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Molecules and Cells

Secretion of EGF-Like Domain of Heregulinβ Promotes Axonal Growth and Functional Recovery of Injured Sciatic Nerve

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Neuregulin 1 (NRG1) and epidermal growth factor receptor (ErbB) signaling pathways control Schwann cells during axonal regeneration in an injured peripheral nervous system. We investigated whether a persistent supply of recombinant NRG1 to the injury site could improve axonal growth and recovery of sensory and motor functions in rats during nerve regeneration. We generated a recombinant adenovirus expressing a secreted form of EGF-like domain from Heregulinβ (sHRGβE-Ad). This virus, sHRGβE-Ad allowed for the secretion of 30-50 ng of small sHRGβE peptides per 10^{7-8} virus particle outside cells and was able to phosphorylate ErbB receptors. Transduction of the concentrated sHRGβE-Ad into an axotomy model of sciatic nerve damage caused an effective promotion of nerve regeneration, as shown by histological features of the axons and Schwann cells, as well as increased expression of neurofilaments, GAP43 and S100 in the distal stump of the injury site. This result is consistent with longer axon lengths and thicker calibers observed in the sHRGβE-Ad treated animals. Furthermore, sensory and motor functions were significantly improved in sHRGβE-Ad treated animals when evaluated by a behavioral test. These results suggest a therapeutic potential for sHRGβE-Ad in treatment of peripheral nerve injury.

INTRODUCTION

Injuries to the peripheral nervous system present a serious problem affecting ~2.8% of all trauma cases and often resulting in poor recovery of function and an impaired quality of life for the patient (Jaquet et al., 2001). Unlike elements in a central nervous system, axons in the adult peripheral nervous system (PNS) are able to regenerate when damaged. Successful axonal regeneration is a key for the functional recovery of injured peripheral nerves. However, satisfactory recovery usually occurs exclusively with minor nerve injuries, where the internal damage is minimal. Surgical repair after an injury is often associated with incomplete and non-specific regeneration, leading to poor recovery of function (Jaquet et al., 2001). Chronic denervation is common in peripheral nerve injuries, mostly because the extensive injury zone prevents axonal outgrowth, resulting in a slow rate of regeneration. As a consequence, the distal nerve segment remains chronically devoid of regenerating axons. The resulting prolonged denervation of Schwann cells appears to be a critical factor which makes them unreceptive for axonal regeneration and is the single most important quantitative contributor to poor end-organ re-innervation (Höke and Brushart, 2010). One strategy to enhance the regenerative efficacy would focus on the delivery of various growth/stimulatory factor(s) to the regenerating nerve (Tannemaat et al., 2008).

Following a nerve injury, the distal part of the axons rapidly degenerate, and the myelin sheaths breakdown (Waller, 1851). Schwann cells in the distal stump divide, migrate, and then differentiate to ensheath the re-growing axons, providing a major trophic support for axonal re-growth and nerve regeneration (Faweett and Keynes, 1990; Lai, 2005). Thus, Schwann cells play a guiding role in axonal regeneration. However, Schwann cells denervated for an extended time, down-regulate their activity and are less capable of promoting axonal regeneration. One of the essential molecular events driving the Schwann cell regeneration during peripheral nerve recovery is known as neuregulin1 (NRG1) signaling (Buonanno and Fischbach, 2001; Corfas et al., 2004; Guertin et al., 2005). NRG1 is primarily expressed by neurons in the peripheral nervous system during development. NRG1 secreted from axons stimulates Schwann cells to reconnect and re-myelinate the severed nerve stumps (Avellana-Adalid et al., 1998; Cai et al., 2004; Chen et al., 1998). However, the level of NRG1 is greatly reduced upon maturation in adults (Lemke, 1996; Marchionni, 1995). The expression of NRG1 quickly increases in Schwann cells of the distal nerves following nerve lesion but less in re-growing axons. The receptor tyr-kinases of NRG, ErbB2, ErbB3 and ErbB4 are expressed by sensory and motor neurons which their axons project into and consist of sciatic nerve, suggesting that Schwann cells regulate axonal re-growth through NRG1 (Bublil and Yarden, 2007; Carroll et al., 1997; Kwon et al., 1997; Pearson and Carroll, 2004). While ErbB2 and ErbB3 are involved in the control of cell proliferation, ErbB4 is known to be expressed in hippocampal neurons, cerebellar granule cells and GABAergic neurons and mediates NRG signaling for neu-

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rite migration and outgrowth in the central nervous system (Fazzari et al., 2010; Gerecke et al., 2004; Rieff et al., 1999).

The effect of erbB signaling appears to be depend not only on the stage of development but also on the specific isoform of the ligand that binds the receptors (Chen et al., 2006). The single *Nrg1* gene produces six major isoforms (I -VI) including neu differentiation factor/heregulin (NDF, HRG-type I), glial growth factor (GGF-type II), sensory and motor neuron derived factor (SMDF-type III) by alternative promoter usage and mRNA splicing (Mei and Xiong, 2008). Isoforms of NRG1 differ substantially in the inclusion of several domains such as an immunoglobulin (Ig)-like domain (type I and II) and a cystein rich domain (CRD, type III) and in other properties, including membrane attachment. However, all NRG1 isoforms contain an epidermal growth factor (EGF)-like signaling domain essential for erbB receptor binding and activation (Birchmeier and Nave, 2008). The EGF-like domain exerts the functional core of NRG-1 and other domains have modulatory effects on erbB signaling through an EGF-like domain (Chen et al., 2006). Recent data indicates that only the type III NRG including CRD, not the type I isoform is presented correctly to Schwann cells, and is therefore able to elicit the proper myelination (Birchmeier and Nave, 2008; Taveggia et al., 2005). Inclusion of an Ig-like domain of type I NRG completely eliminates the effect of an EGF-like domain on expression of myelin protein zero (P0) in Schwann cells, while CRD induces it. Therefore, the use of an EGF-like domain alone would be a better choice for the therapy of axonal regeneration, because other NRG domains can activate a panel of unexpected downstream signaling pathways.

The EGF-like domain is located in the membrane-proximal region of the extracellular domain of each NRG1 protein type, and has alternate splice variants at the most carboxy-terminal region to give α and β isoforms (Mei and Xiong, 2008). EGF-domain β isoforms are prevalent in the nervous system (Meyer and Birchmeier, 1994) and the EGF-like domain of heregulinβ1 (type I) has the highest affinity for erbB2/3, erbB3 and erbB4 among various NRG1s (Buonanno and Fischbach, 2001; Crovello et al., 1998; Jones et al., 1999). It consists of ~50 amino acids, is required for receptor binding and is capable of mimicking many of the biological effects of full-length proteins (Birchmeier and Nave, 2008; Meyer et al., 1997).

Despite the potential for NRG1 to enhance nerve regeneration, there are difficulties in maintaining the growth factor release at a beneficial level over the long duration of nerve regeneration. Therefore, the consistent provision of functional form(s) of NRG1 to an injured site over the duration of an axonal regeneration process is an attractive technique for the treatment of peripheral nerve injury. An effective, targeted delivery vehicle that allows a persistent expression of the peptide at the delivered site would greatly enhance recovery from a nerve injury. In the present study, we investigated (i) whether the EGF-like domain from HRGβ alone is capable of promoting axonal regeneration and (ii) whether adenovirus is an effective vehicle for delivery of this peptide to injured peripheral nerves. We constructed an adenovirus that releases a small peptide including the EGF-like domain of heregulinβ (HRGβ peptide), with the intention of promoting axonal re-growth, and explored whether the administration of the recombinant virus could improve the recovery of sensory and motor functions in the rat axotomy model.

MATERIALS AND METHODS

Cell culture and treatments

Human embryonic kidney cell line 293 and rat glioma cell line C6 were obtained from American Type of Cell Culture (ATCC; USA) and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) penicillin, and streptomycin at 37°C. Human bone marrow mesenchymal stem cells (MSCs) were grown in α-MEM supplemented with 10% FBS, 2.2 g/L $NaHCO_3$, and antibiotics at 37°C. A rat neuronal stem cell line, HiB5, was maintained in DMEM containing 10% FBS and antibiotics at 33°C (Kwon et al., 1997). HiB5 cells were changed to a chemically defined media (N2) and incubated at 39°C, the normal rat body temperature, to induce cell differentiation.

Construction of sHRGβE-Ad

The coding sequence of the HRGβ encompassing amino acids 172 to 232, was amplified from rat nerve tissue by RT-PCR using primers - 5′ gctggtaccatgcatctcataaagtgt3′ and 5′ tcggatccttaatgcttgtagaagct3′. The primer set was designed to flank the coding sequence of EGF-like domain between KpnI and BamHI sites. The oligonucleotides encoding a signal peptide (Delli Bovi et al., 1987; Opalenik et al., 1995), 5′ aattcatgtcgggaccgagaactgcagcaggtac3′ and 5′ ctgctgcagttcctggtcggcacatg 3′, were then annealed and fused to the aforementioned cDNA, which had been cut with KpnI and BamHI, and subsequently inserted into pCA14 (Microbix Biosystems Inc., Canada) between the CMV early promoter and the SV40 polyadenylation site using the EcoRI and BamHI sites. This plasmid was transfected into 293 cells along with pJM17 (Microbix Biosystems Inc.) to generate the recombinant adenovirus. Viral DNA from each plaque was purified and analyzed by PCR to verify the presence of the leader peptide fused to the HRGβEGF-like domain of cDNA, and to show the absence of the adenovirus E1 sequence. DNA molecular weight markers (100 bp ladder, Bioneer, Korea) were used to confirm the size of PCR products. A control recombinant adenovirus expressing *E. coli* β-galactosidase (LacZ-Ad) was used as previously described (Joung et al., 2000). Viruses from a single plaque were amplified in 293 cells and purified by centrifugation in a CsCl gradient, according to the standard protocol (Tollefson et al., 1999). The stock was then dialyzed against phosphate buffered saline (PBS) containing 10% glycerol and maintained as aliquots at -80°C. A plaque forming unit (PFU) of virus particles was determined by a plaque assay using 293 cells.

Measurement of of sHRGβE-Ad peptide in culture media

The secretion of sHRGβE peptide into culture media was measured using a commercial HRGβ ELISA system (R&D Systems, USA) according to the manufacturer's instruction. 3×10^5 MSCs in a 35 mm culture dish either remained uninfected, or were infected with LacZ-Ad, or sHRGβE-Ad at a multiplicity of infection (MOI) of 100 in 2% FBS containing media for 2 h. Medium was replaced with fresh DMEM containing 10% FBS and cells were cultured for an additional 12 h. Media was then was removed and cells were incubated in 1 ml of serum free α-MEM for 24 h. The supernatant was collected and stored at -70°C until analysis. The same experiments were also conducted using differentiated HiB5 cells.

Immunoprecipitation analysis of erbB receptors and immunoblotting analysis

1.2×10^6 HiB5 cells were infected with LacZ-Ad or sHRGβE-Ad at a moi of 100 for 24 h, and the serum-containing media was replaced with serum-free N2 media and incubated for varying time periods to be used as a source of sHRGβE. The conditioned media collected from the aforementioned cultures were treated for 10 min with C6 cells, which were deprived of serum

for 24 h prior to use. The cells used as positive controls were treated with 50 ng/ml of recombinant glial growth factor (HRGβ, R&D Systems). Cell lysates were prepared as previously described (Kwon et al., 1997). The C6 cell lysate (500 μg) was immunoprecipitated with anti-ErbB2 (Oncogene, Germany), ErbB3 (Kim et al., 1999), or erbB4 (Santa Cruz Biotechnology, USA) antibodies, and was separated on 7% SDS polyacrylamide gels and subjected to immunoblot analysis using 4G10 - a monoclonal antibody specific for phosphotyrosine (anti-pTyr; Upstate Biotechnology, USA).

The distal stump of the sciatic nerve was excised and snap-frozen in liquid nitrogen. The lysates were prepared as previously described (Kwon et al., 1997). Proteins were separated in a 7 or 10% polyacrylamide gel, transferred to nitrocellulose paper and immunoblotted with antibodies for NF-200 (Sternberger, USA), GAP43 (Chemicon, USA), β-tubulin isotype III and β-actin (all from Sigma, USA).

Surgical procedures and virus injection

Adult male Sprague-Dawley rats weighing 250-270 g were housed with *ad libitum* access to food and water. All procedures were performed in compliance with relevant laws and institutional guidelines. After a 1-week habituation period, animals were anesthetized by an intraperitoneal injection of Equithensin (0.8-1.2 ml, 2.125 g chloral hydrate, 1.063 g Magnesium Sulfate, 0.405 g phentobarbital Sodium, 19.8 ml propylene glycol, 5 ml ethanol, for total 50 ml). An aseptic technique was used to expose the left sciatic nerve at the sciatic notch. Under the dissection microscope, the major blood vessels were carefully separated from nerve fibers prior to the nerve transection by using #55 forceps and then the nerve fibers were transected. A Hamilton syringe (Bondaduz, Switzerland) was used to administer two injections (2 × 1 μl; each containing 1×10^{11} PFU/ml) of LacZ-Ad, sHRGβE-Ad or saline to each animal. The injections were made very slowly, under the membrane wrapping the sciatic nerve at points 1 cm proximal and distal to the lesion, respectively. Following the surgery, animals were rested on a warm pad until recovered.

Immunohistochemical analysis

The tissue sections for immunohistochemical analysis were prepared as previously described (Kwon et al., 1997). Briefly, the animals were deeply anesthetized, perfused with 4% paraformaldehyde and the dissected distal stumps of the sciatic nerves were post-fixed for 6 h. Cryostat sections (5 μm) from the sciatic nerves were permeabilized with 0.1% Triton-X 100 for 15 min and were blocked with 15% normal goat, horse and donkey serum and 1% BSA for 1 h. The nerve slices were then incubated with the primary antibodies overnight at 4°C and then with FITC or the TRICT-conjugated secondary antibody for 1 h. Images were captured on an LSM 510 confocal microscope (Carl Zeiss, Germany). The primary antibodies used were NF-200 (1:100, Sigma), S100 (1:400, Dako, Denmark), and GAP43 (1:200, Chemicon).

Measurement of axonal length and diameter in sciatic nerves

Twenty-five digital micrographs were taken for each nerve, from the injury sites to the fiber growing sites, of distal stumps using a Confocal Laser Scanning Microscopy LSM 510, to analyze axonal growth after the injury. An image analysis program (Carl Zeiss) was used to measure the length (4 animals per group) and diameter (> 50 axons from each of 3 animals per group) of the individual axons.

Hot- plate test for sensory function recovery

A hot plate test and motor functional assessment were performed in all rats 3 and 5 weeks after the injury and prior to the sacrifice for histochemical analysis. The hot plate test for sensory function was performed as previously described (Baker et al., 2002). Briefly, a mobile transparent acrylic cylinder (diameter: 20 cm, height: 30 cm) was placed on a pre-warmed metal hot plate to form the observation area. The temperature of the hot plate was maintained at 54 ± 0.5°C and constantly monitored. The rats were individually placed on the plate and the measurements were made by noting the response latency with a stopwatch to the nearest 0.1 s. The responses were characterized by licking of either the fore- or hind paws or by jumping.

Motor function index

The animals were rated for their performance in a series of reflexes according to modified descriptions of 3 scoring methods as follows (Bederson et al., 1986; Mittoux et al., 2002). *Gait disturbance;* this test assesses limb coordination and body weight support. The response was scored by the extent to which the hind paw contacts the table. *Postural reflex;* the response was scored by the degree of hindlimb flexion when held by the tail. *Tactile placing;* the response was scored by the degree to which the palms were placed on top of the table when the animal's hind paws contact near a table edge. Each test was repeated 20 times per rat. The neurological scores for each index were based on a scale of 0 to 3 and assessed according to the following: 0 = no function; 1 = severe impairment; 2 = moderate impairment; 3 = normal. A larger score indicated better function. The scores from 3 tests were summed to represent motor function of a rat. Neurological scores, averaged from > 12 rats per group, were used for analysis.

Statistical analysis

The results were subjected to one-way analysis of variance (ANOVA) for repeated measurements across testing sessions. Differences of means between the groups were determined using the student's *t*-test. All data are presented as the mean ± SEM.

RESULTS

Characterization of recombinant adenovirus sHRGβE-Ad

The recombinant adenovirus used in this study, sHRGβ-Ad, was designed to produce the EGF-like domain of HRGβ for effective delivery and synthesis of the peptide in the targeted area. A short signal peptide, MSGPGTAACT, was added upstream to the EGF-like domain of HRGβ (Fig. 1A), which allowed secretion of the EGF-like domain of HRGβ here-after HRGβE peptide). PCR was used to verify the presence of the coding sequence of the signal peptide and the HRGβE, and the absence of the adenoviral E1 sequence (Fig. 1B) These results demonstrated successful insertion of fused DNA in the E1 region of viral genome. The integrity of the inserted sequence was also confirmed by double stranded DNA sequencing of viral DNA (data not shown). Conditioned medium from cell cultures was analyzed for the presence of secreted HRGβE peptide, using standard ELISA 2 days after adenovirus vector transduction. MSCs transduced with sHRGβE-Ad produced HRGβE peptide (~6.5 kDa) at the rate of 80-90 ng per 10^{7-8} virus particles. HRGβ proteins were released in non-transduced and LacZ-Ad infected MSCs cultures at a much lower level (30-40 ng per 10^{7-8} virus particles), and the net increase by infection with sHRGβE-Ad was 30-50 ng/ml. (Fig. 1C). Considering the smaller molecular weight of the peptide compared to the whole

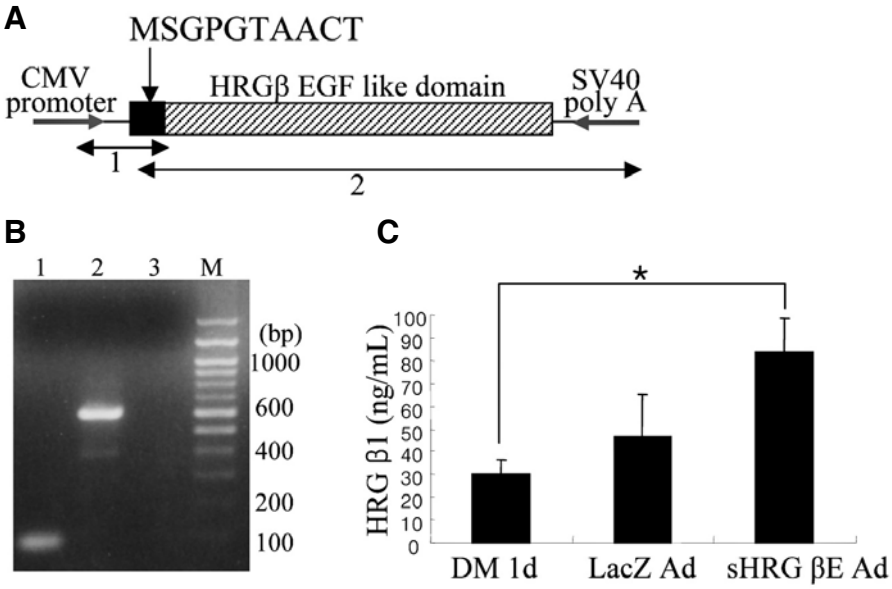

Fig. 1. Characterization of sHRGβE-Ad. (A) The schematic diagram of the expression cassette in viral DNA showing the EGF-like domain of HRGβ fused to N-terminal signal peptide. The sHRGβE was inserted between the CMV early promoter and the SV40 polyadenylation site. The numbered arrows indicate the expected PCR products shown in panel B. (B) PCR analysis of recombinant adenovirus DNA to confirm the insertion of the leader peptide sequence downstream of the CMV promoter (lane 1), for the fusion of the leader peptide to the EGF domain of HRGβ (lane 2), and for the absence of wild type Ad in viral stocks (lane 3). M indicates DNA molecular weight markers. (C) Level of secreted HRGβE peptide in culture media. The conditioned media of uninfected human mesenchymal stem cells (DM 1d) or cells infected with LacZ-Ad, or sHRGβE-Ad at a moi of 100 were harvested at 2 days post-infection and sHRGßE was measured using a commercial HRGβ ELISA. The cells released net HRGβE peptide (~ 6.5 kDa) at the rate of 30-50 ng per 10^{7-8} virus particles (*$p < 0.05$).

HRGβ protein (45 kDa), the molecule numbers of sHRGβE are ~7 fold more than that of full length protein.

Bioactivity of sHRGβE-Ad on receptor phosphorylation

The biological activity of the secreted HRGβ peptide expressed from adenovirus was confirmed by analyzing the stimulation of the tyrosine kinase activity of the ErbB receptors located on C6 cells. When N2 conditioned media collected from HiB5 cell cultures 1, 2, and 3 days after infection was used to treat C6 cells, phosphorylation of ErbB3, the predominant NRG1 receptor on C6 cells (Flores et al., 2000), markedly increased in the presence of the N2 medium collected from sHRGβ-Ad infected HiB5 cultures (Fig. 2A). In contrast, media from either the uninfected or LacZ-Ad infected cell cultures showed minimal effect on ErbB3 phosphorylation (Fig. 2A). The extent of ErbB3 phosphorylation by the sHRGβ-Ad infected culture media markedly increased even at day 1 and was comparable to that of the recombinant HRGβ protein, demonstrating that the peptide generated by sHRGβ-Ad was biologically active. There were no significant differences observed in ErbB3 phosphorylation resulting from treatment with medium that was collected between 1 and 3 days post-infection, and the activity of virus released sHRGβE was maintained over three days. Although only minimal levels of ErbB4 and ErbB2 are present in C6 cells and ErbB4 and ErbB2 are phosphorylated at very low levels, phosphorylation of ErbB4 significantly increased in cells treated with sHRGβE-Ad transduced culture media, to a level comparable to cells treated with recombinant HRGβ (Fig. 2A). This finding suggests that the peptide produced by sHRGβ-Ad effectively binds and activates ErbB4 as well as ErbB3. In contrast, the activation of ErbB2, which does not bind directly to NRG, was shown only at a minimal level, suggesting some specificity of this peptide. Full length NRG directly binds both ErbB3 and ErbB4, and ErbB2 is activated, by dimerization, mainly with ErbB3.

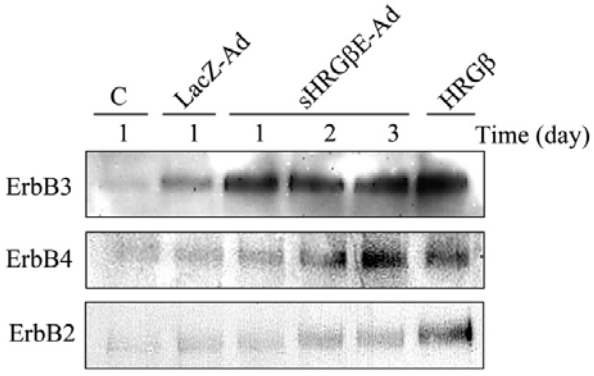

Fig. 2. Biological activity of recombinant sHRGβE. (A) Activation of ErbB receptors by sHRGβE was detected by presence of phosphorylated receptors. Conditioned media from HiB5 cells, either mock-infected (C) or infected with viruses (LacZ-Ad or sHRGβE-Ad) for 1 to 3 days was added to C6 cells for 10 min. Recombinant glial growth factor (50 ng/ml) treatment (HRGβ) was used as a positive control. The C6 cell lysates were equally split into 3 aliquots, immunoprecipitated using anti-ErbB2, B3 and B4 antibodies, and then immunoblotted with an anti-pTyr antibody.

Stimulation of nerve regeneration after injury by administration of sHRGβE-Ad

Many studies have suggested that NRG 1 promotes myelination of Schwann cells (type III), but there are no reports directly showing axonal growth in the peripheral nervous system, in spite of ErbB4 being expressed in axons and NRG1 being released in Schwann cells after injury. We tested the effect of sHRGβE-Ad on nerve regeneration in the rat axotomy model. Rat sciatic nerves were axotomized under the microscope without damage to major blood vessels or nerve bundles, as described in the Materials and Methods. Rats with injured nerves were assigned to three groups: sHRGβE-Ad treatment, LacZ-Ad treatment, and saline treatment. Each rat received two injections into the sciatic nerve at points1 cm proximal and distal to the site of incision. Tissues from the distal stump of the transected nerve were collected at three weeks after the injury

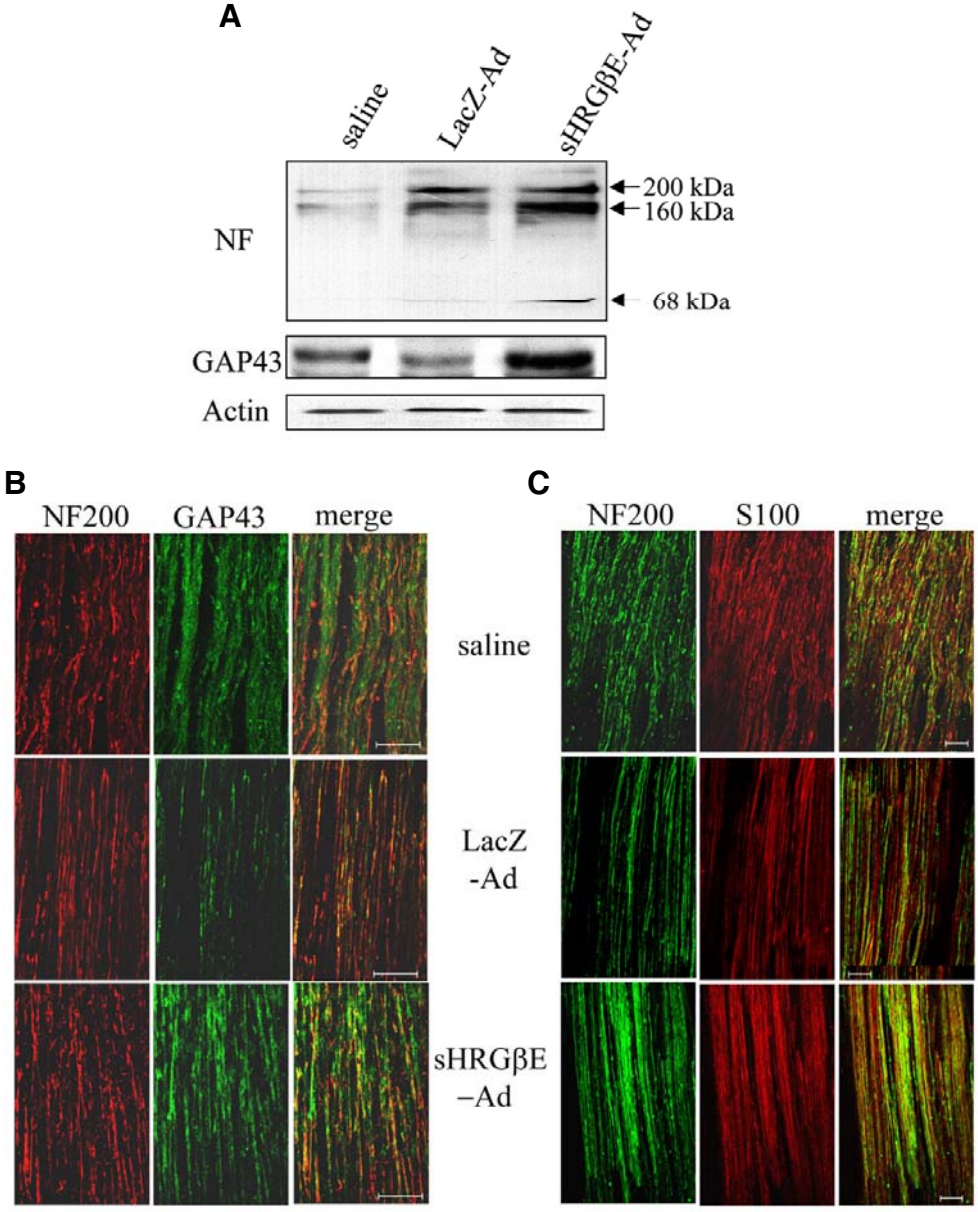

Fig. 3. Improvement of nerve regeneration by injecting sHRGβE-Ad into injured nerves. Expression of neurofilament (NF200) and GAP43 in regenerating axon fibers in sciatic nerve at 3-weeks post-injury was detected by immunoblot analysis (A) and immunohistochemical staining (B). (C) Expression of neurofilament and S100 in sciatic nerve at 3-weeks post-injury. Scale bar, 100 μM.

and the expressions of NF200, the heavy neurofilament protein and GAP43 were observed. GAP43 enables neurons to sprout new terminals and is, therefore, considered to be an intrinsic determinant of axonal growth in the regenerating nervous system (Aigner et al., 1995; Benowitz and Routtenberg, 1997). Immunoblot analysis showed markedly increased expressions of NF200 and GAP43 in the sHRGβE-Ad treated animals (Fig. 3A).

The enhancement of nerve regeneration by sHRGβE-Ad treatment was also confirmed by the immunohistochemical analysis of tissue sections (Fig. 3B). The immunostained axons of NF200 and GAP43 were more organized, parallel, and thicker in the sHRGβE-Ad injected group than in the age-matched control rats that received either saline or LacZ-Ad. GAP43 was detected near the leading ends of newly growing nerve terminals.

Schwann cell regeneration associated with the sciatic nerve recovery was evaluated by immunohistochemical analysis. Three weeks after the surgery, Schwann cells were immunostained with a Schwann cell marker (S100). S100 staining filled the gap in the injury site and surrounded the re-growing axons by proliferation and migration, and it also appeared to differentiate into a long and thin shape with contact to growing axons in the distal stump of the cut site. The S100 staining was found either colocalized with (yellow) or in parallel (red) to the growing axons (NF200, green) in the tissue sections of sHRGβE-Ad treated rats, indicating that Schwann cells were aligned along the re-growing axons or surrounded the axons to re-myelinate them (Fig. 3C). The Schwann cells were, however, aligned to only a portion of the growing axons in the control groups and their aligned length was shorter than the length of the axon, while the Schwann cells were colocalized with most of the full-length axons in the sHRGβE-Ad treated rats.

Measurement of axonal length three weeks after injury showed that sHRGβE-Ad proved to promote axonal growth after injury by (14.49 ± 0.05 mm). In contrast, axonal length in

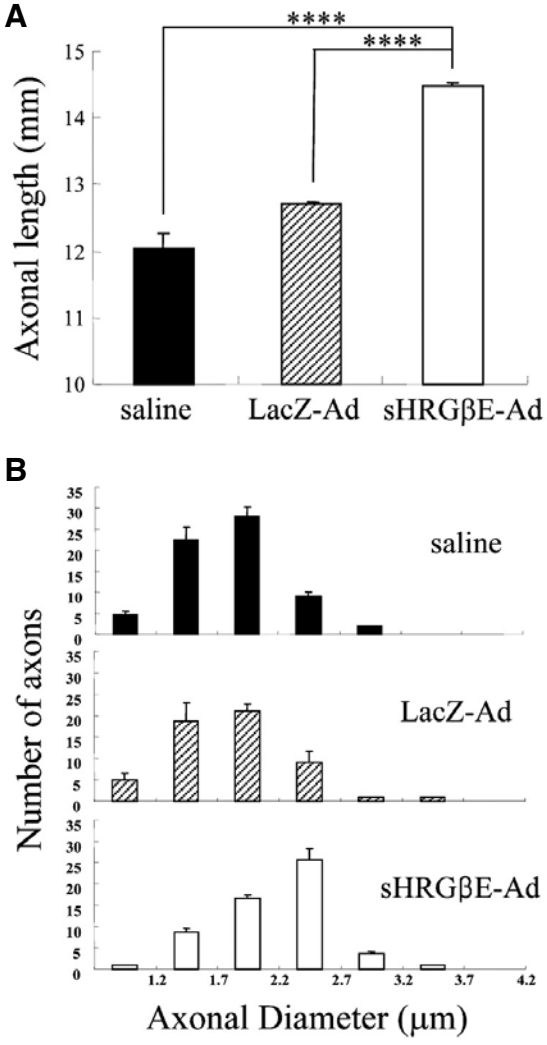

Fig. 4. Axonal growth by injecting sHRGβE-Ad into injured nerves. (A) Axonal length in regenerating nerves at 3 weeks post-injury measured by confocal laser microscopy and an image analyzer (****$p < 0.0001$; $n = 4$ rats). (B) Distribution of axonal caliber in regenerating sciatic nerves. Bars indicate median number of axons within each range (***$p < 0.001$; $n = 3$ rats).

the saline or LacZ-Ad injected groups was shorter (12.03 ± 2.4 mm and 12.72 ± 0.02 mm, respectively; Fig. 4A). Furthermore, as the number of large-diameter axons (> 2.5 μm) increased, small-diameter axons decreased in sHEGβE-Ad treated animals compared to control groups (Fig. 4B). This observation confirms the above results and indicates that the adenovirus releasing the HRGβE peptide improves axonal re-growth and Schwann cell differentiation after nerve injury.

Improved sensory and motor function in injured rats by sHRGβE-Ad

We also investigated the behavioral consequences of the sHRGβE-Ad treatment in axotomized rats at 3 and 5 weeks post-surgery. The withdrawal latency was measured when the rats were individually placed on a heated plate to evaluate the recovery of nociception (Fig. 5). The sHRGβE-Ad injected rats exhibited the shortest mean latencies, at both 3 and 5 weeks post-injury. In addition, only the sHRGβE-Ad treated group

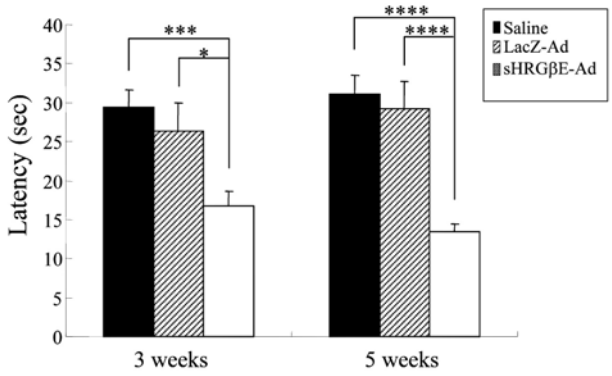

Fig. 5. Recovery of nocieception following axotomy by sHRGβE-Ad. Neurological deficit following axotomy was evaluated with a 54°C hot plate test designed to assess the response to heat. sHRGβE-Ad treatment resulted in faster recovery of nociception after 3 and 5 weeks, respectively, compared to the saline or LacZ-Ad treatment (n = 15 animals for saline and LacZ-Ad, respectively, n = 25 sHRGβE-Ad at 3 weeks; n = 20, 15, and 25 animals for saline, LacZ-Ad, and sHRGβE-Ad, respectively at 5 weeks). (*$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$).

showed a decrease in response latency over the regeneration period, to approximately half the time of the control groups at 5 weeks post-injury (for saline; 29.5 ± 2.2 and 31 ± 2.5 s, LacZ-Ad; 26.3 ± 3.7 and 29.2 ± 3.5 s, and sHRGβE-Ad; 16.7 ± 2.0 and 13.4 ± 0.95 s at 3 and 5 weeks post-injury, respectively).

Motor performance after injury was also assessed using three different neurological tests. The gait of each experimental animal was abnormal after surgery, as they had difficulty in raising their left hind limbs. The sHRGβE-Ad treated groups showed statistically significant improvement over the control groups as measured by neurological scoring at 3 weeks post-injury (Fig. 6). Although the animals did not fully recover by 5 weeks, the total score of axotomized animals in all groups increased due to spontaneous recovery and the sHRGβE-Ad treated animals continued to show better scores.

DISCUSSION

In the present study, we investigated axonal regeneration and its correlation with functional recovery following nerve injury, using an adenovirus to secrete a small peptide of the HRGβEGF-like domain to evaluate its potential usage in nerve regeneration therapy. NRG1 has been reported to play a pivotal role in peripheral nerve development by producing neurons in the cranial ganglia, and by promoting axon guidance and axonal ensheathment in developing nerves. In previously reported studies, neurotrophic and other growth factors were commonly delivered by subcutaneous injection, osmotic mini pumps (Tria et al., 1994), or via nerve conduits (Kemp et al., 2009) of the peripheral nerve injury site, to promote nerve regeneration and speed recovery. Limitations of these methods include inadequate bioavailability and/or bioactivity and inadequate maintenance of a uniform concentration delivered to the target site. Therefore, therapeutic efficacy of a delivery system, including delivery kinetics and optimal dose administration, is crucial to successful peripheral nerve regeneration and subsequent behavioral recovery.

Although there are several reports showing the effects of neuregulins in nerve regeneration, researchers have reported difficulties in maintaining the growth factor release over the long

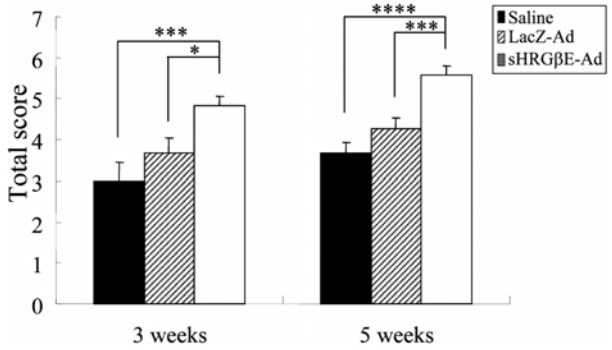

Fig. 6. Recovery of locomotive function following axotomy by treatment with sHRGβE-Ad. Neurological tests to measure the locomotive function in experimental animals were conducted three different ways as described in "Materials and Methods". Locomotive function was severely impaired 3 weeks after surgery in the control groups in comparison to the sHRGβE-Ad treated group (n = 12 animals for saline and LacZ-Ad, respectively, n = 26 animals sHRGβE-Ad at 3 weeks; n = 21 animals for saline and LacZ-Ad, respectively, n = 35 animals sHRGβE-Ad at 5 weeks). The scores in the neurological tests improved in all experimental groups by 5 weeks after surgery (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

duration of nerve regeneration. One strategy for addressing this problem is to develop an efficient delivery system in which HRGβ release is biologically mediated. We have previously demonstrated the ability of adenovirus to produce a reporter gene for up to 7 weeks, which is similar to the period required for axon re-growth in the rat model of an injured sciatic nerve (Joung et al., 2000). Therefore, we assessed the possibility of using a recombinant adenovirus to secrete HRGβE peptide, as a therapy for nerve regeneration. Adenoviral vectors have many merits as suitable gene delivery systems to injured nerves, since they can infect non-dividing nervous system, express a gene of interest for relatively long time periods, and the foreign genetic materials disappear following functional nerve recovery (Shirakawa, 2008). We showed that the adenovirus vector efficiently produced and secreted the small peptide of HRGβE.

We evaluated axonal regeneration following nerve injury. The sHRGβE-Ad treated animals showed improvements in the histological regeneration features and the expression of NF-200 and GAP43 in the distal stump of the transected nerve. sHRGβE-Ad injection also promoted the regeneration of S100-expressing Schwann cells. Furthermore, an increase in the length and caliber of the axons was observed in sHRGβE-Ad treated rats. When injected into injured nerves, the sHRGβE-Ad may easily infect the Schwann cells near the injury sites rather than the cell bodies of sensory and motor neurons located far away in the DRG and spinal cord. It is likely that the HRGβE peptide secreted from the infected Schwann cells could promote the growth of nearby axons. Because axons of sensory and motor neurons in sciatic nerves express ErbB4 (Pearson and Carroll, 2004), this process could be mediated through ErbB4 activation, since sHRGβE-Ad effectively phosphorylates ErbB4, as shown in Fig. 2A. We also found parallel organization of Schwann cells along axons, rather than an excess proliferation of Schwann cells, in the rats transduced with the sHRGβE-Ad. This finding suggests that the HRGβE peptide delivered by adenovirus enhances Schwann cell differentiation, including migration, longitudinal alignment along the axons and the potential re-myelination of the axons. Sensory and motor function recovery is the ultimate result to be obtained in nerve regeneration. We observed that sHRGβE-Ad treatment supported the recovery of motor function as demonstrated by the improved performance in neurological tests. This result is consistent with the immunohistochemical changes in nerve tissues.

In conclusion, this study demonstrated the efficacy of the EGF-like peptide of HRGβ produced by an adenovirus, in lengthening and thickening newly growing axons, as well as stimulating Schwann cell regeneration. It also showed a statistically enhanced functional recovery in rats treated with sHRGβE-Ad compared to control groups, suggesting a new concept to promote regeneration of peripheral nerves, other than surgical repair techniques. The viral vector delivery of the bioactive form of NRG1 would be beneficial since it is generally thought that surgery has reached an optimal technical refinement. Further research is required to confirm the potential of viruses in axonal myelination and to insure the safety of the viruses, thus allowing later therapeutic use of the observed beneficial effects of sHRGβE-Ad.

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