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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Gene Therapy for Laryngeal Paralysis

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Objectives: The surgical options for laryngeal paralysis only achieve static changes of vocal fold position. Laryngeal reinnervation procedures have had little impact on the return of dynamic laryngeal function. The development of a new treatment for laryngeal paralysis, aimed at the return of dynamic function and neurologic restoration and regeneration, is necessary.

Methods: To assess the possibility of gene therapy for laryngeal paralysis aiming for the return of dynamic laryngeal function, we investigated the therapeutic effects of gene therapy using rat laryngeal paralysis models.

Results: In a rat vagal nerve avulsion model, we transferred glial cell line–derived neurotrophic factor (GDNF) gene into the nucleus ambiguus using an adenovirus vector. Two and 4 weeks after the GDNF gene transfer, a significantly larger number of surviving motoneurons was observed. These neuroprotective effects of GDNF gene transfer were enhanced by simultaneous brain-derived neurotrophic factor gene transfer. In a rat recurrent laryngeal nerve crush model, we transferred GDNF gene into recurrent laryngeal nerve fibers after crush injury. Two and 4 weeks after GDNF gene transfer, we observed significantly faster nerve conduction velocity and better vocal fold motion recovery.

Conclusions: These results indicate that gene therapy could be a future treatment strategy for laryngeal paralysis. Further studies will be necessary to demonstrate the safety of the vector before clinical application.

Key Words: BDNF, brain-derived neurotrophic factor, functional recovery, GDNF, gene therapy, glial cell line–derived neurotrophic factor, motoneuron loss, nerve conduction velocity.

INTRODUCTION

Current treatment of recurrent laryngeal nerve (RLN) paralysis focuses on static repair of vocal fold position, rather than on restoration of movement of the paralyzed vocal fold. The results of surgery are accordingly often less than satisfactory. In particular, in patients with bilateral vocal fold paralysis, preserving both airway and voice quality is difficult, and a choice must be made between closing the tracheostomy and better voice quality. Surgery to restore innervation by nerve suturing or transplantation, with the goal of improving dynamic function, is generally ineffective in restoring vocal fold mobility. Therefore, novel therapies must be developed from the neurologic perspective of treating RLN paralysis as a motor nerve paralysis. This requires research aimed at restoring vocal fold mobility.

Several neurologic problems may occur with denervation (Fig 1). Injury of the RLN can cause 1) loss of motoneurons in the nucleus ambiguus, 2) degeneration and poor regeneration of nerve fibers and motor end plates, and 3) laryngeal muscle atrophy. Each of these problems needs to be addressed for restoration of RLN motor function. Furthermore, nonselective regeneration can lead to (4) faulty innervation after nerve regeneration, in which the wrong neurons may innervate other laryngeal muscles (misdirected reinnervation), so that even if innervation is reestablished, proper motor function is not restored (synkinesis).

Although clinicians are aware of each of these problems, definitive therapy has not been available. However, recent advances in neurology have led to the discovery of several neurotrophic factors with potent trophic effects on myoneural function (ie, affecting motoneurons, motor nerve fibers, motor end plates, and muscle tissue), including regenerative and protective effects.[1] They include nerve growth factor, brain-derived neurotrophic factor (BDNF), glial cell line–derived neurotrophic factor (GDNF), fibroblast growth factor, ciliary neurotrophic factor, and insulin-like growth factor I (IGF-I). These neurotrophic factors may be useful in treating RLN paralysis.

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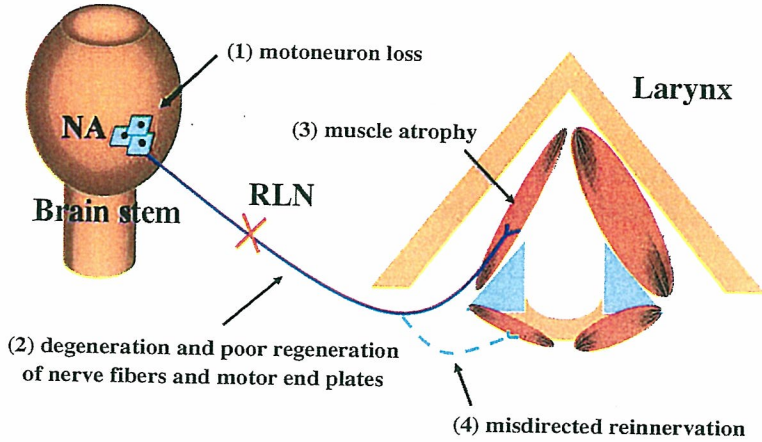


Fig 1. Neurologic problems after injury of recurrent laryngeal nerve (RLN). (1) — loss of motoneurons in nucleus ambiguus (NA); (2) — degeneration and poor regeneration of nerve fibers and motor end plates; (3) — laryngeal muscle atrophy; (4) — misdirected reinnervation (synkinesis). These problems need to be solved for return of vocal fold movement.

For the clinical application of neurotrophic factors, the most convenient route of administration is direct injection of neurotrophic factor proteins into atrophied laryngeal muscle or injured RLN fibers. However, for these neurotrophic factors to have an effect, certain concentrations must be maintained over time. Once they are injected, these neurotrophic factor proteins are rapidly absorbed and eliminated; thus, several injections per day over a long period are required. Systemic administration is also an option, but carries an increased risk of adverse reactions. This is where gene transfer (gene therapy) can be useful.

Gene therapy can be used as a specific type of drug delivery system. Instead of injecting the protein drug (in this case, the neurotrophic factor), the gene (DNA) encoding the protein can be transferred into human cells via a gene carrier (vector), and the proteins can then be made in the cells. Once the vector is injected, proteins will be expressed in high concentration at the delivery site for a few weeks up to 1 month. If an extrachromosomal vector for gene transfer is selected, gene expression will spontaneously decrease, so the risk of expression for longer than required is avoided. In addition, local gene expression carries a lower risk of adverse reactions than systemic administration. Taking the above into consideration, we embarked on research toward the clinical application of gene therapy in RLN paralysis.

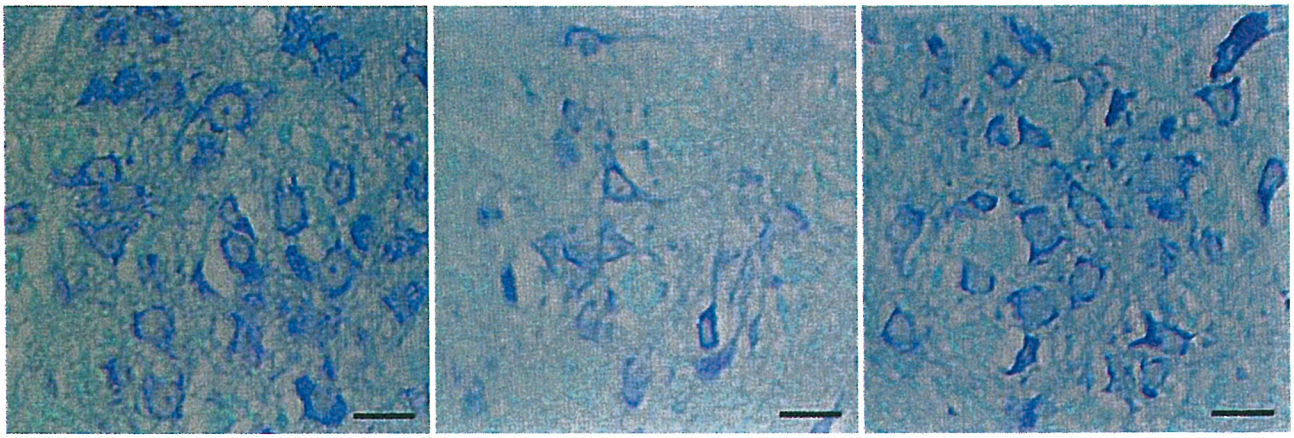
With respect to neurologic problems to be solved in the restoration of vocal fold mobility, as shown in Fig 1, the therapeutic effects for muscle atrophy have already been demonstrated with IGF-I gene. Insulin-like growth factor I has potent trophic effects on both myocytes and neurons. Insulin-like growth factor I gene transfer using a formulated plasmid containing a skeletal muscle–specific actin promoter into the rat thyroarytenoid muscle after transection of the RLN[2-4] prevented muscle atrophy. Four weeks after gene transfer, the gene therapy group, as compared to a control group, exhibited a significant increase in muscle fiber diameter and less muscle atrophy. In addition, the gene therapy group exhibited a significant improvement in peripheral nerve regeneration and protection of motor end plates. These findings showed that IGF-I gene therapy in RLN paralysis was effective in preventing laryngeal muscle atrophy and enhancing peripheral nerve regeneration. Moreover, similar effects were confirmed in a chronic model at 1 month after nerve transection.[5] Myosin heavy chain subunits in laryngeal muscle are known to undergo various changes in composition due to biological demands. IGF-I gene therapy can prevent these denervation-related changes and promote their normalization.[6]

Glial cell line–derived neurotrophic factor offers powerful survival-promoting effects on motoneurons in vitro and in vivo, and promotes nerve regeneration after injury.[7-10] Overexpression of GDNF produces hyperinnervation of neuromuscular junctions,[11] and expression of endogenous GDNF is increased early after peripheral nerve injury.[12] The use of GDNF and other neurotrophic factors may prove beneficial for the treatment of peripheral nerve injuries.

Brain-derived neurotrophic factor has been shown to promote the survival of developing motoneurons in vitro and to rescue motoneurons from axotomy-induced cell death in vivo, and its effects are mediated via 2 classes of receptors (trkB and p75).[13,14]

In adult rodents, administration of BDNF and GDNF proteins has been reported to prevent the loss of spinal motoneurons after spinal root avulsion.[8,15-17] And following avulsion of a spinal ventral root, an adeno-associated viral vector-mediated BDNF and GDNF gene transfer prevents the loss of spinal motoneurons.[18]

In the present article, to assess the neurologic problems to be solved in the restoration of vocal fold



Normal Control AxCAhGDNF

Fig 2. Vagal motoneurons in nucleus ambiguus 4 weeks after vagal nerve avulsion (toluidine blue stain). Photomicrographs show nucleus ambiguus on contralateral (Normal) or ipsilateral side after avulsion and treatment with phosphate-buffered saline solution (Control) or glial cell line–derived neurotrophic factor (GDNF) gene transfer (AxCAhGDNF). GDNF gene expression offers strong protective effect against motoneuron loss. Bars — 50 μm.

mobility (Fig 1) and the possibility of gene therapy for motoneuron loss and functional recovery after vagal nerve or RLN injury, we examined the neuroprotective effect of adenoviral GDNF and BDNF gene transfer using rat laryngeal paralysis models.

METHODS AND RESULTS

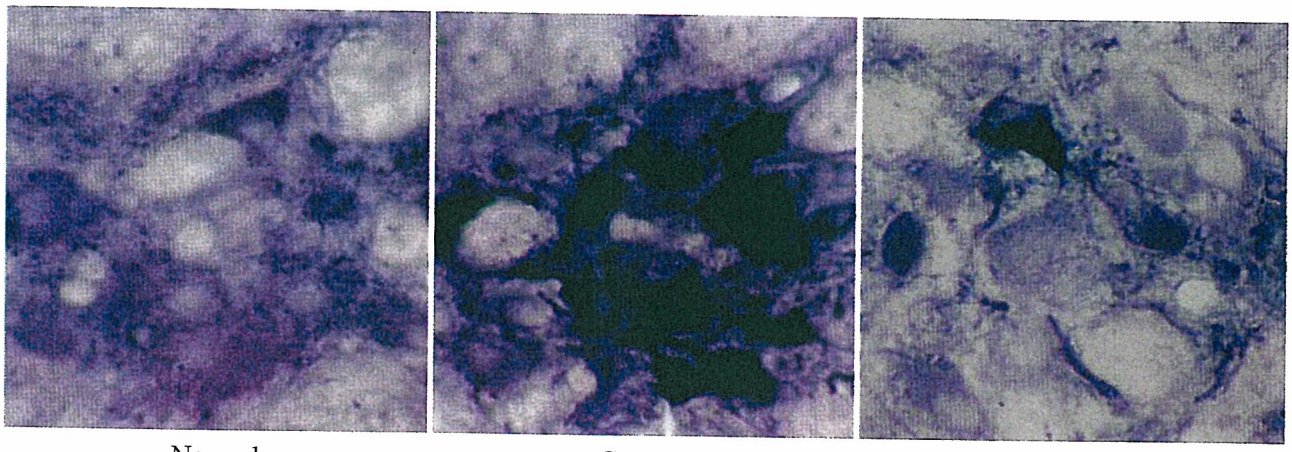
Gene Therapy for Motoneuron Loss in Nucleus Ambiguus. We evaluated the effects of GDNF gene therapy on prevention of denervation-related motoneuron loss in the nucleus ambiguus by injecting GDNF into nucleus ambiguus motoneurons.[19] First, we created a model of induced motoneuron loss in the rat nucleus ambiguus by resection of the vagus nerve at the level of the jugular foramen. Then, using an adenovirus vector (AxCAhGDNF), we selectively delivered the GDNF gene to nucleus ambiguus motoneurons through the jugular foramen. At 2 weeks and 4 weeks after gene transfer, the brain stem tissues were excised, and serial transverse sections were cut at 7 μm. Every fifth section (28-μm interval) was collected and stained with toluidine blue, and ambiguus motoneurons that had nuclei containing distinct nucleoli on both sides of the nucleus ambiguus were counted in 20 sections. We did not apply any correction factors for data analysis, since the ambiguus neurons have a maximum diameter of 21.8 ± 4.96 μm,[20] and these neurons were counted only once in every fifth section at a 28-μm interval. The data are expressed as the mean ± SD from 4 animals (2 and 4 weeks after operation), and statistical significance was assessed between the groups by the one-factor analysis of variance and a Fisher test ($n = 4$). At 2 weeks and 4 weeks after gene transfer, as compared to a control group, the GDNF gene therapy group demonstrated a significantly lower loss of nucleus ambiguus motoneurons (motoneuron survival rate at 4 weeks, 72.0% ± 8.4% in the GDNF gene therapy group versus 56.2% ± 3.7% in the control group; Fig 2). The GDNF gene therapy group also showed inhibition of expression of nitric oxide synthase, which is induced by nerve injury (Fig 3), and preservation of choline acetyltransferase activity, which decreases rapidly after nerve injury (Fig 4).

With concurrent transfer of the BDNF gene and the GDNF gene, the rate of loss of nucleus ambiguus motoneurons was significantly lower than the rates in both the control group and the GDNF gene therapy–only group (motoneuron survival rate at 4 weeks, 84.7% ± 2.2% in GDNF + BDNF gene therapy group; Fig 5).[21]

These findings demonstrate that GDNF gene therapy and BDNF gene therapy prevented the loss of motoneurons in the nucleus ambiguus associated with nerve transection and also promoted nerve regeneration.

Gene Therapy to Restore Nerve Function. The research results described above delineate the morphological effects of gene therapy on laryngeal muscle and the nucleus ambiguus; however, we also need to know to what extent these effects contribute to restoring RLN function. We therefore evaluated the functional effects of GDNF gene therapy in an RLN crush model.[22] Using hemostatic forceps, we crushed the RLN in rats for 60 seconds. After laryngoscopically confirming vocal fold fixation, we injected an adenovirus vector carrying the GDNF gene into the nerve bundle at the crush site.

To assess neurofunctional recovery, we subjected rats at 2 or 4 weeks after operation to measurement



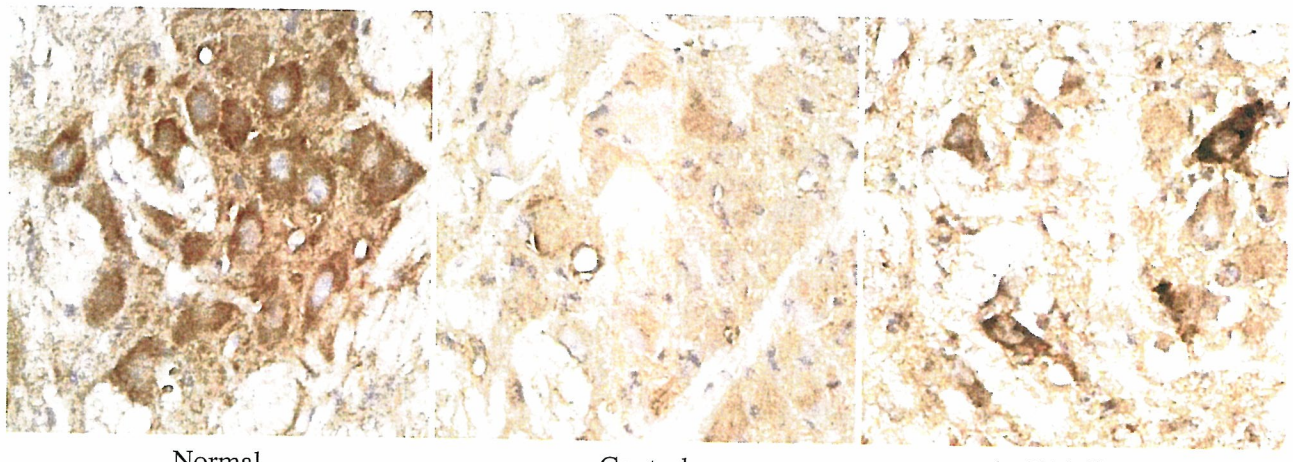
Normal Control AxCAhGDNF

Fig 3. NADPH diaphorase histochemistry of nucleus ambiguus, 14 days after vagal nerve avulsion followed by GDNF gene transfer (AxCAhGDNF) or no treatment (Control). Compared to normal contralateral side (Normal), strong expression of nitric oxide synthase was observed on avulsed side in control animals. However, when AxCAhGDNF was inoculated, induction of nitric oxide synthase was suppressed.

of motor nerve conduction velocity (MNCV). The animals were anesthetized, and the left RLN was exposed. The strap muscles were sectioned to expose the larynx, and a laryngeal fissure was made. The left thyroarytenoid muscle was stabbed through the fissure with a needle concentric electrode for recording. To stimulate the left RLN, we placed 2 bipolar hook electrodes and hooked them into the left RLN. One was placed inferior to the left lobe of the thyroid as a distal stimulator, and the other was placed at 16 mm proximal to the distal electrode as a proximal stimulator. The nerve was maximally stimulated, and compound muscle action potentials in the TA muscle were recorded with a Power Lab computer-assisted electromyography machine (AD Instruments, Colorado Springs, Colorado). Maximal stimulation was achieved by increasing current output until no further change in amplitude of the compound action potential occurred. A current impulse of 0.01-ms duration was delivered. Maximum MNCVs were calculated from the derived latencies and the distance between the 2 stimulating points (16 mm).

At the time of laryngeal fissure creation, recovery of vocal fold movement was also assessed. Recovery was only considered present when vocal fold movement on the denervated side was observed to be equal to that on the contralateral, non-denervated side. When vocal fold movement on the denervated side was limited (not fixed), recovery was not considered present.

Representative evoked electromyograms obtained by left RLN stimulation are shown in Fig 6. The electromyogram in an AxCAhGDNF-injected rat showed a large action potential wave and a short-



Normal Control AxCAhGDNF

Fig 4. Choline acetyltransferase (ChAT) immunohistochemistry of nucleus ambiguus, 7 days after vagal nerve avulsion followed by GDNF gene transfer (AxCAhGDNF) or no treatment (Control). Compared to normal contralateral side (Normal), decrease in ChAT immunoreactivity was observed on avulsed side in control animals (Control). However, when AxCAhGDNF was inoculated, ChAT immunoreactivity was preserved on avulsed side.

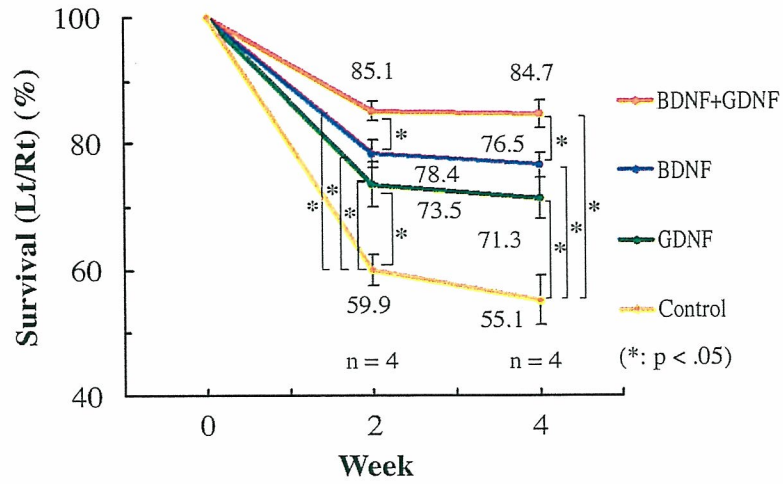


Fig 5. Time course of motoneuron loss in nucleus ambiguus after operation. Percentages of surviving motoneurons in nucleus ambiguus (treated side/contralateral side) are plotted. Data following vagal nerve avulsion (n = 4) and treatment with phosphate-buffered saline solution, LacZ (control) gene transfer, GDNF gene transfer, brain-derived neurotrophic factor (BDNF) gene transfer, or GDNF and BDNF gene transfer are shown. Data are mean ± SD (bars). Statistical comparisons were done by Fisher test.

er latency, as compared to that in the control rat (AxCALacZ). At 2 weeks and 4 weeks after gene transfer, the MNCVs of the RLN in the GDNF gene therapy group were 31.49 ± 7.03 m/s and 35.59 ± 6.28 m/s, respectively. Taking into account an RLN conduction velocity of about 40 m/s in normal rats, we concluded that the conduction velocity had almost normalized. In the control group the RLN conduction velocity was less than 20 m/s at 2 weeks and at 4 weeks. The RLN conduction velocity was thus significantly faster in the GDNF gene therapy group (Fig 7). Concerning vocal fold mobility (see Table), in the control group the degree of restoration of vocal fold mobility at 2 weeks and 4 weeks after gene transfer was 12.5% and 37.5%, respectively. In the GDNF gene therapy group there was 100% restoration at both 2 weeks and 4 weeks. This demonstrated that treatment had a significant effect on restoring vocal fold mobility. Furthermore, the diameters of axons were measured from left RLN sections. Representative cross sections of RLN samples stained with Epon–toluidine blue are shown in

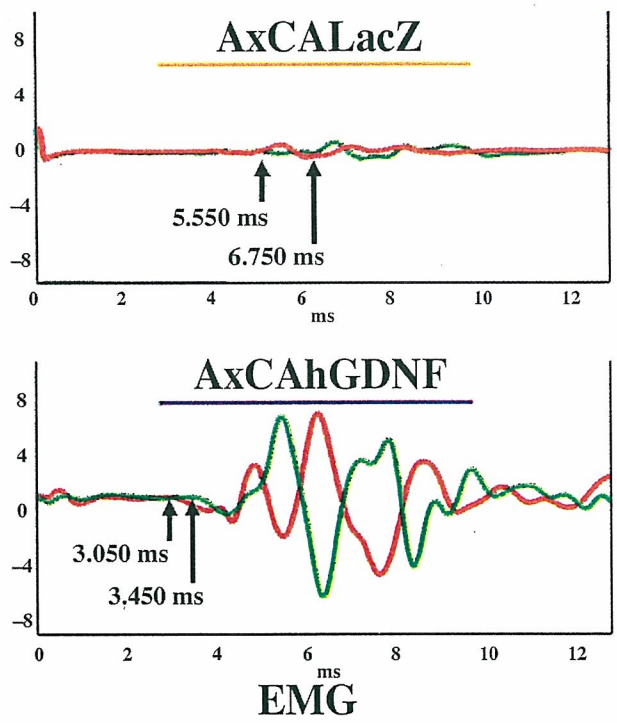


Fig 6. Electromyography (EMG) of thyroarytenoid muscle. Representative evoked EMG traces obtained by left recurrent laryngeal nerve stimulation in LacZ gene–transferred (AxCALacZ) animals and GDNF gene–transferred (AxCAhGDNF) animals. EMG in AxCAh-GDNF-injected rat revealed large action potential wave and shorter latency. Conversely, EMG in control rat (AxCALacZ) showed small, unclear action potentials and delayed latency.

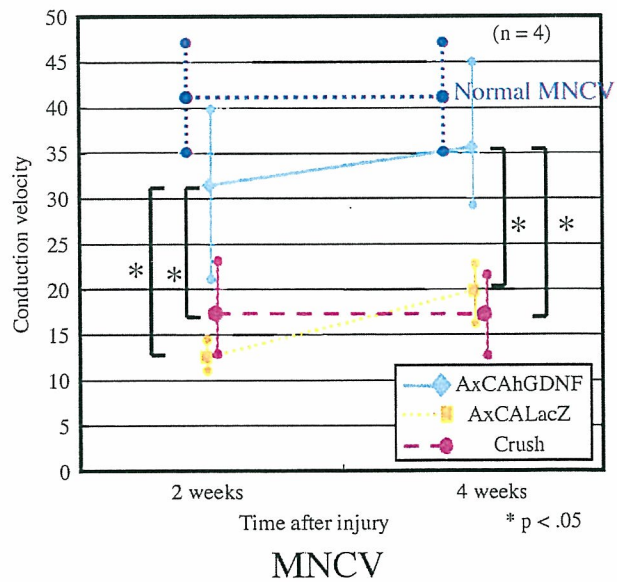


Fig 7. Motor nerve conduction velocity (MNCV) at 2 and 4 weeks after injury (n = 4). Significant differences are apparent between GDNF gene (AxCAhGDNF)–transferred and LacZ gene (AxCALacZ)–transferred animals, and between GDNF gene–transferred and RLN crush animals at 2 and 4 weeks after injury. MNCV in GDNF gene–transferred animals recovered favorably and was comparable to that of normal rat. GDNF gene expression offers strong protective and regenerative effects against motor nerve injury.

RECOVERY OF VOCAL FOLD MOVEMENT IN EACH GROUP

	2 Weeks		4 Weeks	
AxCAhGDNF	4/4	*	4/4	*
AxCALacZ	1/4		1/4	
Crush	0/4		2/4	

Numerators show numbers of animals with vocal fold movement recovery. Denominators show numbers of animals observed. Glial cell line–derived neurotrophic factor (GDNF) gene–transferred (AxCAhGDNF) animals showed significantly better recovery of vocal fold movement than did control animals (AxCALacZ-injected and recurrent laryngeal nerve crush) at 2 and 4 weeks after injury, indicating strong functional recovery following GDNF gene transfer.

*$p < .05$.

Fig 8. The mean axon diameter was 6.28 ± 0.43 μm in normal rats, 4.76 ± 0.62 μm in the GDNF gene therapy group, and 2.67 ± 0.10 μm in the control group. The mean axon diameter in the GDNF gene therapy group had recovered to an almost normal level — significantly larger than that in the control group ($p < .05$).

DISCUSSION

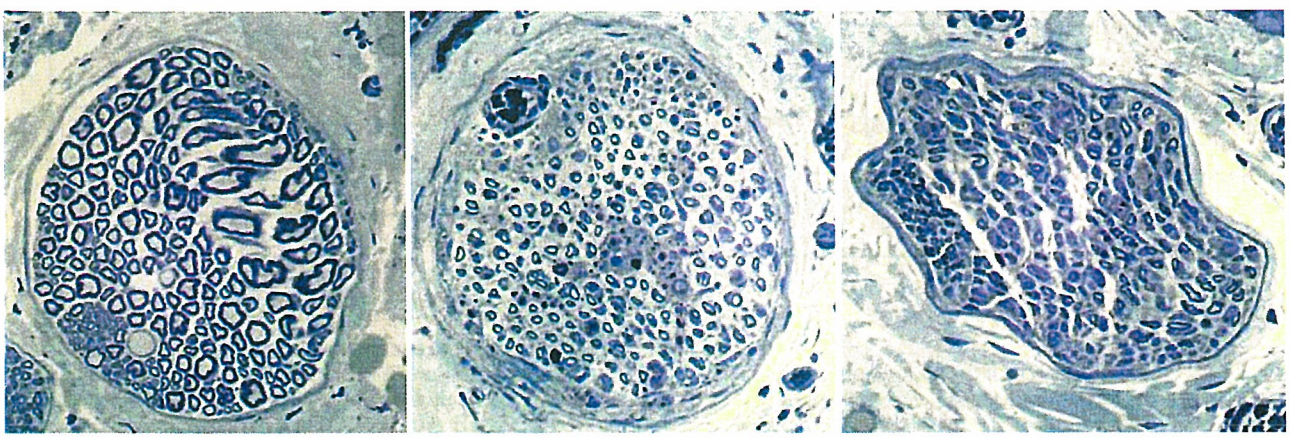
Treatment with GDNF gene transfer significantly prevented the loss of vagal motoneurons in comparison to the control in our study. In BDNF gene–transferred animals, a significantly larger number of surviving motoneurons were observed in the nucleus ambiguus as compared to phosphate-buffered saline solution–treated controls and GDNF gene–transferred animals 2 to 4 weeks after inoculation. In addition, the survival of motoneurons was further improved by treatment with a combination of BDNF and GDNF gene transfer, as compared to treatment with either BDNF gene transfer or GDNF gene transfer.

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 RLN injury.[23,24] Adenoviral BDNF and GDNF gene therapy may be useful not only for preventing motoneuron loss in the nucleus ambiguus, but also for decreasing the risk of laryngeal synkinesis after vagal nerve injury or RLN injury by inoculation of the vector at the injured site of the nerve during head and neck surgery.

Our study also assessed neurofunctional recovery 2 and 4 weeks after injury, demonstrating enhancement of motor nerve recovery according to neurofunctional and laryngeal functional data. The timing of assessment was appropriate for the regenerative process of peripheral nerves, and our results demonstrate that early neurofunctional recovery might preserve good laryngeal function.

Measurement of the MNCV of the injured nerve is a commonly used physiological measure to evaluate peripheral nerve function in the rat nerve injury model.[25-27] In this study, control animals injected with or without AxCALacZ displayed a markedly slowed MNCV that did not change between 2 and 4 weeks after injury. These results are consistent with limited regeneration of the nerve in control animals that had not improved even 4 weeks after injury.

We also demonstrated that GDNF gene–transferred animals showed significant improvements in recovery of vocal fold movement and axonal diameter and myelination compared to control animals. Thickening of axon diameters and enhancement of myelination are considered to contribute to increases in MNCV. These regenerative effects on the nerve



Normal Control AxCAhGDNF

Fig 8. Photomicrographs of sectioned recurrent laryngeal nerve stained with Epon–toluidine blue at 5 mm distal from crush site at 2 weeks after injury. Control animal with crush injury (Control) displays atrophied and demyelinated axons. However, in GDNF gene–transferred (AxCAhGDNF) animal, better myelination and thick axons are observed, comparable to those in normal rat.

might finally achieve recovery of dynamic laryngeal function (recovery of vocal fold movement). We have not investigated the therapeutic effect of the combination of GDNF and BDNF gene transfer on MNCV and recovery of vocal fold movement. However, this issue is interesting, and we will investigate it in future studies.

To minimize the deleterious effects of early trauma and promote and guide axonal regrowth, the delivery of neurotrophic factors has emerged as a promising strategy to manipulate axonal regrowth in the early phase. This study demonstrated the enhancement of neurofunctional recovery after remote injection of adenovirus vector coding for the GDNF gene into crushed RLNs over the course of a few weeks. The vocal folds are extremely delicate structures, and imperceptible injuries can result in excessive vocal complications. Extended injury results in atrophy of the laryngeal muscles, motoneuron loss in the nucleus ambiguus, and decreases in both motor axon density and nerve–end plate contact. Early recovery from axonal degeneration is important for preservation and recovery of laryngeal function. Again, the present methods achieved good preservation and facilitated recovery of laryngeal function.

Laryngeal paralysis most often occurs clinically as a result of vagal nerve or RLN injury after surgical ablation of a tumor involving the head and neck region. If the nerve is injured during surgery, direct injection of the vector into the nerve might prevent paralysis. Alternatively, when paralysis becomes apparent on extubation, the vector can be injected into the nerve after reintubation and opening of the wound.

The adenovirus vector was used in this study. For clinical applications, controversy remains regarding the potential risks of virus-mediated gene therapy,[28-30] particularly when applied to nonlethal benign diseases such as laryngeal paralysis. To overcome this problem, the safety of the vector must be demonstrated before clinical application. Preliminary experiments of highly safe viral[31] and nonviral gene transfer systems are also currently under way.

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Title page

A novel drug therapy for recurrent laryngeal nerve injury using T-588.

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Abstract

Objectives/Hypothesis: We have previously shown that gene therapy using Insulin-like growth factor (IGF)-I, glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF), or a combination of these trophic factors, is a treatment option for recurrent laryngeal nerve (RLN) palsy. However, there remain some difficulties preventing this option from becoming a common clinical therapy for RLN injury. Thus, we need to develop novel treatment option that overcomes the problems of gene therapy.

R(-)-1-(benzo[b]thiophen-5-yl)-2-[2-N,N-diethylamino]ethoxy]ethanol hydrochloride (T-588), a synthetic compound, is known to have neuroprotective effects on neural cells. In the present study, the possibility of new drug treatments using T-588 for recurrent laryngeal nerve (RLN) injury was assessed using rat models.

Study Design: Animal study.

Methods: Animals were administered T-588 for 4 weeks. The neuroprotective effects of T-588 administration after vagal nerve avulsion and neurofunctional recovery after recurrent laryngeal nerve crush were studied by motoneuron cell counting, evaluation of choline acetyltransferase immunoreactivity, the electrophysiological examination and the re-mobilization of the vocal fold.

Results: T-588 administration successfully prevented motoneuron loss and ameliorated the choline acetyltransferase immunoreactivity in the ipsilateral nucleus ambiguus after vagal nerve avulsion.

Significant improvement of motor nerve conduction velocity (MNCV) of the RLN and vocal fold movement were observed in the treatment group when compared to controls.

Conclusion: These results indicate that oral administration of T-588 may be a promising therapeutic option in treating peripheral nerve injury.

Keywords

T-588, Avulsion, Crush, Nucleus ambiguus, Laryngeal paralysis, Motoneuron

1. Introduction

Laryngeal paralysis most often occurs as a result of vagal nerve injury or recurrent laryngeal nerve injury after surgical ablation of a tumor involving the head and neck region. Injury to the recurrent laryngeal nerve occurs in 1-2% of all thyroid surgeries. Other surgical procedures utilizing a cervical approach carry a similar incidence of laryngeal paralysis. Patients with unilateral laryngeal paralysis typically present with disabling symptoms related to aspiration, dysphagia and loss of voice (dysphonia). Except for laryngeal reinnervation procedures, surgical options for the management of patients with unilateral laryngeal paralysis (vocal fold injection, thyroplasty and arytenoid adduction) only achieve vocal fold medialization due to static changes in the vocal fold tissue or laryngeal framework, and such deficits can never be neurologically restored.[1] Laryngeal reinnervation procedures have had little impact on the return of dynamic laryngeal function and are still not widely accepted as a treatment option. The failure of reinnervation after recurrent laryngeal nerve injury may be attributed to multiple factors, including decreases in motor fiber density, atrophy of laryngeal muscle, loss of motoneurons in the nucleus ambiguus, and inappropriate or misdirected innervation by antagonistic motoneurons.[2, 3] Gene therapy is a potential treatment option for recurrent laryngeal nerve (RLN) palsy. The potential of gene therapy using insulin-like growth factor (IGF)-I [4, 5], glial cell line-derived neurotrophic factor (GDNF) [6, 7] and brain-derived neurotrophic factor (BDNF), or a combination of these trophic factors [8], has been reported previously. Although we have demonstrated

that gene therapy is very useful for RLN palsy, there are several difficulties preventing it from becoming a common clinical therapy, including ethical problems, technical difficulties and toxicity of viral vectors. Low molecular weight compounds that are safe and convenient to administer are thus desirable alternatives for clinical applications.

Steroids are one of the common agents currently used for the treatment of peripheral nerve palsy, including RLN palsy and idiopathic facial palsy, as a result of their anti-inflammatory and anti-edematous effects. However, these agents produce little benefit in recurrent laryngeal nerve palsy.[9] Donepezil, Galantamine, Memantine and R(-)-1-(benzo[b]thiophen-5-yl)-2-[2-N,N-diethylamino)ethoxy]ethanol hydrochloride (T-588) are recently developed neuroprotective agents against Alzheimer's disease.[10] Among these, we focused on T-588, a novel neuroprotective compound, that delays progression of neuromuscular dysfunction in wobbler mouse motoneuron disease.[11] It has also been demonstrated that oral administration of T-588 improves the survival of injured motoneurons and supports their neuronal function after facial nerve avulsion.[12] In this study, the potential of oral administration of T-588 for RLN injury was assessed using rat models. The neuroprotective effects of T-588 after vagal nerve avulsion and RLN crush were also studied.

2. Materials and Methods

2.1. Animals and surgical procedures

Forty-two Sprague-Dawley male rats (12-weeks-old, 340-360 g) were used in this study. Animals were anesthetized with ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) during all surgical procedures. The vagal nerve avulsion model was utilized to assess the neuroprotective effects of T-588 in the nucleus ambiguous after severe vagal/RLN surgery. 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 and skin was suture closed[7]

The nerve crush model was utilized to assess the potential of T-588 to promote neurofunctional recovery of RLN after detrition injury. Following a midline vertical cervical incision, the left RLN was exposed and dissected circumferentially just inferior to the left lobe of the thyroid gland. At 10 mm proximal from the inferior of the thyroid gland, the nerve was crushed with a forceps for exactly 60 seconds. To confirm left vocal fold paralysis, direct laryngoscopy was performed. Subsequently, strap muscles and overlying fascia were replaced, and the skin was suture closed. Animals were cared for and used in accordance with protocols approved by the Animal Care and Use Committee of Keio University School of Medicine (Tokyo, Japan).

2.2. T-588 administration

T-588 was supplied by Toyama Chemical Co. Ltd. (Tokyo, Japan). After avulsion or crush of the left vagal nerve, rats were freely administered water containing 0.05% T-588 for 4 weeks. The total daily amount of T-588 consumed by the rats thorough freely available 0.05% solution was set to be equivalent to the daily dose for humans.[12, 13]

2.3. Histological analysis

For motoneuron cell counting, rats were anesthetized with a lethal doze of ketamine and were transcardially perfused with PBS followed by 4% paraformaldehyde in 0.1 M phosphate buffer at 4 weeks after vagal nerve avulsion. Brain stem tissue was harvested and immersion fixed in the same fixative for 2 h. Subsequently, samples were embedded in paraffin, and serial transverse sections were cut at 7 μm. Every fifth section (28-μm interval) was collected, deparaffinized and stained with Toluidine Blue, and ambiguus motoneurons having nuclei containing distinct nucleoli on both sides of the nucleus ambiguus were counted in 20 sections. We did not apply any correction factors for data analysis, as the ambiguus neurons have a maximum diameter of 21.8±4.96 μm [14], and these neurons were counted only once in every fifth section, at a 28-μm interval. Data are expressed as means ± S.D. from eleven animals and statistical significance was assessed between the treatment group and control group by Mann-Whitney U test.

For immunostaining of Choline acetyltransferase (ChAT), animals were perfused at 4 weeks after