contralateral intact motoneurons (Fig. 3f) when an antibody that recognizes only rat HGF was used. These immunohistochemical results suggest that endogenous rat HGF was preserved in injured motoneurons after avulsion, while adenovirus-induced exogenous human HGF was successfully expressed in these neurons. Immunoreactivity for HGF receptor c-Met was consistently demonstrated in both ipsilateral and contralateral motoneurons after avulsion and AxCAhHGF or AxCALacZ treatment (Figs. 3g–i). No significant immunoreactivity for HGF and c-Met was observed in astrocytes, oligodendrocytes or microglia.

We further examined the expression of exogenous human HGF and endogenous rat HGF in brain stem tissue containing facial nuclei after facial nerve avulsion and adenovirus treatment by human-specific (Funakoshi and Nakamura, 2003) or rat-specific (Sun et al., 2002) ELISA (Table 1). Rat HGF levels measured by ELISA showed no significant difference between injured and contralateral sides. Human HGF levels were more than twofold compared with endogenous rat HGF levels after AxCAhHGF infection. Human HGF was also detectable in the tissues at the contralateral side after AxCAhHGF infection, which was considered to originate from injured and infected motoneurons at the ipsilateral side (Table 1).

One week after facial nerve avulsion and the treatment with AxCAhHGF, RT-PCR analysis showed that virus-induced human HGF mRNA transcripts were expressed in the brainstem tissue containing the facial nucleus on the ipsilateral, but not the contralateral side, whereas endogenous rat HGF mRNA was consistently detected in the tissues on both ipsilateral and contralateral sides after avulsion (Fig. 4).

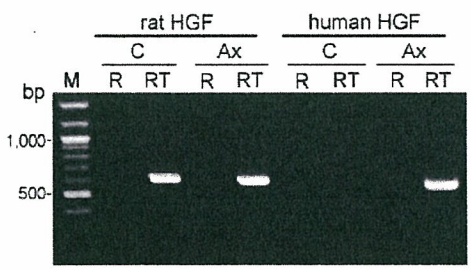

Fig. 4 – RT-PCR analysis of HGF mRNA transcripts in ipsilateral (Ax) and contralateral (C) sides of the brain stem tissue containing facial nuclei 7 days after facial nerve avulsion and AxCAhHGF treatment. The PCRs were performed on RNA without (R) or with (RT) reverse transcription. Primers that amplify rat or human HGF mRNA transcripts were used as described in the text. M=DNA size marker.

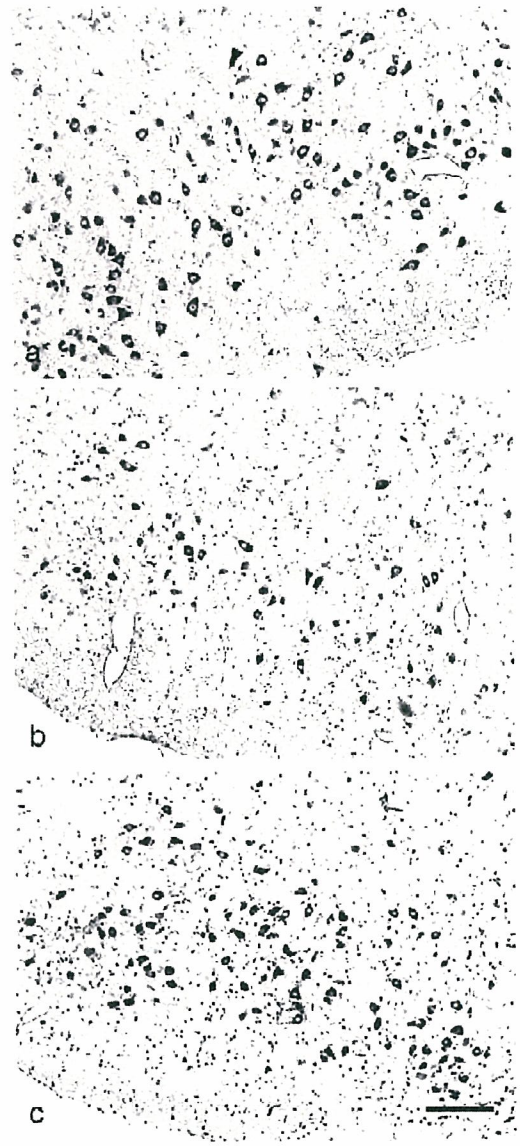

Fig. 5 – Photomicrographs of facial motoneurons at the contralateral (a) and ipsilateral (b, c) side 4 weeks after the right facial nerve avulsion and the treatment of AxCALacZ (b) or AxCAhHGF (c). Pictures (a) and (c) were taken from the same section. Nissl stain. Scale bar=200 μm.

2.3. *Neuroprotective effects of HGF gene transfer*

Four weeks after facial nerve or C7 spinal root avulsion and treatment with phosphate-buffered saline (PBS) or AxCALacZ, the number of surviving facial or spinal motoneurons declined to 30–50% of that on the contralateral side as described previously (Sakamoto et al., 2000, 2003a, 2003b; Watabe et al., 2000). The treatment with AxCAhHGF prevented the loss of facial (58.8±5.9% survival) and spinal (75.4±4.4% survival)

motoneurons after avulsion compared with the treatment with PBS (30.2±6.7% survival of facial motoneurons; 44.6±9.3% survival of C7 motoneurons) or AxCALacZ (32.4±4.3% survival of facial motoneurons; 46.0±5.3% survival of C7 motoneurons) (Sakamoto et al., 2000) (Figs. 5, 6; Table 2). The treatment with AxCAhHGF after avulsion attenuated the decrease of choline acetyltransferase (ChAT) immunoreactivity in injured facial motoneurons compared with the treatment with PBS or AxCALacZ (Fig. 7). We found no perivascular or intrathecal lymphocytic/mononuclear cell infiltration in the facial nuclei and the spinal cord tissues that would be histologically defined and identified in case of immunogenic reaction against adenovirus infection (Figs. 5, 6).

Table 2 – Survival of motoneurons after facial nerve and spinal root avulsion and treatment with adenoviral vectors

Treatment (n=animal number)	Ipsilateral motoneuron number	Contralateral motoneuron number	% Survival
Facial nerve avulsion			
PBS (n=8)	213±41	712±38	30.2±6.7
AxCALacZ (n=4)	239±29	741±73	32.4±4.3
AxCAhHGF (n=7)	441±87*	745±38	58.8±5.9*
Spinal root avulsion			
PBS (n=4)	66±22	144±20	44.6±9.3
AxCALacZ (n=4)	69±9	150±12	46.0±5.3
AxCAhHGF (n=4)	108±15**	143±14	75.4±4.4**

Numbers of facial motoneurons and the percent survival at the ipsilateral (lesion) side relative to the contralateral (control) side 4 weeks after avulsion and treatment with phosphate-buffered saline (PBS), AxCALacZ and AxCAhHGF. Results are presented as the mean±SD. Statistical comparison was done by Mann–Whitney *U* test. *$P<0.01$ vs. PBS- and AxCALacZ-treated groups. **$P<0.05$ vs. PBS- and AxCALacZ-treated groups.

3. Discussion

HGF binds to tyrosine kinase receptor c-Met and triggers diverse biological responses that include cell motility, proliferation, morphogenesis, neurite extension and anti-apoptotic activities in a variety of cells (Funakoshi and Nakamura, 2003; Maina and Klein, 1999). Although the function of HGF in the nervous system has not been fully elucidated, it has recently been shown that HGF plays a strong neuroprotective

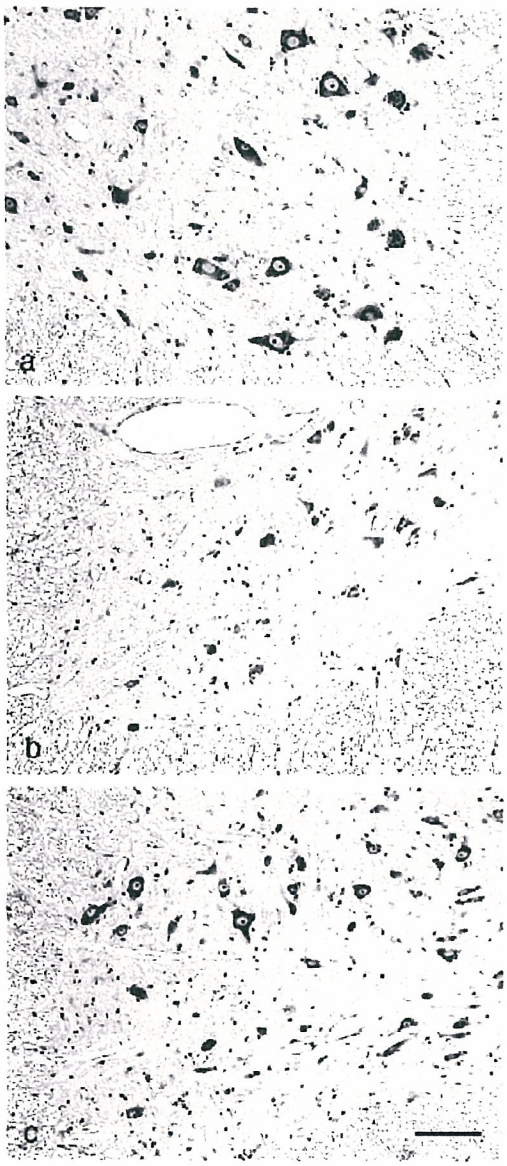

Fig. 6 – Photomicrographs of C7 spinal motoneurons at the contralateral (a) and ipsilateral (b, c) side 4 weeks after the right C7 spinal nerve avulsion and the treatment of AxCALacZ (b) or AxCAhHGF (c). Pictures (a) and (c) were taken from the same section. Nissl stain. Scale bar=100 μm.

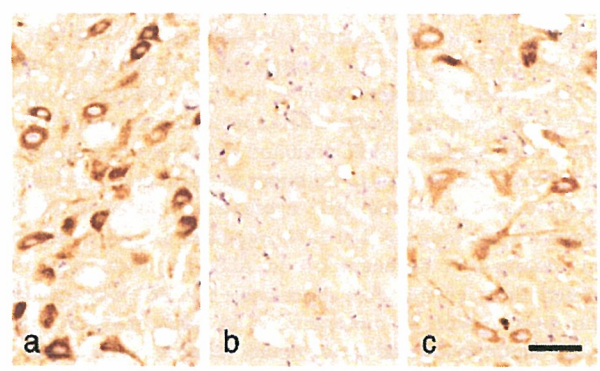

Fig. 7 – Photomicrographs of ChAT immunohistochemistry (a–c) of facial motoneurons at the contralateral (a) and ipsilateral (b, c) side 7 days after the facial nerve avulsion and the treatment of AxCALacZ (b) or AxCAhHGF (c). Pictures (a) and (c) were taken from the same section. Scale bar=50 μm.

role for motoneurons both in vitro and in vivo (Caton et al., 2000; Ebens et al., 1996; Honda et al., 1995; Koyama et al., 2003; Naeem et al., 2002; Novak et al., 2000; Okura et al., 1999; Sun et al., 2002; Wong et al., 1997; Yamamoto et al., 1997). It has been demonstrated that HGF-c-Met receptor coupling leads anti-apoptotic activities via MAP kinase (Hamanoue et al., 1996) and phosphatidylinositol-3 kinase/Akt (Hossain et al., 2002; Zhang et al., 2000) pathways and prevents caspase-1 and inducible nitric oxide synthase induction in motoneurons (Sun et al., 2002). In addition, HGF up-regulates the expression of excitatory amino acid transporter 2/glutamate transporter 1 (EAAT2/GLT1) in primary cultured astrocytes, which may improve glutamate clearance and reduce glutamate-mediated neurotoxicity (Sun et al., 2002).

In the present study, we investigated whether the treatment of AxCAhHGF can prevent the degeneration of motoneurons in adult rats after facial nerve and spinal root avulsion. We produced AxCAhHGF that induced bioactive HGF protein in infected COS1 cells in vitro as demonstrated by ELISA, Western blot analysis and MDCK scatter assay. Immunohistochemistry and RT-PCR results indicated that AxCAhHGF successfully infected injured motoneurons after facial nerve avulsion, suggesting the autocrine and paracrine neurotrophic effects of exogenous HGF on injured motoneurons after avulsion. Subsequently, we demonstrated that the treatment of AxCAhHGF delayed the loss of injured facial and spinal motoneurons. In addition, peripheral nerve avulsion as well as axotomy induces rapid decrease of ChAT immunoreactivity in injured motoneurons (Sakamoto et al., 2000; Watabe et al., 2000). In the present study, AxCAhHGF treatment after facial nerve avulsion improved ChAT immunoreactivity in injured motoneurons. We have previously shown that the treatments of recombinant adenoviral vectors encoding GDNF, BDNF, TGFβ2 and GIF promote the survival of motoneurons and attenuated ChAT immunoreactivity in the same avulsion model (Sakamoto et al., 2000, 2003a, 2003b; Watabe et al., 2000). Similarly, the present results clearly indicate that HGF have neuroprotective effects on injured adult motoneurons.

It has been reported that HGF mRNA is up-regulated in the spinal cord of human sporadic amyotrophic lateral sclerosis (ALS) (Jiang et al., 2005), and certain residual anterior horn cells in the spinal cord of ALS patients co-express both HGF and c-Met with the same or even stronger intensity compared with those of normal subjects (Kato et al., 2003). Transgenic mice expressing human mutant Cu/Zn superoxide dismutase (G93A mice) overexpressing HGF exhibited significant prolongation in survival and decreased motoneuron death compared with G93A mice with normal HGF expression (Sun et al., 2002). These reports indicate that HGF may have protective effects on motoneuron degeneration in ALS. Together with the present data, it is therefore conceivable that HGF may prevent the degeneration of motoneurons in adult patients with motoneuron injury and motor neuron diseases such as ALS.

In conclusion, we examined neuroprotective effects of HGF on injured adult motoneurons. The treatment of an adenoviral vector encoding HGF after facial nerve and spinal root avulsion significantly improved the survival of injured facial and spinal motoneurons and ameliorated ChAT immunoreactivity in these neurons. These results indicate that HGF may be a potential neuroprotective agent against motoneuron injury and motor neuron diseases in adult humans.

4. Experimental procedures

4.1. *Adenovirus preparation*

The human HGF cDNA was excised from pBS-hHGF with deletion of 15 base pairs (Seki et al., 1990) and subsequently cloned into *Swa*I cloning site of a cassette cosmid pAxCAwt (TaKaRa, Osaka, Japan) carrying an adenovirus type-5 genome lacking the E3, E1A and E1B regions to prevent the virus replication. The cosmid pAxCAwt contains the CAG (cytomegalovirus-enhancer-chicken β-actin hybrid) promoter on the 5′ end and a rabbit globin poly (A) sequence on the 3′ end. The cosmid was then cotransfected to 293 cells with the adenovirus genome lacking the E3 region (Miyake et al., 1996). A recombinant adenoviral vector encoding HGF (AxCAhHGF) was propagated and isolated from 293 cells and purified by two rounds of CsCl centrifugation. Generation of recombinant adenovirus containing bacterial β-galactosidase gene (AxCA-LacZ) has been described elsewhere (Kanegae et al., 1996).

4.2. *Analysis of HGF expression in COS1 cells infected with AxCAhHGF*

COS1 cells were infected with AxCAhHGF at a multiplicity of infection (moi) of 100 in serum-free Dulbecco's minimum essential medium (DMEM) (Invitrogen, Carlsbad, CA) for 1 h and incubated with serum-free DMEM in 5% CO_2 at 37 °C. The conditioned media (CMs) were harvested at 3 days postinfection for ELISA and Western blot analysis. The ELISA was performed as described (Sun et al., 2002; Funakoshi and Nakamura, 2003). For Western blot analysis, CM was treated with heparin beads to concentrate HGF and the CM or rhHGF (Nakamura et al., 1989; Seki et al., 1990) was electrophoresed on 4–20% gradient sodium dodecyl sulfate (SDS)/polyacrylamide gels under reduced condition and transferred to PVDF membrane (Atto, Tokyo, Japan). The blotted membrane was then blocked with 3% skim milk and incubated overnight with rabbit anti-HGF (1:500; Tokusyu Meneki, Tokyo, Japan) followed by incubation with goat anti-rabbit IgG–HRP conjugate (1:1,000; DAKO, Glostrup, Denmark). Reactions were visualized by enhanced chemiluminescence detection using an ECL Western blotting detection kit (Amersham, Piscataway, NJ).

4.3. *Bioassay of adenoviral HGF; MDCK scatter assay*

MDCK cells cultured in DMEM with 10% fetal bovine serum (FBS) were trypsinized, seeded on 24-well plate (5000 cells/well) in the presence or absence of AxCAhHGF-infected COS1 CMs or rhHGF in DMEM with 5% FBS and incubated for 24 h at 37 °C. The cell scattering was viewed under a phase contrast microscope.

4.4. *Animals and surgical procedures*

The experimental protocols were approved by the Animal Care and Use Committee of the Tokyo Metropolitan Institute for Neuroscience.

4.4.1. *Facial nerve avulsion*

Adult Fischer 344 male rats (12–14 weeks old, 200–250 g) were anesthetized with intraperitoneal injection of pentobarbital sodium (40 mg/kg). Under a dissecting microscope, the right facial nerve was exposed at its exit from the stylomastoid foramen. Using microhemostat forceps, the proximal facial nerve was avulsed by gentle traction and removed from the distal facial nerve as described previously (Sakamoto et al., 2000). Immediately following the avulsion, microsyringe was inserted into the stylomastoid foramen and 20 μl solution of AxCAhHGF (1×10^8 pfu), AxCALacZ (1×10^8 pfu) or PBS was injected through the facial canal. The wounds were covered with a small piece of gelatin sponge (Gelfoam; Pharmacia Upjohn, Bridgewater, NJ) and suture closed, and the animals were sacrificed at 1 and 4 weeks postoperation as described below.

4.4.2. *Spinal root avulsion*

Anesthetized animals were placed in a supine position. Under a dissecting microscope, the right seventh cervical segment (C7) nerve was exposed by separating the surrounding cervical muscles and connective tissue until the point where the vertebral foramen was identified. Using microhemostat forceps, the C7 ventral and dorsal roots and dorsal root ganglia (DRG) were avulsed and removed from the peripheral nerve as described previously (Watabe et al., 2000). Immediately following avulsion, a small piece of Gelfoam presoaked with 10 μl solution of AxCAhHGF (1×10^8 pfu), AxCALacZ (1×10^8 pfu) or PBS was placed in contact with the lesioned vertebral foramen. The wounds were suture closed and animals were sacrificed at 4 weeks postoperation as described below.

4.5. *HGF ELISA of brain stem tissue containing facial nucleus*

One week after facial nerve avulsion and the treatment with AxCALacZ or AxCAhHGF, the animals ($n=3$) were euthanized with a lethal dose of pentobarbital sodium and the brain stem tissue containing the facial nucleus (11–14 mg wet weight) was collected. ELISA for rat HGF and human HGF was performed as described (Sun et al., 2002; Funakoshi and Nakamura, 2003).

4.6. *Reverse transcription followed by polymerase chain reaction (RT-PCR)*

One week after facial nerve avulsion and the treatment with AxCAhHGF, the brain stem tissue containing the facial nucleus ($n=3$) was collected as described above. Total RNA was isolated from the tissue using RNA isolation reagent (TRIZOL, Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and treated with RNase-free DNase (Roche, Penzberg, Germany) in transcription buffer for 30 min. First strand cDNA was synthesized from 250 ng of total RNA using random primer and Superscript II reverse transcriptase (Invitrogen) for one PCR analysis. The PCR reactions were carried out in PCR buffer containing cDNA template, 200 μM dNTPs, 2 mM $MgCl_2$, 0.2 μM of each primer and 25 unit/ml of ExTaq DNA polymerase (TaKaRa, Osaka, Japan). Specific oligonucleotide primers for PCR were designed to amplify rat HGF cDNA (Tashiro et al., 1990; GenBank Accession no. NM_017017; forward, 5′-GCCAAAACAAAA-CAACTG-3′; reverse, 5′-GACACCAAGAACCATTCTCA-3′) that yield 615 bp amplified products, and human HGF cDNA (Seki et al., 1990; M60718; forward, 5′-AAACATATCTGCGGAGGATC-3′; reverse, 5′-ACGATTTGGAATGGCACATC-3′) that give 561 bp amplified products. The PCR amplification program consisted of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min for 40 cycles. For negative control reactions, non-reverse transcribed RNA samples were processed for PCR to exclude the possibility of the contamination of genomic or adenoviral DNA as a source of amplified products. The PCR products were subjected to electrophoresis on a 1.5% agarose gel stained with ethidium bromide. To confirm the sequence identity of the amplified products, the PCR fragments were subcloned into pCRII (Invitrogen) and sequenced by a model 373A sequencer and the ABI PRISM BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

4.7. *Histological analysis*

Rats were anesthetized with a lethal dose of pentobarbital sodium and transcardially perfused with 0.1 M phosphate buffer, pH 7.4 (PB) followed by 4% paraformaldehyde in 0.1 M PB. The brain stem tissue containing facial nuclei and their intramedullary nerve tracts after facial nerve avulsion or the C7 spinal cord tissue after spinal root avulsion was dissected and immersion fixed in the same fixative for 2 h. As for facial nerve avulsion, we routinely checked the absence of extra-axial portion of facial nerve on the avulsed side under a dissecting microscope and confirmed the absence of any peripheral nerve tissues at the level of facial nerve outlet from the brain stem in microscopic sections prepared from every animal as described below. As for C7 spinal root avulsion, the absence of C7 ventral and dorsal roots as well as DRG on the lesioned side was confirmed under the dissecting microscope. A small longitudinal incision was made in the anterolateral white matter through the level of C7 ventral root outlets on the contralateral side in aid of identifying the level of C7 spinal ventral horn in histological sections.

For immunohistochemistry, the brain stem tissues were either embedded in paraffin or cryoprotected in 30% sucrose in 0.1 M PB and serial paraffin or cryostat sections were made. For immunostaining for HGF, deparaffinized sections were pretreated with 0.3% H_2O_2 in methanol, incubated with 0.05% trypsin for 15 min at 37 °C and preincubated with 3% heat-inactivated goat serum in 0.1% Triton-X100 in phosphate-buffered saline (T-PBS). Sections were then incubated overnight at 4 °C with rabbit anti-human HGFα antibody (H55; recognizes human and rat HGFα; IBL, Fujioka, Japan) or rat HGFα antibody (H56; recognizes rat, but not human, HGFα; IBL) diluted 1:200 in T-PBS followed by the incubation with biotinylated goat anti-rabbit IgG at a dilution of 1:200 and with ABC reagent (Vector). Immunostaining for ChAT on cryostat sections was performed using rabbit polyclonal antibody to ChAT (1:1000; Chemicon, Temecula, CA) and ABC method as described previously (Watabe et al., 2000). Sections were visualized by 3,3′-diaminobenzidine tetrahydrocholoride (DAB)–H_2O_2 solution and counterstained with hematoxylin.

For negative controls, the primary antibodies were omitted or replaced by non-immunized animal sera.

For motoneuron cell counting, serial paraffin-embedded brain stem or C7 spinal cord sections were made. Every fifth section (6 μm thickness; 24 μm interval) was picked up, deparaffinized and stained with cresyl violet (Nissl staining). Facial motoneurons having nuclei containing distinct nucleoli on both sides of the facial nuclei were counted in 25 sections. For spinal motoneuron cell counting, ventral horn motoneurons located in Rexed's lamina IX having nuclei greater than 15 μm in diameter with distinct nucleoli on both sides of the C7 spinal cord were counted in 35 sections. The data were then expressed as the mean ± SD from 4 to 8 animals, and statistical significance was assessed by Mann–Whitney U test.

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Hepatoma-derived growth factor, a new trophic factor for motor neurons, is up-regulated in the spinal cord of PQBP-1 transgenic mice before onset of degeneration

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Abstract

Hepatoma-derived growth factor (HDGF) is a nuclear protein homologous to the high-mobility group B1 family of proteins. It is known to be released from cells and to act as a trophic factor for dividing cells. In this study HDGF was increased in spinal motor neurons of a mouse model of motor neuron degeneration, polyglutamine-tract-binding protein-1 (PQBP-1) transgenic mice, before onset of degeneration. HDGF promoted neurite extension and survival of spinal motor neurons in primary culture. HDGF repressed cell death of motor neurons after facial nerve section in newborn rats *in vivo*. We also found a significant increase in p53 in spinal motor neurons of the transgenic mice. p53 bound to a sequence in the upstream of the *HDGF* gene in a gel mobility shift assay, and promoted gene expression through the *cis*-element in chloramphenicol acetyl transfer (CAT) assay. Finally, we found that HDGF was increased in CSF of PQBP-1 transgenic mice. Collectively, our results show that HDGF is a novel trophic factor for motor neurons and suggest that it might play a protective role against motor neuron degeneration in PQBP-1 transgenic mice.

Keywords: degeneration, motor neuron, polyglutamine, polyglutamine-tract-binding protein-1, trophic factor.

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Hepatoma-derived growth factor (HDGF) was originally purified from the conditioned medium of the human hepatoma-derived cell line, HuH7, by monitoring the growth-stimulating activity on Swiss 3T3 cells (Nakamura *et al.* 1994). Molecular cloning of the cDNA revealed that HDGF is homologous (32% in amino acid sequences) to high mobility group protein-B1 (HMGB1) (Nakamura *et al.*

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Abbreviations used: ALS, amylotrophic lateral sclerosis; BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; CAT, chloramphenicol acetyl transfer; ChAT, choline acetyltransferase; CNTF, ciliary neurotrophic factor; Cont, control; DMEM, Dulbecco's modified Eagle's medium; E*n*, embryonic day *n*; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; GFAP, glial fibrillary acidic protein; GST, glutathione transferase; HDGF, hepatoma-derived growth factor; HEK, human embryonic kidney; HMG, high-mobility group; IFN, interferon; IL, interleukin; JNK, c-Jun N-terminal kinase; MMLV, Moloney murine leukemia virus; PBS, phosphate-buffered saline; PC, phase contrast; PQBP, polyglutamine tract-binding protein-1; RAGE, receptor for advanced glycation end-products; SDS, sodium dodecyl sulfate; SSC, saline sodium citrate buffer; SOD, superoxide dismutase; Tg, transgenic.

1994), although HDGF lacks the HMG box, a DNA-binding domain shared by HMG proteins. HDGF contains a nuclear localization signal sequence, and it is actually located in the nucleus (Kishima *et al.* 2002). On the other hand, HDGF is released from cells in a similar manner to the way in which HMGB proteins are released in necrosis (Scaffidi *et al.* 2002). The released HDGF promotes proliferation of fibroblasts, HuH7 cells (Nakamura *et al.* 1989), endothelial cells (Oliver and Al-Awqati 1998) and smooth muscle cells (Everett *et al.* 2000). These findings collectively suggest that HDGF is a growth factor involved in the tissue damage response.

We have investigated molecular mechanisms underlying neurodegeneration of polyglutamine diseases and have found a candidate mediator molecule of the pathology, polyglutamine-tract-binding protein-1 (PQBP-1) (Waragai *et al.* 1999; Okazawa *et al.* 2002). Interestingly, transgenic (Tg) mice overexpressing PQBP-1 show a late-onset motor neuron disease phenotype (Okuda *et al.* 2003). We performed microarray analysis of gene expression profiles in PQBP-1 Tg mice and reported that a major group of changed genes are those transcribed from the mitochondrial genome (Marubuchi *et al.* 2005). Simultaneously, we found up-regulation of HDGF in the spinal cord of PQBP-1 Tg mice, and analyzed the pathophysiological significance in this study.

Western blot and immunohistochemistry showed that HDGF is increased in the nuclei of motor neurons of presymptomatic PQBP-1 Tg mice. HDGF had trophic effects on motor neurons in our analyses with primary motor neurons and facial motor neurons after nerve section. In addition, p53, which was increased in spinal motor neurons of PQBP-1 Tg mice, activated transcription from the *HDGF* gene through binding sites in the upstream region. Furthermore, HDGF was increased in the CSF of these Tg mice. Collectively, these results suggest that HDGF might be a new motor neuron trophic factor that plays a protective role against motor neuron degeneration in PQBP-1 Tg mice.

Materials and methods

Total RNA preparation

For each microarray analysis, spinal cords at the level from L1 to L2 were removed from three PQBP-1 Tg mice or from three age-matched littermates at 2 and 12 months. The anterior halves of the spinal cords were dissected using razor blades under microscopy. Dissected tissues from each group were transferred into a glass homogenizer and homogenized with Trizol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was prepared according to the manufacturer's protocol.

Cy3- or Cy5- labeled amplified RNA preparation for microarray analyses

Labeling and amplification of RNA was performed using the Agilent Fluorescent Linear Amplification kit (Agilent Technologies, Palo Alto, CA, USA) according to the manufacturer's protocol. First, double-stranded cDNAs with T7 promoter were synthesized from 2 μg total RNA by Moloney murine leukemia virus (MMLV) reverse transcriptase using oligo dT primer, which contains the T7 promoter sequence, and random hexamers (40°C, 4 h). Then, using these double-stranded cDNAs as templates, cRNA was synthesized by T7 RNA polymerase using Cy3- or Cy5- labeled CTP (40°C, 1 h). cRNAs from PQBP-1 Tg mouse and age-matched littermates were labeled with Cy3 and Cy5 respectively. Synthesized cRNA was precipitated with lithium chloride, rinsed with ethanol and dissolved in nuclease-free water. To check the quality of cRNA, OD_{260}, OD_{280}, A_{552} (for Cy3) and A_{650} (for Cy5) were measured. Then, OD_{260}/OD_{280}, amplification rate and dye incorporation rate (pmol/μg RNA) of the cRNA were calculated. Our samples were of high quality based on these criteria (OD_{260}/OD_{280} 2.0 >, amplification rate 400 >, Cy3 incorporation > 15 pmol/μg RNA and Cy5 incorporation > 12 < pmol/μg RNA). Microarray analysis with the mixture of total RNA from three mice was repeated twice.

Hybridization of microarrays

Hybridization procedures were performed using *In Situ* Hybridization Plus kit (Agilent Technologies) according to the manufacturer's manual. First, Cy3- and Cy5- labeled cRNAs (1 μg each) were mixed and incubated with fragmentation buffer (Agilent Technologies) at 60°C for 30 min. Then, Mouse Development Oligo Microarray (Agilent technologies), which contains 20 371 60mer oligonucleotides from mouse cDNA, was hybridized with fragmented cRNA target at 60°C for 17 h. Hybridized microarrays were rinsed twice and dried by spraying N_2 gas (99.999%) using a filter-equipped air-gun (Nihon mycrolis KK, Tokyo, Japan).

Signal detection and data analysis of microarrays

The fluorescence signal was read using a microarray scanner, CRBIO® IIe (Hitachi Software Engineering Co., Ltd, Tokyo, Japan). Data were analyzed using the software DNASIS® array (Hitachi Software Engineering Co., Ltd). Briefly, data either from control spots or from spots containing high intensities of artificial signals were removed. Then, the signal intensity of each spot was normalized to equalize total signal intensity. The normalized signal intensity of each spot was plotted on a scatter plot with Cy3 fluorescence on the *y*-axis, and Cy5 fluorescence on the *x*-axis. The ratio of Cy3 fluorescence (gene expression in PQBP-1 Tg mice) to Cy5 fluorescence (gene expression in age-matched littermates) were calculated, and genes with a Cy3/Cy5 ratio of more than 1.5 or less than 0.67 were listed (Marubuchi *et al.* 2005). To identify genes of interest, the sequences of 60mer oligonucleotides on the spots of interest were retrieved from Agilent Technology and the sequences were searched for in a mouse cDNA database using National Center for Biotechnology Information at NIH (NCBI) BLAST. Most of the selected spots of interest were identified with aid of the manufacturer's annotation information, although there were several unidentified genes.

Northern blot analysis

Northern blot analysis was performed basically as described previously (Okamoto *et al.* 1990; Okazawa *et al.* 1991). Some 15 μg total RNA was separated on a 1% agarose gel and blotted to Hybond-N (Amersham Biosciences, GE Health Care BioSciences,