Kwai Chung, Hong Kong). Hybridization was performed in 5 × saline sodium citrate buffer (SSC) containing 0.5% sodium dodecyl sulfate (SDS), 5 × Denhardt's solution and 20 μg/mL salmon sperm DNA at 60°C for 24 h. The membrane was washed twice in 2 × SSC–0.1% SDS at 50°C for 20 min and twice in 0.1 × SSC–0.1% SDS at 55°C for 20 min. The membrane was exposed to Hyperfilm ECL (Kodak, New York, USA) for 72 h.

Generation of antibody against HDGF

An antiserum against HDGF was raised by immunizing rabbits with the peptide KEEAEAPGVRDHESL (C-terminal 15 amino acids of mouse HDGF) or with the peptide KEDAEAPGIRDHESL (C-terminal 15 amino acids of human HDGF) conjugated to the carrier protein by cysteine. The specificity of these antibodies in western blot and immunohistochemistry was reconfirmed by preabsorption with glutathione transferase (glutathione S-transferase [GST])-HDGF (data not shown).

Immunohistochemistry

PQBP1 Tg and control mice at 7, 16 and 20 months were transcardinally perfused with cold 4% paraformaldehyde. The brains and the spinal cords were post-fixed in 4% parafolmaldehyde for 24 h. Paraffin sections (5–10 μm) were de-paraffinized in xylene and rehydrated through an ethanol dilution series. Endogenous peroxidase was inactivated with 0.3% hydrogen peroxide in phosphate-buffered saline (PBS) for 30 min. Paraffin sections were treated with 3% goat serum for 30 min. For HDGF staining, anti-HDGF C-terminal polyclonal rabbit serum (1 : 1000 dilution) with or without anti-glial fibrillary acidic protein (GFAP) rabbit polyclonal antibody (1 : 500; Chemicon, Temecula, CA, USA) was used as primary antibody. For p53 staining, p53 rabbit polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was used at 1 : 100 dilution and another rabbit polyclonal anti-p53 antibody (CM5; Novocastra) was used at 1 : 200 dilution. Horseradish peroxidase-conjugated anti-rabbit antibody (Envision; DAKO, Glostrup, Denmark) was used as a secondary antibody according to the protocol and visualized with diaminobenzidine (Sigma, St Louis, MO, USA). Antibodies against phospho-p53 at Ser15, Ser20 or Ser389 (Cell Signaling, Beverly, MA, USA) were diluted at 1 : 200 and used for immunohistochemistry. The first two antibodies did not show any signal in the spinal cord of control and Tg mice.

Generation and purification of recombinant HDGF

To express GST-HDGF fusion protein, full-length mouse HDGF cDNA amplified by PCR with the primers HDGF-F (AAAGGGATCCGATCCAACCGGCAGAAAGAG) and HDGF-R (AAAGAATTCTACAGGCTCTCATGATCTCT) was subcloned between *Bam*HI and *Eco*RI restriction sites of pGEX-3X (Amersham Biosciences). As a result of this subcloning the first two N-terminal amino acids of HDGF were changed from M-S to G-I. After 5 h of induction with Isopropyl-beta-D-thiogalactoside (IPTG) at 0.1 mM, *Escherichia coli* cells were collected by centrifugation and sonicated. GST-HDGF was recovered from the lysate using a glutathione sepharose 4B column (Amersham Biosciences). HDGF was cleaved from the fusion protein by Factor Xa, and purified with a heparin-sepharose column (HiTrap Heparin HP; Amersham Biosciences).

Spinal cord tissue culture and analyses of neurite outgrowth

Spinal cords of embryonic day 14 (E14) Sprague–Dawley rat embryos were dissected and dorsal root ganglia and meninges were stripped away. The ventral half of the spinal cord was separated under the microscope and cut into a 1-mm cube of tissue, whereas the dorsal half of the spinal cord was discarded. The ventral tissue was plated on to a poly-L-lysine-coated 35-mm dish (Falcon, BD Biosciences, San Jose, CA, USA) which had been incubated overnight with Dulbecco's modified Eagle's medium (DMEM), and cultured in 5% CO_2 at 37°C. Three HDGF concentrations, 3, 10 and 30 ng/mL, were examined for neurite-promoting activity, and 6–10 explants were used at each concentration. The culture medium was exchanged every other day. Neurite outgrowth of explants was evaluated after 7 days by using WinROOF software (Mitani Corporation, Tokyo, Japan). For anti-choline acetyl transferase (ChAT) antibody staining, the tissues were fixed with 0.1% paraformaldehyde and incubated with goat anti-ChAT polyclonal antibody (Chemicon) at a dilution of 1 : 500 for 12–24 h at 4°C. The stain was visualized with donkey anti-goat IgG labeled with Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA) and observed by fluorescence microscopy (IX-71; Olympus, Tokyo, Japan) and Aquacosmos software (Hamamatsu Photonics, Hamamatsu, Japan).

Primary culture of spinal motor neurons and analysis of survival effects

Rat embryo (E14) spinal cords were dissected, and the dorsal half of the spinal cord was removed under the microscopy. The ventral half was chopped into small pieces using razor blades, incubated in PBS containing 0.05% trypsin for 15 min at 37°C, and dissociated mechanically. After filtration, the cells were plated in 24-well plates (Greiner, Kremsmünster, Austria) that had been coated with polyornithine (Sigma) and laminin (Invitrogen) at a density of 6×10^4 cells/well. The cells were cultured in neurobasal medium (Gibco, Rockville, MD, USA) supplemented with B27 (Gibco), glutamate (Wako, Tokyo, Japan), glutamine (Wako), 3-mercapto-ethanol (Nakarai, Tokyo, Japan) and Gentamicin (Gibco). Recombinant mouse HDGF (3 or 30 ng/mL) or recombinant human BDNF (30 ng/mL; Pepro Tech, London, UK) were added to the culture medium simultaneously. Twelve hours after plating, cytosine arabinoside (Sigma) was added to the medium at 4 M final concentration. The cells were cultured at 37°C in 5% CO_2 for 7 days, fixed with 2% paraformaldehyde in 0.1 M phosphate buffer, and stained with anti-ChAT antibody (Chemicon). ChAT-positive cells were counted as motor neurons.

Facial nerve section of newborn rats and survival of facial motor neurons

The main trunk of the facial nerve was sectioned unilaterally in newborn pups (P1). The proximal nerve stump was treated with a piece of Spongel (Yamanouchi, Astellas, Tokyo, Japan) containing 5 μg HDGF in 4 μL PBS with 1% bovine serum albumin (Sigma). Human BDNF was applied in a similar manner as positive control and a piece of Spongel soaked in 4 μL PBS with 1% bovine serum albumin was implanted as a negative control. After 7 days, the animals were transcardially perfused with ice-cold 20 mL 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were dissected and soaked in the similar solution for 12 h. The brainstem

was embedded in paraffin, and 6 μm-thick serial coronal plane sections were prepared using a microtome. The serial sections were stained with cresyl violet and the neurons in the facial nucleus were counted on injured and uninjured sides. The percentage of motor neurons on the injured side compared with the uninjured side was calculated in each mouse.

Primary culture of cortical neurons

Cerebral cortical tissues were isolated from E17 Wistar rat embryos, minced using razor blades, and treated with 0.25% trypsin (Gibco) in PBS (pH 7.5) at 37°C for 20 min, with gentle shaking every 5 min. After stopping the reaction with DMEM containing 50% fetal bovine serum, Dnase I (Boehringer Mannheim, Indianapolis, IN, USA) was added to the solution at a final concentration of 100 μg/mL, and tissues were dissociated gently by pipetting with blue tips. Cells filtered through nylon mesh (pore size 70 mm; Falcon) were collected by centrifugation, resuspended in DMEM supplemented with 20 mM glucose, 16 mM sodium bicarbonate, 4 mM glutamine, 25 μg/mL gentamicin and 10% fetal bovine serum, and then plated on 24-well dishes (Corning, Corning, NY, USA) coated with polylysine (Sigma) at 3×10^5 cells/well. Twelve hours after plating, cytosine arabinoside was added to the culture medium at 4 M final concentration to prevent growth of glial cells.

Construction of adenovirus vector

Adenovirus vectors for expression of PQBP-1 proteins were constructed by subcloning full-length PQBP-1 cDNA (Waragai *et al.* 1999) into the *SwaI* site of a cosmid vector, pAxCAwt (Takara, Tokyo, Japan). These cosmids were transfected into HEK 293 cells with the fragmented adenovirus DNA (DNA-TPC) by the calcium phosphate method, so that adenovirus containing the insert was generated by recombination. The transfected cells were harvested after 12 h, dissociated and cultured in 96-well collagen-coated dishes for 3 weeks. From wells in which cells showed lysis between 7 and 15 days after transfection, the medium was recovered as the primary virus solution. The solution (Ax-PQBP1) was amplified two or three times in 293 cells, and the working virus solution was prepared by sonicating the final 293 cells 3 days after transfection. We confirmed the insert in adenovirus vector by PCR before use. These steps were performed according to the protocol of Adenovirus Expression Vector kit (Takara).

Gel mobility shift assay

Gel mobility shift assay was performed as described previously (Okamoto *et al.* 1990; Okazawa *et al.* 1991). To make the probes, sense and antisense oligonucleotides of the sequences shown in Supplementary figure 2 and possessing additional 5′-GGG were synthesized, annealed and radiolabeled with [α-^{32}P]dCTP and Klenow enzyme (Takara). Some 10 000 cpm of probes were incubated with 5 ng human recombinant p53 protein that was purified by affinity chromatography and gel filtration (Active Motif, Carlsbad, CA, USA).

Chloramphenicol acetyl transfer (CAT) assay

CAT assay was performed according to the method described previously (Okamoto *et al.* 1990; Okazawa *et al.* 1991). In brief, 1×10^7 HEK293 or P19 cells were cultured in 10-cm dishes and after 12 h they were transfected with plasmids using Superfect (Qiagen, Valencia, CA, USA). Transfection efficiency was verified by pCH110 (Promega, Madison. WI, USA), a eukaryotic expression vector containing the simian virus early promoter and the *E. coli* β-galactosidase (LacZ) structural gene. After further 24 h, cells were harvested and used for CAT assay. pHDGFe2-IFN-CAT, containing the e1/2 sequence upstream of the interferon (IFN) promoter and *CAT* gene, was constructed by subcloning the region around the e2 sequence amplified by PCR between *NotI* and *XbaI* sites of pIFN-CAT (Okazawa *et al.* 1991). The PCR reaction was performed with mouse genomic DNA using the primers SF1 (CAGCGGC-CGCCTTTAAGTCAGGATCTT) and SF2 (GTTCTAGAAGGA-GCAGAAGTTCCAGGCCAT). p53 expression vector, pCI-p53, was constructed by subcloning full-length rat p53 cDNA between *EcoRI* and *SalI* sites of pCIneo (Promega).

CSF collection

CSF was collected from three Tg and three littermate mice under deep anesthesia by tapping cisterna magna with a 23-G needle.

Mutant SOD1

A human SOD1 transgenic strain, B6SJL-TgN (SOD1-G934) 1 Gu (The Jackson Laboratories) was used for morphological analyses.

Results

HDGF expression is up-regulated in the spinal cord of motor neuron degeneration model mice

PQBP-1 Tg mice show progressive weakness of hind limbs when they become more than 18 months old. Pathological analyses have revealed that motor neurons in the lumbar spinal cord are degenerated (Okuda *et al.* 2003). To understand the molecular mechanisms underlying the pathology, we analyzed gene expression in the lumbar spinal cord of presymptomatic PQBP-1 Tg mice (2 and 12 months old) using Mouse Development Oligo Microarray (Agilent Technologies). We did not perform microarray analysis with older Tg mice after the onset of symptoms because dysfunction and loss of motor neurons prevented us from getting primary changes in gene expression profiles. The probes were synthesized from RNA samples of three Tg or age-matched littermate mice, and the microarray experiments were repeated twice. We compared the expression profiles, and selected 14 genes whose expression was constantly changed more than 1.5-fold in Tg mice (Marubuchi *et al.* 2005). Thirteen genes were up-regulated whereas one gene was down-regulated. The up-regulated group included six mitochondrial genes, suggesting that a kind of mitochondrial stress is involved in the pathology of PQBP-1 Tg mice (Marubuchi *et al.* 2005). The down-regulated gene was neural tropomodulin. All these data were reported previously (Marubuchi *et al.* 2005). Interestingly, the list included a trophic factor, HDGF, which was increased to 2.301 fold. We performed northern blot analysis and confirmed that HDGF

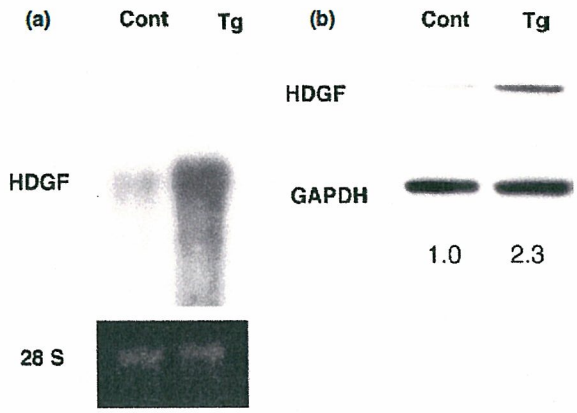

Fig. 1 HDGF mRNA and protein expression is increased in the spinal cord of PQBP-1 Tg mice. (a) Northern blot analysis confirmed up-regulation of HDGF mRNA in the spinal cord of PQBP-1 Tg mice at 12 months. Cont, age-matched littermates; Tg, PQBP-1 Tg mice; 28S, 28S ribosomal RNA. (b) Western blot analysis of the anterior horn of spinal cord from Tg mice (12 months old) and age-matched littermates (each $n = 3$). The expression ratios corrected with respect to glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) are shown below the panels. HDGF protein expression was increased in the spinal cord of Tg mice.

mRNA was actually increased (Fig. 1a) HDGF was not increased at 2 months (Marubuchi *et al.* 2005).

To investigate the protein expression level of HDGF in Tg mice, we generated antibodies against human and rat C-terminal sequences. In parallel, we made recombinant mouse HDGF to test the specificity of these antibodies. HDGF was cleaved from GST-HDGF fusion protein by factor Xa, and purified with heparin column (Supplementary Fig. S1a). We generated specific antibodies against mouse and human HDGF C-terminal peptides (Fig. S1b), and confirmed by western blot that the 37-kDa band of HDGF was increased in the spinal cord of Tg mice (Fig. 1b).

The genes changed in the spinal cord of Tg mice include mitochondrial genes tRNA(Cys), tRNA(Glu), cytochrome C oxidase (CCO)1, CCO2, and Protein4, RNA polymerase I (Pol I) transcription-related factor [rRNA promoter-binding protein (Ribin)] (Kermekchiev and Ivanova 2001), heterogeneous nuclear ribonucleoproteins methyltransferase-like 2/ protein arginine methyltransferase (Hrmtl2/PRMT) (Gary *et al.* 1996; Lin *et al.* 1996), a growth factor (HDGF) (Nakamura *et al.* 1994), receptor type Z protein tyrosine phosphatase (Krueger *et al.* 1990; Krueger and Saito 1992), MOR 6.5 (mouse genomic sequence relevant to ouabain resistance) (Zhou *et al.* 1993), and two unknown genes. We reported previously that PQBP-1 overexpression leads to a kind of mitochondrial stress and up-regulation mitochondrial genes (Marubuchi *et al.* 2005). The pathological relevance of the other genes will be reported elsewhere (Marubuchi *et al.*, unpublished observations).

HDGF protein is up-regulated in spinal motor neurons before degeneration

To identify the spinal cord cells that up-regulate HDGF protein, we performed immunohistochemical analysis of the lumbar spinal cords of PQBP1 Tg mice and littermates at 16 months (Fig. 2a). HDGF antibody (mC15-1) stained the largest neurons in the anterior horn that were not stained with GFAP antibody, i.e. motor neurons (Fig. 2a). Interestingly, nuclei of the anterior horn neurons were stained strongly in Tg mice but not in control mice (Fig. 2a). These data showed that HDGF expression in the nuclei of spinal motor neurons was remarkably up-regulated in PQBP-1 Tg mice. It is of note that anti-HDGF antibody stained some medium- or small-sized cells, which could be interneurons (Fig. 2a, thin arrows). HDGF was also expressed in the other neurons of the CNS including cortical neurons, as reported previously (Abouzied *et al.* 2004). However, no remarkable change in immunostaining was detected in neurons of PQBP-1 Tg mice other than spinal motor neurons (data not shown).

To understand the relevance of HDGF to amylotrophic lateral sclerosis (ALS), we asked whether the increased HDGF expression is observed in the motor neurons of mutant superoxide dismutase I (SOD1) mice (G93A). At 2 months, when the mutant SOD1 mice have not yet shown symptoms, HDGF was strongly stained in a part of the motor neuron nuclei, whereas staining in motor neurons was very weak in the age-matched control mice (Fig. 2b).

HDGF promotes neurite extension of motor neurons *in vitro*

HDGF is known to act as a trophic factor for dividing cells (Nakamura *et al.* 1989; Oliver and Al-Awqati 1998; Everett *et al.* 2000). Therefore, up-regulation of HDGF in spinal motor neurons of the mouse model of neurodegeneration prompted us to test whether HDGF acts as a trophic factor for motor neurons.

First, we observed its effect on neurite extension of spinal motor neurons. We synthesized recombinant mouse HDGF and added it to slices of lumbar spinal cord of E14 rat embryos cultured in medium with neither serum nor growth factors. As a control, mock solution, which was prepared from non-transformed *E. coli* cells exactly in the same manner as HDGF, was used. The comparison revealed that HDGF remarkably enhanced neurite extension (Fig. 3a). To distinguish neurites of motor neurons, we stained the slices with ChAT antibody. Extension of ChAT-positive neurites was also remarkably enhanced by HDGF (Fig. 3a). Quantitative analyses confirmed the effect of HDGF on neurite extension of spinal motor neurons (Fig. 3b). Most ChAT-positive neurons prepared from the anterior horn were considered to be motor neurons because E14 is the developmental stage at which motor neurons increase very rapidly and because we carefully separated the anterior horn from the

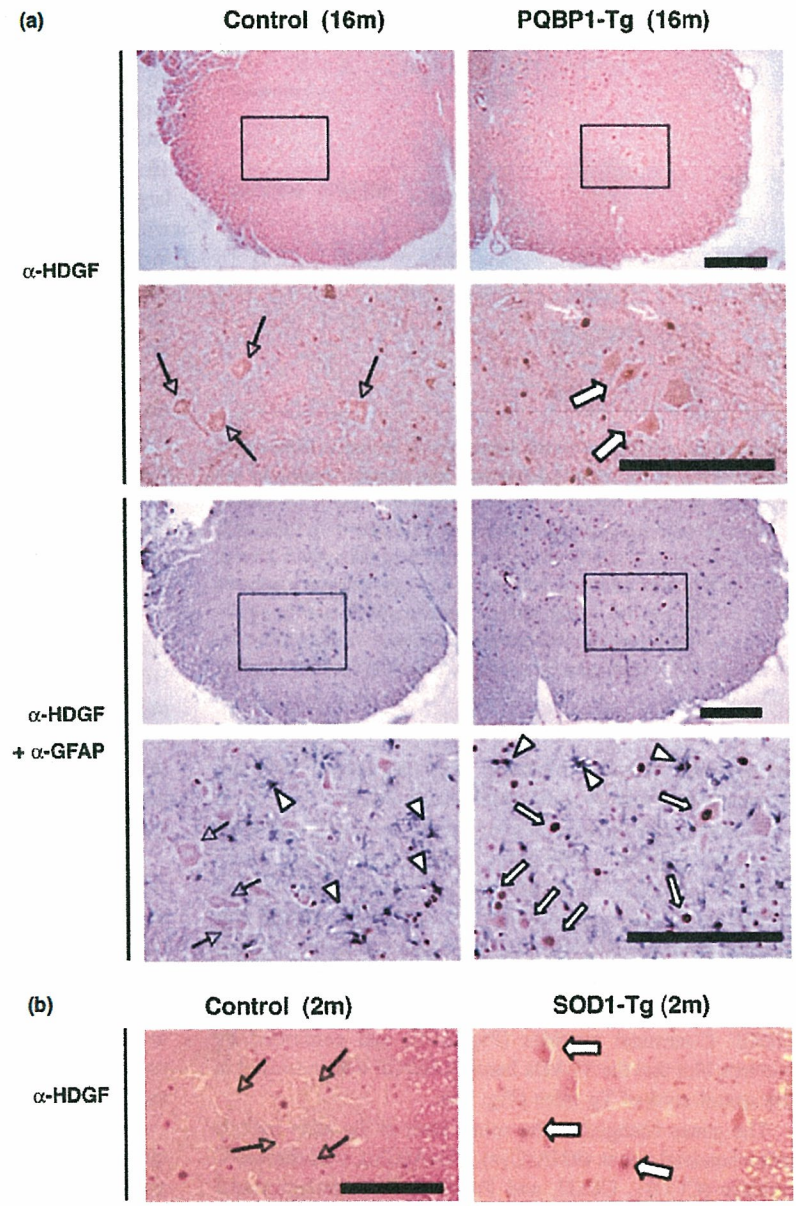

Fig. 2 HDGF is increased in spinal motor neurons of Tg mice. (a) Immunohistochemistry showing that HDGF protein was increased in spinal motor neurons of PQBP-1 Tg mice at 16 months. The upper panels show single staining with anti-HDGF antibody (mC15-1) and the lower panels show double staining with anti-HDGF (brown) and anti-GFAP antibody (blue). Higher magnifications (2nd and 4th rows) show that HDGF was increased in the nuclei of motor neurons (thick white arrows) and some other cells (thin white arrows) of Tg mice. On the other hand, the nuclei were not stained in most motor neurons of control mice (black arrows). GFAP staining (4th row) revealed that the large HDGF-positive cells were neurons. Glial cells (black) are indicated by white arrowheads. Scale bars 50 μm. (b) HDGF expression was increased in the motor neurons of mutant SOD mice (G93A). At 2 months when no symptoms were observed, HDGF was strongly stained in some of the motor neuron nuclei of mutant SOD mice (right panel, white arrows), whereas the nuclei of motor neurons were very weakly stained in the age-matched control mice (left panel, black arrows). Smaller cells other than motor neurons were stained by HDGF antibody in control mice. Scale bar 50 μm.

lateral horn under the microscope to prevent contamination by preganglionic autonomic neurons in the lateral horn.

We next compared the neurite-promoting activities of HDGF and other trophic factors, and found that the activity of HDGF was equivalent to that of ciliary neurotrophic factor (CNTF), BDNF, interleukin (IL)-6 and basic fibroblast growth factor (bFGF) (Fig. 3c). The trophic effect of HDGF was increased additively by any one of these trophic factors (data not shown), indicating that HDGF bound to a distinct receptor.

It is of note that a number of ChAT-positive neurons spread around the slice tissue in the presence of HDGF (Fig. 3a). This suggests enhanced migration by HDGF, although further investigations are necessary to confirm this assumption.

HDGF promotes survival of motor neurons *in vitro*

To test whether HDGF promotes survival of motor neurons in primary culture, spinal cord tissues of E14 rat embryos were dissected, mechanically dispersed, cultured for 7 days in serum-free medium with or without HDGF, and stained with ChAT antibody (Fig. 4a). As a control, we used the mock solution described above. Survival of the ChAT-positive neurons was clearly increased by addition of 3 or 30 ng/mL HDGF, and the effect of HDGF was almost equivalent to that of BDNF (Fig. 4a). Quantitative analyses

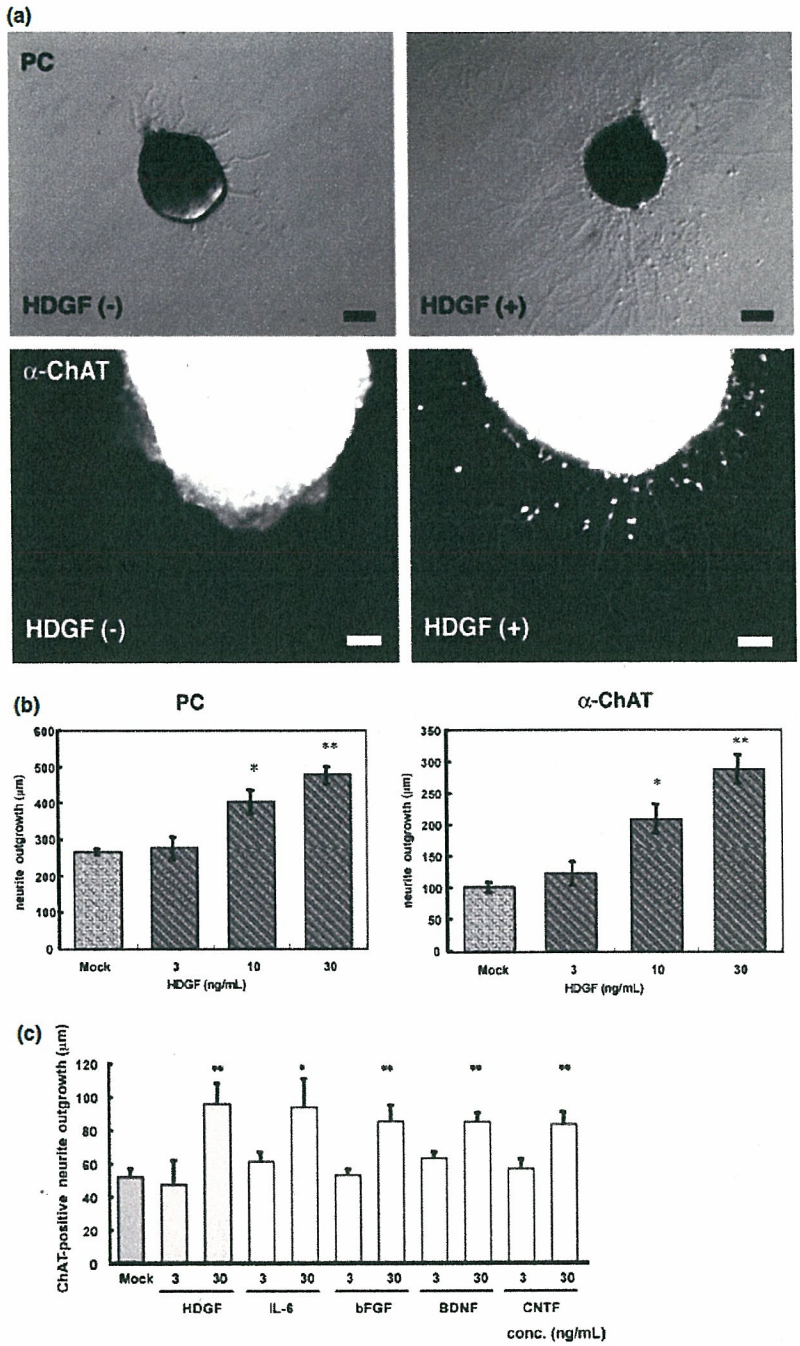

Fig. 3 HDGF promotes neurite extension of spinal motor neurons *in vitro*. (a) Recombinant mouse HDGF was added to slice cultures of rat embryonic spinal cord (E14). Phase-contrast images (PC) show that neurite extension was remarkably promoted 2 days after addition of HDGF (upper panels). HDGF also promoted neurite extension of ChAT-positive motor neurons (lower panels). Scale bars 10 μm. (b) Quantitative analysis by WinROOF (Mitani Corporation) confirmed dose-dependent neurite outgrowth in response to HDGF. Mock solution prepared from non-transformed *E. coli* cells was used as a control. Mean = SD, $^{*}p < 0.05$, $^{**}p < 0.01$ versus control (Dunnet test). (c) Comparison of the ChAT-positive neurite outgrowth between HDGF and other trophic factors. Mean = SD, $^{*}p < 0.05$, $^{**}p < 0.01$ versus mock control (Student's *t*-test).

of ChAT-positive motor neurons in 20 visual fields conformed that HDGF elongated survival of motor neurons (Fig. 4b). Together with the result of Fig. 3, these results strongly suggest that HDGF is a trophic factor for spinal motor neurons.

We also performed the neurite extension and neuron survival assays with spinal cords from E14 rat embryos of PQBP-1 Tg mice. Motor neurons from the transgene-positive embryos and those from negative embryos showed a similar response to HDGF in both assays (data not shown).

HDGF promotes survival of facial motor neurons *in vivo*

We examined the effect of HDGF on the survival of motor neurons in newborn rats after facial nerve section, which has been used to evaluate the effect of CNTF and BDNF on motor neurons (Sendtner *et al.* 1990, 1992). The proximal

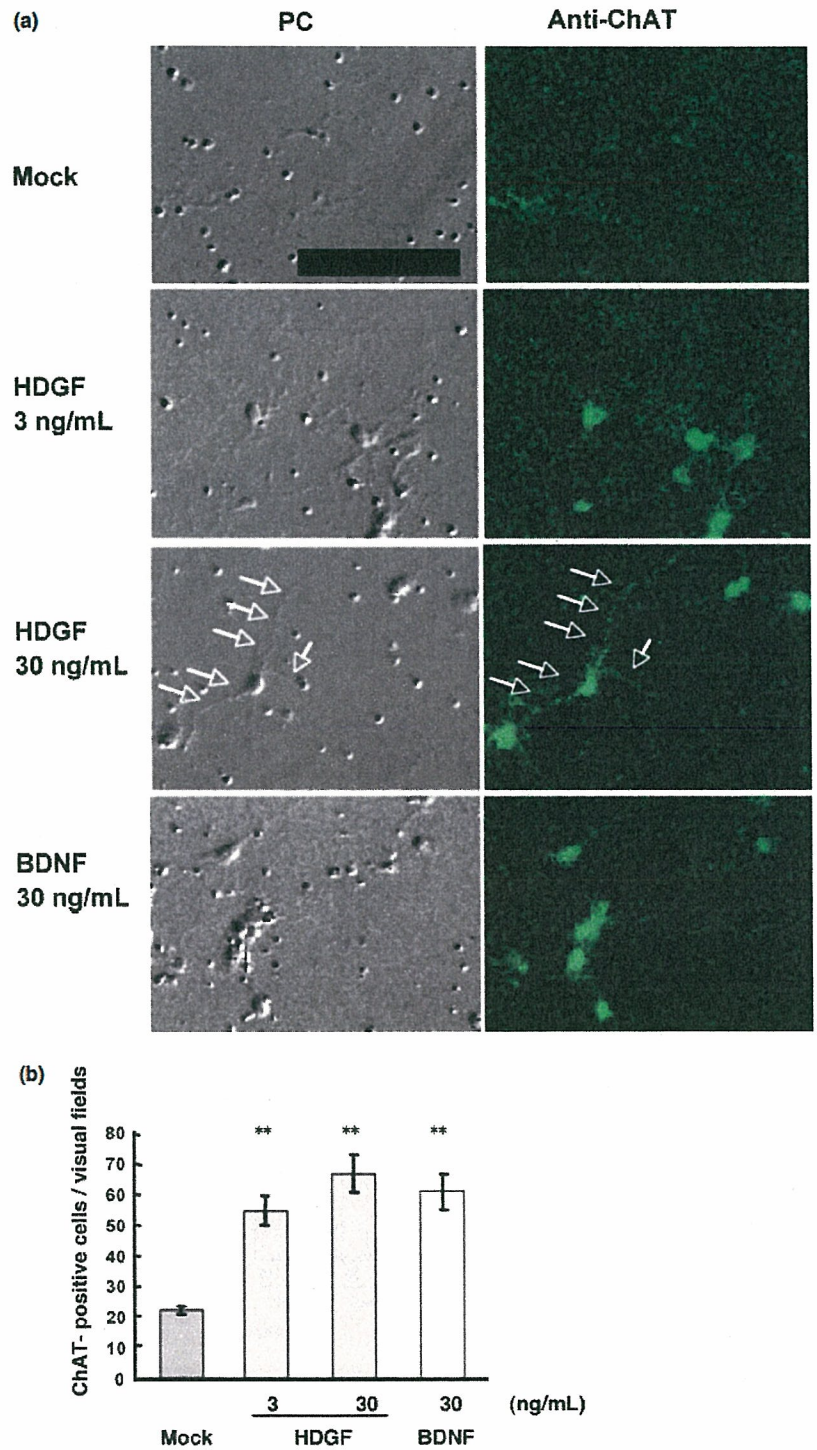

Fig. 4 HDGF promotes survival of spinal motor neurons *in vitro*. (a) Embryonic rat (E14) neurons form the anterior horn were mechanically dissociated and cultured in medium containing no serum or trophic factors. Seven days after addition of mock solution, HDGF or BDNF, survival of motor neurons was estimated by immunostaining with ChAT antibody. The number of ChAT-positive neurons was increased by addition of HDGF or BDNF. HDGF clearly promoted neurite outgrowth (white arrows). Scale bar 10 μm. (b) Quantitative analysis of survival of ChAT-positive neurons in 20 visual fields. Mean ± SD $^{**}p < 0.01$ versus mock control (*t*-test).

nerve end after operation was treated with a piece of gel foam soaked with mock, HDGF or BDNF solution (each $n = 5$) and the survival of motor neurons in the facial nerve nucleus was analyzed morphologically after 7 days (Fig. 5). We made serial sections of 6 μm thickness from paraffin-embedded blocks of the brainstem, stained one every six sections with cresyl violet (Nissl staining), counted neurons in the facial nerve nucleus on injured and uninjured sides,

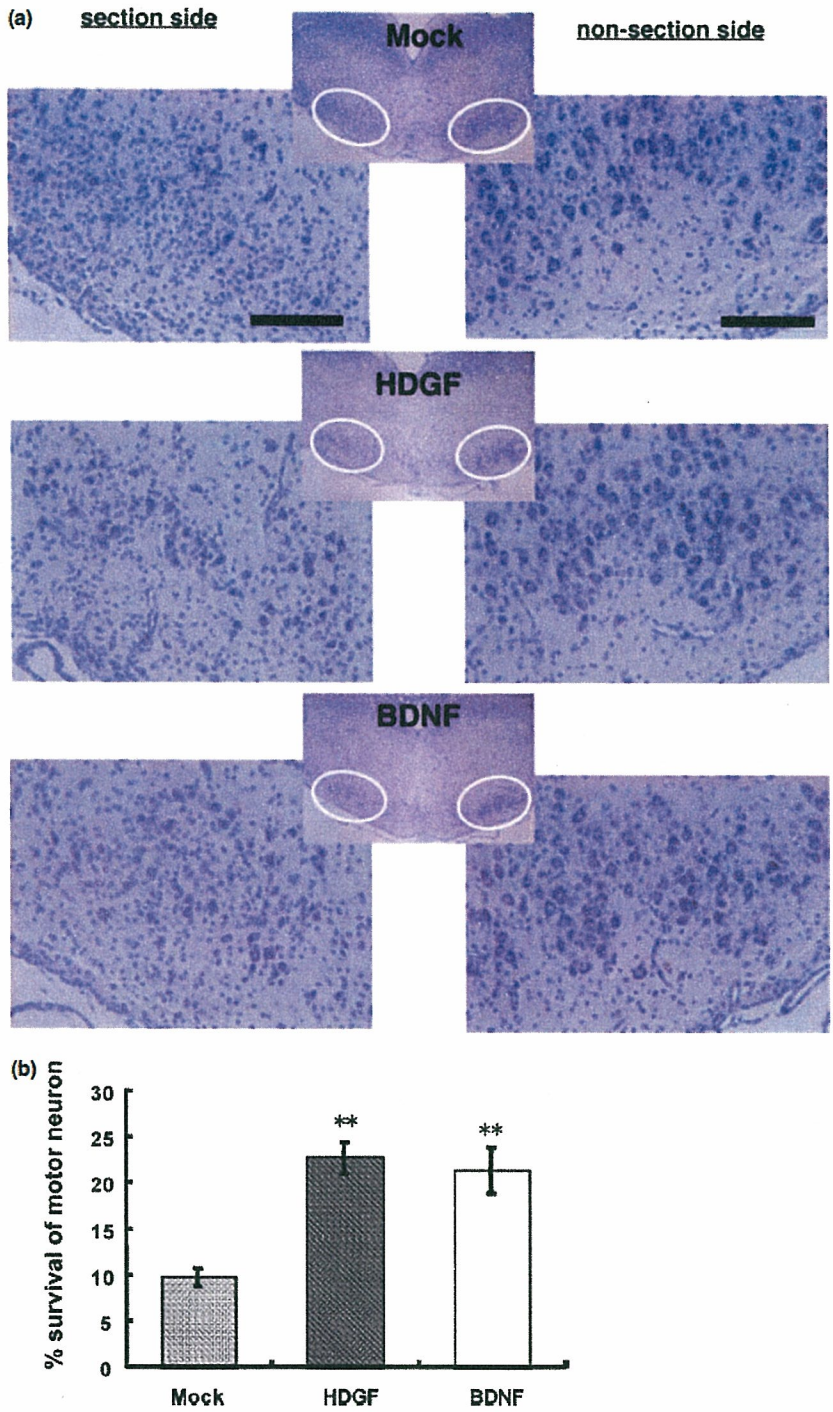

Fig. 5 HDGF promotes survival of facial motor neurons *in vivo*. (a) Representative pictures of the facial nuclei in mock-, HDGF- and BDNF-treated newborn rats (each $n = 5$). The slides were stained by cresyl violet to visualize rough endoplasmic reticulum (Nissl body) of neurons. High-power magnification of facial nuclei on sectioned and non-sectioned sides (left and right panels respectively) shows facial motor neurons distributed in the areas indicated by white circles in the low-power magnification images (central panels). Residual neurons became small and pyknotic. Proliferation of small reactive astrocytes (gliosis) was remarkable especially in mock-treated animals. Scale bars 10 μm. (b) The percentage survival was calculated as the ratio of the total number of facial motor neurons on sectioned side to that on non-sectioned side in each animal. A nucleolus was judged as a neuron. Mean = SD $^{**}p < 0.01$ versus mock control (*t*-test).

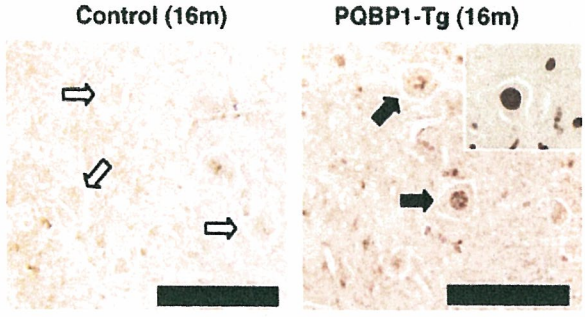

Fig. 6 p53 is increased in spinal motor neurons of PQBP-1 Tg mice. Expression of p53 in the nuclei of spinal motor neurons of PQBP-1 Tg mice at 16 months. Paraffin sections of the spinal cord were stained with p53 antibody. Motor neurons (largest neurons in the anterior horn) with nuclear staining were clearly increased in PQBP-1 Tg mice (right panel). The inlay shows immunohistochemistry with anti-phospho-p53 (Ser389) antibody, indicating that the nuclear staining corresponded to an increase in p53 phosphorylated at Ser389. Unstained neurons in control mice and stained neurons in PQBP-1 Tg mice are indicated with white and black arrows respectively. Scale bars 10 μm.

and calculated the survival ratio, i.e. the total number of neurons on the injured side as a percentage of that on the uninjured side in the same animal. By using a nucleolus with a diameter smaller than 36 μm as a hallmark of a neuron, we excluded overlapped counting of neurons in serial sections. A remarkable reduction in neurons was found throughout the facial nucleus on the lesioned side, supporting the accuracy of the nerve section model. The number of residual neurons was decreased to about 9% of that on the control side in mock-treated animals. The loss of facial motor neurons was clearly repressed by HDGF (Fig. 5). Although residual neurons became small and pyknotic, the survival rate was more than 20% with HDGF and BDNF treatments (Fig. 5). These results support the trophic effect of HDGF on motor neurons *in vivo*.

p53 is increased in spinal motor neurons under degeneration

We next investigated the molecular mechanisms underlying the up-regulation of HDGF in spinal motor neurons. Because several studies have reported an increase in p53 immunoreactivity in spinal motor neurons in patients with ALS (see review by Sathasivam and Shaw 2005) and because we previously found mitochondrial dysfunction and delayed activation of caspase 3 in PQBP1 Tg mice (Marubuchi *et al.* 2005), we focused on this molecule and examined whether p53 is increased in presymptomatic PQBP-1 Tg mice at 12 months by using p53 antibody. Spinal motor neurons of Tg mice showed an increase in p53 from 12 to 16 months. At 16 months, we found that p53 strongly stained the nuclei of motor neurons of PQBP-1 Tg mice (Fig. 6).

Furthermore, we examined spinal cord tissues with antibodies against phosphorylated p53: anti-phospho-Ser15, -Ser20 and -Ser 389 antibodies. Anti-phospho-Ser389 antibody clearly stained the nuclei of spinal motor neurons in the Tg mice at 16 months (Fig. 6, inlay). Because phosphorylation at Ser389 is known to regulate transcriptional activity of p53 (Bruins *et al.* 2004), these findings suggest the involvement of p53 in the transcriptional regulation of HDGF in spinal motor neurons.

p53 up-regulates *HDGF* gene expression

We searched the upstream region of the *HDGF* gene for consensus binding sequences of transcription factors, and found five sequences that partially match the p53-binding consensus (e1–e5; Fig. S2a). We synthesized double-strand oligonucleotides (Fig. S2b) and tested binding of purified recombinant p53 to these sequences in gel mobility shift assays. p53 bound to the e2 sequence with a similar affinity to the p53 consensus sequence in the upstream of the *p21/waf1* gene (Figs 7a and b). The e1 sequence including the e2 sequence showed a weak interaction in which the shift of the e1 probe was similar to that of the e2 probe (Figs 7a and b), indicating that the binding core motif in the e1 probe is the e2 sequence.

We then constructed a CAT reporter plasmid containing the e1/2 sequence in the upstream of the *IFN* promoter and *CAT* gene (Fig. S2a). CAT assay with the reporter plasmid clearly showed that p53 up-regulated transcription of the *HDGF* gene through this *cis* element both in P19 and HEK293 cells (Figs 7c and d). These data suggest that p53 is a factor regulating *HDGF* gene expression and that the increase in p53 in spinal motor neurons of PQBP-1 Tg mice up-regulates transcription of the *HDGF* gene.

HDGF is increased in CSF

Finally, we investigated the connection between the findings that HDGF is a trophic factor for motor neurons (Figs 3–5) and that HDGF is increased in spinal motor neurons of PQBP-1 Tg mice (Figs 1 and 2). HDGF was originally purified from conditioned medium of the human hepatoma-derived cell line, HuH-7 (Nakamura *et al.* 1994), indicating that HDGF is released from cells although HDGF does not possess any signal sequence for secretion. In addition, Zhou *et al.* showed that HDGF is released from primary hippocampal neurons into the medium (Zhou *et al.* 2004). Therefore, we examined at 12 months whether the up-regulation of HDGF leads to an increase in CSF HDGF levels. Western blot analysis of age-matched littermates and Tg mice clearly showed an increase in HDGF in CSF (Fig. S3), suggesting that the increase in HDGF in spinal cord tissue leads to an increase in the CSF. The increase in HDGF in the CSF might directly lead to an increase in HDGF in the extracellular fluid of the CNS inside the blood–brain barrier, and it may support spinal motor neurons. We performed a similar experiment with PQBP-1 Tg mice at 2 months, but HDGF was not yet increased at this age

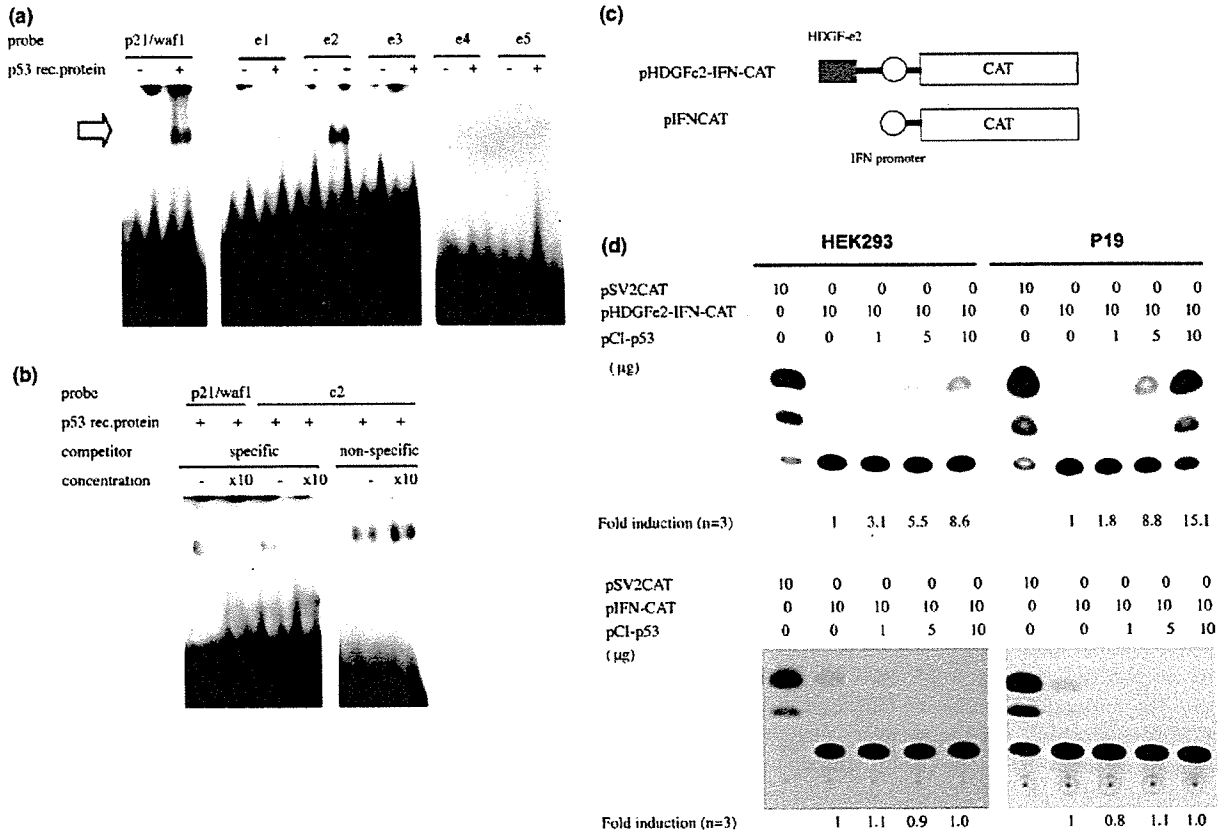

Fig. 7 p53 enhances gene expression via a binding site in the upstream region of the *HDGF* gene. (a) Gel mobility shift assays show definite binding of p53 to e2 and weak binding to e1. Human recombinant p53 protein (5 ng) was incubated with 10 000 cpm of each probe. The p53 consensus sequence in the upstream of *p21/waf1* gene was used as a positive control. (b) Competition by cold probe (10 times the concentration of hot probe) showed that the affinity of p53 for e2 was similar to that for the consensus from the *p21/waf1* gene. Non-specific cold probe did not impair the binding of p53 to e2. (c) Structures of pHDGFe2-IFN-CAT and pIFN-CAT. (d) CAT assays with P19 and HEK293 cells revealed that p53 transactivates gene expression from pHDGFe2-IFN-CAT (upper panels) but not from pIFN-CAT (lower panels). Together with gel mobility shift assay data, the CAT assay showed that p53 transactivated the *HDGF* gene through the e2 element. Total amounts of plasmids used for transfection were adjusted to 21 μg by addition of pBluescriptKS+. Three independent transfections were performed and the fold increase in p53 expression is shown beneath each panel.

(Fig. S3). This means that PQBP-1 expression at 2 months does not promote spinal neurons to up-regulate HDGF. We also tried to obtain CSF from PQBP-1 Tg mice at 20 months, but this was technically impossible because they were unable to tolerate the surgical procedure.

Discussion

We have shown in this study that HDGF is a novel trophic factor for motor neurons *in vitro* and *in vivo*. The effects of HDGF on survival and neurite extension of motor neurons (Figs 3 and 4) are equivalent to those of well known neurotrophic factors including CNTF and BDNF (Arakawa *et al.* 1990; Sendtner *et al.* 1990, 1992; Oppenheim *et al.* 1991, 1992; Yan *et al.* 1992; Henderson *et al.* 1993; Koliatsos *et al.* 1993; Mitsumoto *et al.* 1994). In addition, HDGF clearly supports survival of facial motor neurons in newborn rats after facial nerve section (Fig. 5). As far as we know, this is the first report that has proved HDGF to be a trophic factor for motor neurons.

HDGF is homologous to HMGB1 protein that is released from necrotic cells (Scaffidi *et al.* 2002). Zhou *et al.* recently reported that HDGF is released from primary hippocampal neurons under physiological conditions and that the release is accelerated in necrosis (Zhou *et al.* 2004). Like other HMG family proteins (Scaffidi *et al.* 2002), HMGB1 is not released in apoptosis (Zhou *et al.* 2004), suggesting that HMGB proteins have an extracellular function in a limited type of cell death. HDGF might also be released from neurons in a specific type of cell death distinct from apoptosis.

Regarding the pathological relevance of HGDF to motor neuron degeneration in PQBP-1 Tg mice, we have shown

that (i) HDGF is increased in the cytoplasm of spinal motor neurons of presymptomatic PQBP-1 Tg mice, (ii) HDGF protects spinal motor neurons *in vitro* and *in vivo*, and (iii) the release of HDGF into CSF is increased in PQBP-1 Tg mice. Collectively these data suggest that HDGF seems to play a protective role against motor neuron degeneration in presymptomatic PQBP-1 Tg mice.

We used mice of several ages of (2, 7, 12, 16 and 20 months) and obtained the following data. At 2 months, microarray analysis did not show an increase in HDGF (Marubuchi *et al.* 2005). The increase in HDGF was detected by microarray at 12 months (Fig. 1) and supported by immunohistochemistry at 12 and 16 months (Fig. 2). After 20 months, the increase in HDGF was not confirmed because of the neuronal loss (data not shown). Therefore, HDGF seems to increase transiently during the presymptomatic period and the transient increase might support survival of motor neurons. We also found expression of HDGF in motor neurons of mutant SOD1 Tg mice. However, general pathological roles of HDGF in other motor neuron neurodegenerations remain unknown, and we need further analyses to answer these questions.

The receptor and signaling pathways of HDGF are not yet known. The molecular structure of HDGF is distinct from those of neurotrophins, and the receptor is supposed to be completely different from trk family membrane tyrosine kinases (Barbacid *et al.* 1991; Meakin and Schooter 1992; Ip and Yancopoulos 1994; Chao 2003; Reichardt and Mobley 2004). Meanwhile, RAGE, the receptor for advanced glycation end-products, is known to be the receptor of HMGB proteins that are partially homologous to HDGF (Hori *et al.* 1995). RAGE activates intracellular signaling molecules such as extracellular signal-regulated kinases, p38 mitogen-activated protein kinase, c-Jun N-terminal kinase (JNK) and nuclear factor-κ (Simm *et al.* 2004). The binding of HMGB proteins to the RAGE induces the release of various cytokines including IL-6, tumor necrosis factor-α and transforming growth factor-β from macrophage/monocytes, fibroblasts and vascular smooth muscle cells (Rauvala *et al.* 1988), leading to transcriptional up-regulation of genes involved in apoptosis. Interestingly, however, RAGE-mediated signals also support neurite extension (Rauvala *et al.* 1988; Parkkinen and Rauvala 1991). However, it is still possible that HDGF, which lacks the HMG box conserved among HMG family proteins, interacts with completely different receptors and mediates totally distinct cell signals. HDGF does not possess any typical signal peptide; thus, although the detail is not known, secretion of HDGF from cells might be analogous to that of CNTF without signal peptide.

It is also possible that HDGF is taken up by some membrane transporter(s) and further transferred to the nucleus. We tested this hypothesis by immunostaining motor and cortical neurons cultured with recombinant HDGF or GST-HDGF fusion protein. However, no definite increase in nuclear staining was observed by immunocytochemistry with anti-HDGF or anti-GST antibody (data not shown), refuting this possibility. However, further analyses are required to enable us to understand the molecular mechanisms underlying the biological functions of HDGF.

p53 is implicated in various neurodegenerative disorders including ALS, a typical motor neuron degeneration (de la Monte *et al.* 1998; Martin 2000; Sathasivam and Shaw 2005). It is of interest that spinal motor neurons of presymptomatic PQBP-1 Tg mice showed a similar increase in p53 proteins in this study (Fig. 6) to those of patients with ALS (de la Monte *et al.* 1998; Martin 2000). The mechanisms underlying the increase in p53 protein in ALS are not yet known. The increase in p53 in PQBP-1 Tg mice was not due to transcriptional up-regulation because the level of p53 mRNA was not changed more than 1.5 fold at any time point (data not shown). Therefore, post-transcriptional regulation including translation and/or protein degradation might lead to the increase in p53 in PQBP-1 Tg mice. At 16 months, total p53 and Ser389-phosphorylated p53 (equivalent to phophorylation of human p53 at Ser392) were increased in the nuclei of spinal motor neurons of PQBP-1 Tg mice (Fig. 6). Phospho-p53 was weakly stained at 12 months (data not shown). Collectively, these results suggest post-transcriptional modification and/or degradation of p53 to increase expression of HDGF.

The role of p53 in motor neuron degeneration is still under debate (see review by Sathasivam and Shaw 2005). Increased p53 might trigger apoptotic cell death signaling, whereas cross-breeding of mutant SOD1 Tg mice with p53 knockout mice did not show any protective effect on neurodegeneration (Kuntz *et al.* 2000; Prudlo *et al.* 2000). On the other hand, cell-protective roles of p53 in various types of DNA repair have been recognized recently (see review by Sengupta and Harris 2005). Our finding that increased p53 in motor neurons up-regulates a trophic factor may shed light on the role of p53 in neurodegeneration and may promote understanding of the controversial functions of p53 in neurodegenerative disorders.

In summary, starting with microarray analysis of a mouse model of motor neuron disease, we found a novel trophic factor for motor neurons, HDGF, that is endogenously up-regulated in the affected neurons. The change in spinal motor neurons and CSF of presymptomatic Tg mice suggest that HDGF plays a role in the motor neuron degeneration in this mouse model. In addition, some of our results suggest a relevance of HDGF to ALS. Our data suggest that HDGF and its derivatives may be developed as candidate drugs to inhibit progression of motor neuron degeneration.

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Supplementary material

The following material is available for this paper online.

Fig. S1 (a) Preparation of recombinant mouse HDGF. Samples at various steps of production and purification were stained with CBB. G, GST protein expressed in E. coli; M, molecular weight marker; F, GST-HDGF fusion protein. GST-HDGF fusion protein was digested by Factor X from 3 to 16 h and recovered by heparin column. FT, flow through; W, wash; E, elute; PE, postelution wash. Arrow indicates recombinant HDGF. (b) Four antibodies against HDGF were characterized by western blotting using extracts of either fusion protein-expressing or non-expressing bacteria (upper panel). Arrow indicates the specific band of HDGF. The lower panel is a Coomassie-stained gel showing the amounts of sample blotted. C, non-transformed E. coli cells; H, recombinant HDGF-expressing E. coli cells.

Fig. S2 (a) Schematic structure of the upstream region of the mouse HDGF gene. Five possible binding sites for p53 (e1–e5) are indicated by white boxes. Exon 1 is indicated as a black box. (b) The sequences of e1–e5 used for the gel mobility shift assay are shown. The p53 consensus sequence in the upstream of the p21/waf1 gene was used as positive control.

Fig. S3 Western blot analysis of CSF protein with anti-HDGF antibody showed that HDGF is increased in the CSF of PQBP-1 Tg mice. CSF was centrifuged to exclude cells and then separated by SDS–polyacrylamide gel electrophoresis. We performed western blot with three Tg and littermate (C) mice. Hela cells expressing HDGF were used as a positive control (PC). (b) Band intensities were quantified by image analyzer and corrected with respect to those for β-actin. A representative case is shown in the left panel. The fold increase of HDGF in Tg mice (n = 3, 12 months) was calculated by defining the mean intensity of HDGF in the control group as 1.0. Right panel shows the mean ± SEM fold increase in HDGF in CSF of Tg mice.

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Research Report

Adenoviral gene transfer of BDNF and GDNF synergistically prevent motoneuron loss in the nucleus ambiguus

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ABSTRACT

We have previously shown that neuroprotective effects of an adenoviral glial cell line-derived neurotrophic factor (GDNF) gene transfer on the lesioned adult rat motoneurons in the nucleus ambiguus. In the present study, we examined neuroprotective effects of adenoviral gene transfer of brain-derived neurotrophic factor (BDNF) or/and GDNF to motoneurons in nucleus ambiguus using an adult rat vagal nerve avulsion model. The animals avulsed and inoculated with adenoviral vectors encoding BDNF (AxCAmBDNFME) or/and GDNF (AxCAhGDNF) showed immunolabeling for BDNF or/and GDNF in the nucleus ambiguus on the treated side, respectively, and expression of virus-induced BDNF or/and GDNF mRNA transcripts in the brainstem tissue that contained the nucleus ambiguus of the treated side. The treatment with AxCAhGDNF or AxCAmBDNFME significantly prevented the loss of vagal motoneurons in comparison to the control; the protective effect of AxCAmBDNFME was greater than that of AxCAhGDNF. The combined treatment with AxCAmBDNFME and AxCAhGDNF acted synergistically and significantly larger number of vagal motoneurons was preserved as compared to either AxCAmBDNFME treatment or AxCAhGDNF treatment. The treatment with AxCAmBDNFME or/and AxCAhGDNF after avulsion also suppressed the activity of nitric oxide synthase in lesioned motoneurons in the nucleus ambiguus. These results indicate that adenovirus-mediated BDNF and GDNF gene transfer may prevent the degeneration of motoneurons in humans after either vagal nerve injury or recurrent laryngeal nerve injury.

1. Introduction

Laryngeal paralysis most often occurs as a result of iatrogenic vagal or recurrent laryngeal nerve injury in oncologic surgery of the head and neck. Injury to the recurrent laryngeal nerve occurs in 1–2% of all thyroid surgery (Eisele, 1993). Other surgical operations utilizing a cervical approach carry a similar substantial incidence of true vocal cord paralysis. Patients with unilateral laryngeal paralysis can present with disabling symptoms related to aspiration, dysphagia and dysphonia. Except for laryngeal reinnervation procedures, surgical options for the management of patients with unilateral laryngeal

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paralysis (vocal fold injection, thyroplasty, and arytenoid adduction) only achieve vocal fold medialization due to static change in the vocal fold tissue or laryngeal framework, and such defects can never be neurologically restored.

Laryngeal reinnervation procedures have had little impact on the return of dynamic laryngeal function and are still not widely accepted as a treatment option. Failure of reinnervation procedures results from a decrease in motor fiber density, existing laryngeal muscle atrophy, motoneuron loss in the nucleus ambiguus and inappropriate or misdirected reinnervation by antagonistic motoneurons (Crumley, 1989, 1994, 2000; Flint et al., 1991).

Several neurotrophic factors, including brain-derived neurotrophic factor (BDNF), neurotrophin (NT)-3, NT-4/5, ciliary neurotrophic factor (CNTF), glial cell line-derived neurotrophic factor (GDNF), have been shown to prevent the death of facial and spinal motoneurons after axotomy in neonatal rodents (Houenou et al., 1996). BDNF has been shown to promote the survival of developing motor neurons in vitro and to rescue motor neurons from axotomy-induced cell death in vivo, and its effects are mediated via two classes of receptors (trkB and p75) (Friedman and Greene, 1999; Yano and Chao, 2000). GDNF mediates its effects via a single receptor complex composed of a GDNF-family receptor alpha 1 and a Ret (Saarma and Sariola, 1999). GDNF has been shown to promote axonal regeneration in adult rats (Chen et al., 2001).

In adult rodents, administration of BDNF and GDNF proteins has been reported to prevent the loss of spinal motoneurons after spinal root avulsion (Kishino et al., 1997; Li et al., 1995; Oppenheim et al., 1995; Chai et al., 1999). And following avulsion of a spinal ventral root, an adeno-associated viral (AAV) vector-mediated BDNF and GDNF gene transfer prevents the loss of spinal motoneurons (Blits et al., 2004). We have shown that an adenoviral vector-mediated BDNF and GDNF gene transfer prevents the loss of adult facial motoneurons (Sakamoto et al., 2000; Saito et al., 2003). These data suggesting the potential uses of these viral vectors as new approaches in treating patients with adult-onset motoneuron injury and motor neuropathies (Yuan et al., 2000; Zurn et al., 1996; Gimenez y Ribotta et al., 1997; Novikov et al., 2000; Clatterbuck et al., 1994).

The potential of gene therapy in the treatment of laryngeal paralysis has been demonstrated. Insulin-like growth factor I (IGF-I) gene transfer to the denervated thyroarytenoid muscle has been shown to prevent muscle atrophy while improving motor endplate morphology in a rat laryngeal paralysis model (Shiotani et al., 1998, 1999; Flint et al., 1999). Recently we have shown that adenoviral LacZ gene transfer can transduce the β-galactosidase gene in injured adult motoneurons in the nucleus ambiguus after vagal nerve avulsion, and the successful selective induction of virus-induced foreign gene in these neurons (Saito et al., 2003). We have also demonstrated adenoviral GDNF gene transfer after vagal nerve avulsion prevented the loss of lesioned motoneurons in the nucleus ambiguus compared to the adenoviral LacZ gene transfer or PBS-treated control (Saito et al., 2003).

In the present study, to assess the possibility of BDNF gene therapy for motoneuron loss in after vagal or recurrent laryngeal nerve injury, one of the main problems in the treatment of laryngeal paralysis, we examined the neuroprotective effect of adenoviral BDNF gene transfer on motoneurons in the nucleus ambiguus using an adult rat vagal nerve avulsion model. We also investigated the enhancement of neuroprotective effect of simultaneous adenoviral GDNF gene transfer in addition to BDNF to motoneurons in nucleus ambiguus using the same model.

2. Results

2.1. *Adenoviral vector-mediated BDNF and GDNF gene expression in nucleus ambiguus*

We inoculated AxCAmBDNFME or/and AxCAhGDNF into the avulsed animals and examined the expression of exogenous BDNF and GDNF by immunohistochemistry and RT-PCR analysis for BDNF and GDNF. One week after the vagal nerve avulsion and treatment with AxCAmBDNFME or/and AxCAhGDNF, distinct immunostaining for BDNF and GDNF was demonstrated in the nucleus ambiguus on the operated side (Fig. 1), being observed by 4 weeks after these treatments (data not shown) as in the case of facial nerve and cervical root avulsions that have been previously reported (Sakamoto et al., 2000; Watabe et al., 2000; Holstege et al., 1998). There was no definite immunostaining for these factors in vagal motoneurons in the side contralateral to the adenovirus treatment as well as those in unoperated control animals or operated animals without adenoviral gene transfer (data not shown). These results indicate that the treatment with AxCAmBDNFME or/and AxCAhGDNF after vagal nerve avulsion induces positive immunolabeling for BDNF and GDNF in lesioned vagal neurons.

Three days after the vagal nerve avulsion and treatment with AxCAmBDNFME or/and AxCAhGDNF, RT-PCR analysis showed the expression of virus-induced mouse BDNF and human GDNF mRNA transcripts in the tissue of the nucleus ambiguus on the treated side. The infection of the vector to the contralateral nucleus ambiguus was not detected by RT-PCR for BDNF and GDNF (Fig. 2).

2.2. *Neuroprotective effect of AxCAmBDNFME or/and AxCAhGDNF on injured adult motoneuron*

We then inoculated AxCAmBDNFME, AxCAhGDNF, AxCAmBDNFME and AxCAhGDNF, AxCALacZ or PBS after left vagal nerve avulsion to examine the neuroprotective effect. Two to 4 weeks after the vagal nerve avulsion followed by the treatment of PBS, there was marked atrophy of nucleus ambiguus and the loss of vagal motoneurons with prominent degeneration (Fig. 3). The number of vagal motoneurons gradually decreased and reached almost 55% of the contralateral side by 4 weeks after vagal nerve avulsion. The treatment with AxCAhGDNF significantly prevented the loss of vagal motoneurons (73.5 ± 3.4% survival at 2 weeks, $n = 4$, 71.3 ± 3.2% survival at 4 weeks, $n = 4$) in comparison to the treatment with PBS (59.9 ± 2.4% survival at 2 weeks, $n = 4$, $P < 0.001$, 55.1 ± 4.0% survival at 4 weeks, $n = 4$, $P < 0.001$) or LacZ (61.8 ± 3.2% survival at 2 weeks, $n = 4$, $P < 0.001$, 56.3 ± 4.3% survival at 4 weeks, $n = 4$, $P < 0.001$), as we had shown in the previous study (Saito et al., 2003). The treatment with

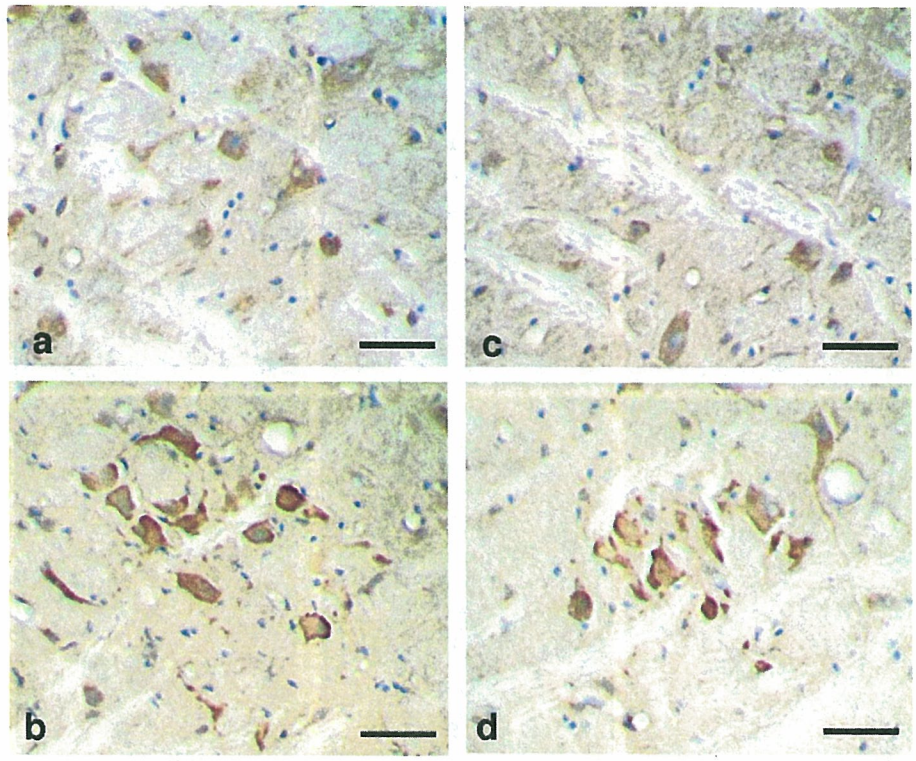

Fig. 1 – Immunohistochemistry for BDNF and GDNF of contralateral (a, c) and ipsilateral (b, d) nucleus ambiguus after vagal nerve avulsion. One week after vagal nerve avulsion and AxCAmBDNFME treatment (a, b), motoneurons were immunoreactive for BDNF in the ipsilateral (treated) side (b). One week after vagal nerve avulsion and AxCAhGDNF treatment (c, d), motoneurons were immunoreactive for GDNF in the ipsilateral (treated) side (d). Photographs (a) and (b), and (c) and (d) are taken from the same section. Scale bar = 50 μm.

AxCAmBDNFME significantly prevented the loss of vagal motoneurons (78.4 ± 2.2% survival at 2 weeks, n = 4, 76.5 ± 2.0% survival at 4 weeks, n = 4) in comparison to the treatment with PBS (at 2 weeks, $P < 0.001$, at 4 weeks, $P < 0.001$) and even to the treatment with AxCAhGDNF (at 2 weeks, $P < 0.001$, at 4 weeks, $P < 0.001$). The treatment with

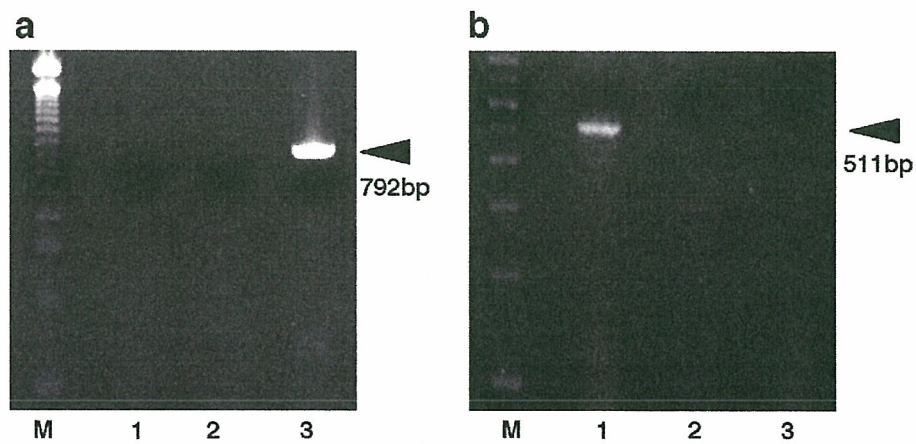

Fig. 2 – Gene expression detected by RT-PCR for virus-induced BDNF and GDNF mRNA transcripts in nucleus ambiguus 3 days after the vagal nerve avulsion and AxCAmBDNFME (a) or AxCAhGDNF (b) treatment. Lane M, size markers; lane 1, brainstem tissue on the contralateral side with reverse transcription; lane 2, brainstem tissue on the ipsilateral side without reverse transcription; lane 3, brain stem tissue on the ipsilateral side with reverse transcription. A positive band for BDNF mRNA was observed in lane 3. (b) Lane M, size markers; lane 1, brainstem tissue on the ipsilateral side with reverse transcription; lane 2, brainstem tissue on the ipsilateral side without reverse transcription; lane 3, brain stem tissue on the contralateral side with reverse transcription. A positive band for GDNF mRNA was observed in lane 1.

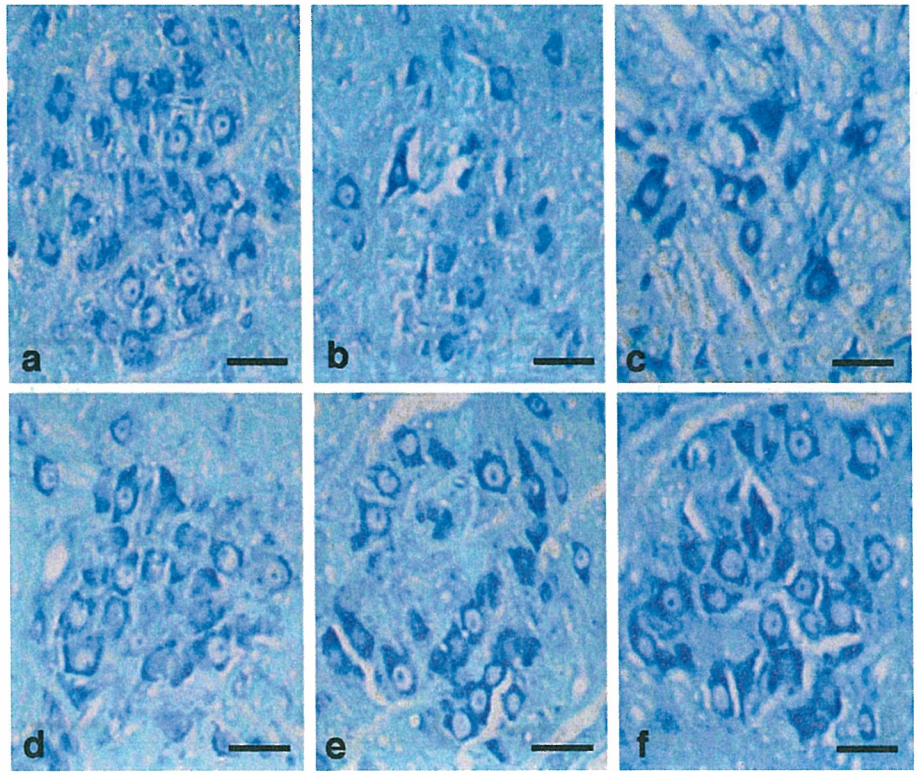

Fig. 3 – Vagal motoneurons in the nucleus ambiguus 4 weeks after vagal nerve avulsion. Photomicrographs show the nucleus ambiguus on the contralateral (a) or ipsilateral (b–f) side after avulsion and the treatment of PBS (a, b), AxCALacZ (c), AxCAhGDNF (d), AxCAmBDNFME (e), or AxCAmBDNFME and AxCAhGDNF (f). There was no infiltration or vascular proliferation in the nucleus ambiguus that would reflect a immunogenic reaction against adenoviral infection (c–f). Photographs (a) and (b) are taken from the same section. Toluidine blue stain. Scale bar = 50 μm.

AxCAmBDNFME and AxCAhGDNF strongly prevented the loss of vagal motoneurons (85.1 ± 1.6% survival at 2 weeks, $n = 4$, 84.7 ± 2.2% survival at 4 weeks, $n = 4$) and significantly larger number of vagal motoneurons was preserved as compared to either AxCAmBDNFME treatment (at 2 weeks, $P < 0.003$, at 4 weeks, $P < 0.003$) or AxCAhGDNF treatment (at 2 weeks, $P < 0.001$, at 4 weeks, $P < 0.001$) (Fig. 4).

The neuronal NADPH diaphorase activity has been reported to be ascribed to NOS (Dawson et al., 1991; Hope et al., 1991) and is induced after nerve injury being concomitant with the degeneration of motoneurons (Estevez et al., 1998; Gonzalez et al., 1987; Jia et al., 1994; Ruan et al., 1995; Wu, 1993). The treatment with PBS or AxCALacZ did not suppress the induction of NOS activity after vagal nerve avulsion. The treatment with AxCAmBDNFME or/and AxCAhGDNF prevented the induction of NOS activity in lesioned nucleus ambiguus, as demonstrated by NADPH diaphorase histochemistry (Fig. 5). We found no histological evidence of either inflammation or cell infiltration in the nucleus ambiguus that would reflect immunogenic reaction against adenovirus infection.

3. Discussion

In the previous report, we have demonstrated that AxCALacZ can transduce β-galactosidase gene in injured adult vagal motoneurons after vagal nerve avulsion in the nucleus ambiguus, suggesting the diffusion of the virus through the jugular foramen, adsorption to avulsed axons, retrograde

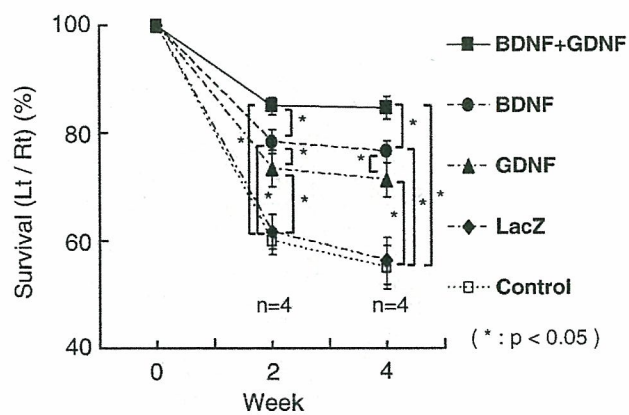

Fig. 4 – Time course of the motoneuron loss in the nucleus ambiguus after the operation. The percentages of surviving motoneurons in nucleus ambiguus (treated side/ contralateral side) are plotted. The data following vagal nerve avulsion ($n = 4$) and treatment of PBS, AxCALacZ, AxCAhGDNF, AxCAmBDNFME, or AxCAhGDNF and AxCAmBDNFME is shown. The data are the mean ± S.D. (bars). Statistical comparisons was done by Fisher test (*$P < 0.05$).

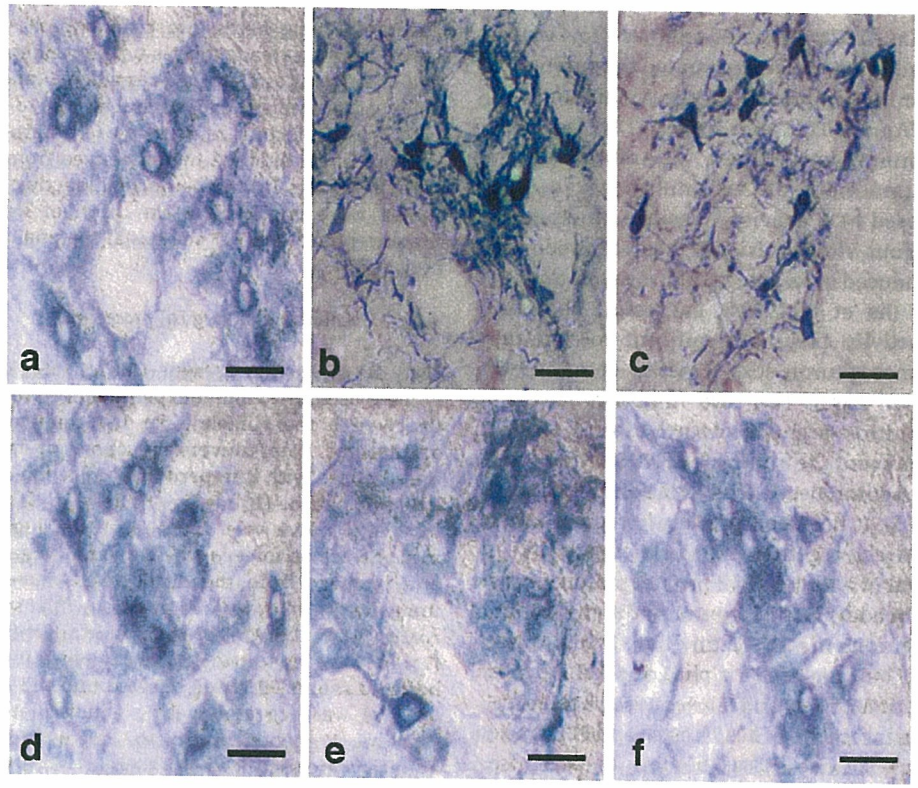

Fig. 5 – NADPH diaphorase histochemistry of the nucleus ambiguus, 14 days after vagal nerve avulsion and treatment of PBS (b), AxCALacZ (c), AxCAhGDNF (d), AxCAmBDNFME (e), or AxCAhGDNF and AxCAmBDNFME (f). Compared to the normal contralateral side (a), strong expression of NOS (b) and (c) were observed after PBS and AxCALacZ administration on the avulsed side. When AxCAhGDNF or/and AxCAmBDNFME was inoculated, induction of NOS was suppressed (d–f) on the avulsed side. Photographs (a) and (b) are taken from the same section. Scale bar = 50 μm.

transport of the virus from the treated site to soma of the vagal motoneurons and successful induction of the virus-induced foreign gene in vagal motoneurons. We have also demonstrated that the treatment with AxCAhGDNF after vagal nerve avulsion induced intense immunolabeling for GDNF in injured vagal motoneurons, indicating that the transduced GDNF gene can operate to rescue the avulsed vagal motoneurons. The treatment with AxCAhGDNF after avulsion remarkably prevented the loss of lesioned vagal motoneurons, ameliorated ChAT immunoreactivity, and suppressed the NOS activity in the nucleus ambiguus (Saito et al., 2003). We observed remarkable motoneuron loss after the vagal nerve avulsion in nucleus ambiguus similar to that in the facial nucleus after facial nerve avulsion and the ventral horn after spinal root avulsion that described in previous reports (Sakamoto et al., 2000; Watabe et al., 2000). In the present study, we investigated whether the treatment with AxCAmBDNFME or/and AxCAhGDNF can prevent the degeneration of vagal motoneurons in adult rats after vagal nerve avulsion.

One week after the vagal nerve avulsion and local treatment of adenoviral vectors used in this study, we demonstrated vagal motoneurons immunolabeled for BDNF and GDNF. RT-PCR analysis also confirmed the expression of virus-induced BDNF and GDNF mRNA transcripts in the brainstem tissue containing vagal motoneurons 3 days after avulsion and treatment. These results indicate that these adenoviral vectors successfully infect the lesioned motoneurons. Diverse degenerative changes in the neurons begin two to four postoperative days after either avulsion, transection, or crush injury of the cranial nerve (Soreide, 1981a,b,c). These immunostaining and RT-PCR analysis data suggest that AxCAmBDNFME or/and AxCAhGDNF can transduce BDNF or/and GDNF in lesioned motoneurons from the onset of their degenerative change after vagal nerve avulsion. Since adult motoneuron death of peripheral nerves can be induced by avulsion but not transection or crush (Koliatsos et al., 1994; Lowrie and Vrbova, 1992; Ruan et al., 1995; Soreide, 1981a,b,c; Wu, 1993), we consider the vagal nerve avulsion deserved to be an excellent model system to examine the effects of adenoviral vectors expressing various neurotrophic factors and neuroprotective molecules on the survival of adult motoneurons. Subsequently, we observed significant motoneuron loss 2 and 4 weeks after the vagal nerve avulsion in control animals as described in our previous report (Saito et al., 2003).

The treatment with AxCAhGDNF significantly prevented the loss of vagal motoneurons in comparison to the control as examined in this study as well as our previous study using AxCAhGDNF (Saito et al., 2003). In AxCAmBDNFME-treated animals, a significantly larger number of survived motoneurons in nucleus ambiguus were observed as compared to PBS-

treated control and AxCAhGDNF-treated animals 2 to 4 weeks after inoculation. In addition, we demonstrated the survival of motoneurons was further improved by the treatment with combination of AxCAmBDNFME and AxCAhGDNF as compared to the treatment with either AxCAmBDNFME or AxCAhGDNF. The treatment of AxCAmBDNFME or/and AxCAhGDNF suppressed NOS activity in lesioned motoneurons in nucleus ambiguus after avulsion. It has been reported that NOS activity is induced in lesioned adult vagal motoneurons after vagotomy (Jia et al., 1994), suggesting that the induction of NOS activity plays a significant role in the initiation of adult motoneuron loss (Estevez et al., 1998; Ruan et al., 1995; Wu, 1993). The NOS inhibitors such as nitroarginine or Nù-nitro-L-arginine methyl ester (L-NAME) have been shown to prevent the induction of NOS activity and the subsequent motoneuron death after avulsion (Wu and Li, 1993; Ruan et al., 1995). It has been previously reported that BDNF and GDNF can act together either synergistically in promoting the survival of axotomized neonatal motoneurons (Vejsada et al., 1998), or additively in promoting differentiation of embryonic motoneurons in vitro (Zurn et al., 1996). At the cell membrane, GDNF binding to GFR alpha1 and Ret induces tyrosine phosphorylation of Ret, and subsequent phosphorylation of MEK and Akt, indicative of MAP kinase and PI3 kinase activity, respectively. Likewise, BDNF binding to trkB also induces trkB phosphorylation, and downstream phosphorylation of MEK and Akt. Thus there is both molecular and functional overlap of signaling pathways induced by both BDNF and GDNF stimulation, which may explain the large beneficial effect of combined BDNF and GDNF (Dolcet et al., 1999; Soler et al., 1999). Taken together, the present results indicate the surviving effects of AxCA.mBDNFME and AxCAhGDNF on the injury and death of adult vagal motoneurons.

The neuronal depopulation results in the innervation of a relatively large number of muscle units by the residual neurons and thus is considered to contribute to the development of laryngeal synkinesis after laryngeal nerve injury (Baumgartner and Shine, 1998; Chen et al., 2001). Adenoviral BDNF and GDNF gene therapy may be useful for not only preventing motor neuron loss in the nucleus ambiguus but also laryngeal synkinesis after vagal nerve injury or recurrent laryngeal nerve injury by inoculation of the vector at the injured site of the nerve during the head and neck surgery. For clinical applications, controversy remains regarding the potential risks of virus-mediated gene therapy (St George, 2003; Check, 2005; Raper et al., 2003), particularly when applied to non-lethal benign diseases such as laryngeal paralysis. To overcome this problem, safety of the vector must be demonstrated prior to clinical application. Preliminary experiments of non-viral gene transfer systems are also currently underway.

4. Experimental procedures

4.1. *Adenovirus preparation*

Replication-defective recombinant adenoviral vectors encoding human GDNF (AxCAhGDNF) and mouse BDNF fused with Myc epitope-His/IRES-EGFP at the 3′ end (AxCAmBDNFME) were generated using a cassette cosmid pAxCAwt (TaKaRa, Osaka, Japan) carrying an adenovirus type-5 genome lacking the E3, E1A, and E1B regions as described elsewhere (Kanegae et al., 1995). The ability of recombinant adenoviral vectors to induce expression of neurotrophic factors in vitro was confirmed by Western blot analysis of conditioned media derived from COS1 cells infected with the vectors. In vitro neurotrophic activities of the conditioned media were checked by the survival assays using E14 rat mesencephalic and E15 rat spinal motoneuron cultures (Miyake et al., 1996).

4.2. *Animals and surgical procedures*

Forty-six male adult Sprague–Dawley rats (12 weeks old, 340–360 g) were used. All animal experiments were conducted in accordance with the guideline for the care and use of laboratory animals of Keio university school of medicine. Rats were anesthetized with intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Under a dissecting microscope, the left vagal nerve was exposed at the jugular foramen. Using microhemostat forceps, the proximal vagal nerve was avulsed and removed from the distal vagal nerve by gentle traction. Immediately following the avulsion, 10 μl solution of AxCALacZ, AxCAhGDNF, AxCAmBDNFME, AxCAmBDNFME and AxCAhGDNF (1×10^8 pfu each for single and combined injection) or phosphate buffered saline (PBS) was injected into the jugular foramen. The wounds were covered with a small piece of gelatin sponge (Spongel, Yamanouchi, Tokyo, Japan) and suture closed. The animals were sacrificed at 3 days ($n = 9$), 1 week ($n = 9$), 2 weeks ($n = 35$) and 4 weeks ($n = 20$) after the operation as described below.

4.3. *Reverse transcription-polymerase chain reaction (RT-PCR)*

Three days after vagal nerve avulsion and the treatment with AxCAmBDNFME or/and AxCAhGDNF ($n = 3$ per group), the animals were euthanized with a lethal dose of ketamine, and the brainstem tissue containing the nucleus ambiguus was collected. 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, (Invitrogen). First strand cDNA was synthesized from 380 ng of total RNA using a random primer and Superscript II reverse transcriptase (Invitrogen) for one PCR analysis. The PCR reactions were performed in PCR buffer containing cDNA template, 200 μM dNTPs, 2 mM MgCl2, 0.2 μM of each primer and 25 U/ml of Taq DNA polymerase (TaKaRa, Osaka, Japan). Specific oligonucleotide primers for PCR were designed to amplify adenovirus-derived mouse BDNF cDNA (forward, 5′-ATGACCATCCTTTTCCTTAC-3′; reverse, 5′-CAGATCCTGTTCTGAGATGA-3′), which produces 792 bp amplified products and human GDNF cDNA (forward, 5′-ATGAAGTTATGGGATGTCGT-3′; reverse, 5′-TCACCAGCCTTC-TATTTCTG-3′), which produces 511 bp amplified products (Watabe et al., 2000).

The PCR amplification program consisted of denaturation at 95 °C for 1 min, annealing at 55 °C for 1.5 min, and extension at 72 °C for 1.5 min, for 40 cycles. For negative control reactions, non-reverse transcripted RNA samples and reverse transcripted brain tissue of non-treated side were processed for PCR. The PCR products were subjected to electrophoresis on 2% agarose gel stained with ethidium bromide.

4.4. *Histological analysis*

For motoneuron cell counting, immunohistochemistry and histochemistry, the rats were anesthetized with a lethal dose of ketamine and transcardially perfused with PBS followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brain stem tissue was dissected and immersion fixed in the same fixative.

For motoneuron cell counting, the brain stem tissues were dehydrated and embedded in paraffin, and serial transverse sections (7 μm thickness) were made. Every fifth section (28 μm interval) was collected, deparaffinized and stained with Toluidine Blue, and nucleus ambiguus motoneurons having nuclei containing distinct nucleoli on both sides were counted in 20 sections. We did not apply any correction factors for data analysis, because the ambiguus neurons had a maximum diameter of 21.8 ± 5.0 μm in the previous study and these neurons were counted only once in every fifth section with 28 μm intervals. The data are expressed as the mean ± SD from 4 animals (2 or 4 weeks post operation) and statistical significance was assessed between five groups by Fisher test.

For immunohistochemistry and histochemistry, the brain stem tissues were cryoprotected in 30% sucrose in PBS and serial transverse frozen sections were cut at 10 μm.

For immunostaining of BDNF and GDNF, the animals (n = 3 per group) were perfused 1 week after treatment, and sections were pretreated with 0.3% H_2O_2 in PBS, rinsed in 0.1% Triton X-100 in PBS (T-PBS) and preincubated in 3% normal goat serum in T-PBS. After the treatment with ABC blocking kit (Vector, Burlingame, CA) according to the manufacturer's instructions, the sections were incubated overnight at 4 °C with rabbit polyclonal antibody to BDNF (Santa Cruz Biotech., CA) or GDNF (Santa Cruz Biotech., CA) at dilution of 1:500, followed by incubation with biotinylated anti-rabbit IgG at a dilution of 1:100 and ABC reagent (Vector), visualized by 3,3′-diaminobenzidine tetrahydrochloride (DAB)-H_2O_2 solution and counterstained with hematoxylin.

For NADPH diaphorase histochemistry that visualizes NOS activity (Ruan et al., 1995; Wu, 1993), the animals (n = 3 per group) were perfused 2 weeks after treatment, and frozen sections were rinsed in 0.1% Triton X-100 in 0.1 M Tris–HCl buffer, pH 8.0, and incubated for 3 h at 37 °C in 1 mg/ml NADPH (Sigma, St. Louis, MO) and 0.2 mg/ml nitroblue tetrazolium (Sigma) in 0.1 M Tris–HCl buffer, pH 8.0.

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