RESEARCH ARTICLE

Skeletal muscle regeneration after insulin-like growth factor I gene transfer by recombinant Sendai virus vector

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We scrutinized the applicability and efficacy of Sendai virus (SeV) vectors expressing either LacZ or human insulin-like growth factor-I (hIGF-I) in gene transfer into skeletal muscle. Seven days after the intramuscular injection of LacZ/SeV X-gal labeled myofibers were demonstrated in rat anterior tibialis muscle with/without bupivacaine treatment and the transgene expression persisted up to 1 month after injection. Recombinant hIGF-I was detected as a major protein species in culture supernatants of a neonatal rat myoblast cell line L6 and thus induced the cells to undergo myogenetic

differentiation. The introduction of hIGF-I/SeV into the muscle showed a significant increase in regenerating and split myofibers which were indicative of hypertrophy, and also an increase in the total number of myofibers, in comparison to that seen in the LacZ/SeV-treated control muscle. These results demonstrate that SeV achieves high-level transgene expression in skeletal muscle, and that hIGF-I gene transfer using SeV vector may therefore have great potential in the treatment of neuromuscular disorders. Gene Therapy (2001) 8, 1043–1050.

Keywords: Sendai virus vector; IGF-I; muscle regeneration

Introduction

Skeletal muscle is an attractive site for the delivery and expression of exogenous genes encoding therapeutic proteins for the treatment for systemic diseases and neuromuscular disorders. In this study, we investigated the feasibility of the novel gene transfer system, recombinant Sendai virus (SeV) vectors containing LacZ reporter gene and human insulin-like growth factor-I (hIGF-I) gene, respectively, for gene delivery into skeletal muscle.

The virus subfamily *Paramyxovirinae* comprises three genera, Rubulavirus, Paramyxovirus and Morbillivirus, which are enveloped viruses with non-segment negative strand RNA genomes. The genome functions both as a template for the synthesis of mRNA and as a template for the synthesis of the anti-stranded genome.^{1,2} SeV belongs to Paramyxovirus and has not yet been reported to be a human pathogen since early studies in virology. SeV has a strict cytoplasmic life cycle in mammalian cells and its genomic RNA is maintained in a cytoplasmic state without an interaction with the chromosomes of the host cells. Therefore, SeV may be free from genotoxicity and thus may be safely used in human gene therapy for a wide range of purposes. The entire genome nucleotide

sequence of the SeV Z strain used here had been determined by Shioda *et al* in 1986.³ Recent success in the recovery of infectious viruses from the transfected cDNA of SeV had opened the way to the genetic engineering of SeV.⁴ Using the method, a variety of foreign genes with additional transcription units have been inserted at 3' end of the genomic position and are expressed at extremely high level.^{5,6} As a result, SeV vectors are expected to be used as a new type of gene transfer system with high safety and efficacy for gene therapy.

IGF-I plays an important role in the development, maintenance and regeneration of skeletal muscle. The effects of IGF-I on myogenic cells include stimulation of myoblast replication, myogenic differentiation and myotube hypertrophy.^{7,8} In muscle regeneration, a proliferation of muscle precursor cells, fusion into myotubes, and reinnervation are involved. In the process, IGF-I produced in satellite cells acts as a powerful stimulant for proliferation and differentiation of muscle precursor cells.⁹

The IGF-I gene transfer to skeletal muscle has been applied to the treatment of the denervated skeletal muscle atrophy using a non-viral system¹⁰ and the age-related loss of skeletal muscle function using AAV vector.¹¹ We herein report the efficient transgene expression in skeletal muscle mediated by a novel vector system based on SeV and the significant induction of muscle regeneration and new myofiber formation in mature rat skeletal muscle due to the high expression level of hIGF-I protein achieved by this new class vector.

Results and discussion

hIGF-I expression in vitro

IGF-I has been shown to be closely related with proliferation, differentiation and hypertrophy in both rat^{12,13} and mouse^{14,15} cell lines. We investigated whether or not SeV-vector-mediated hIGF-I gene transfer can induce morphological change to L6 cells, a neonatal rat myoblast cell line. The newly constructed recombinant SeV vector harboring human IGF-I (hIGF-I) gene, designated as hIGF-I/SeV, and the vector carrying β -galactosidase gene (*LacZ/SeV*)¹⁶ were used and investigated to clarify the following: (1) the detection of hIGF-I expression in the cell culture supernatant (Western blotting analysis); (2) the kinetics of hIGF-I expression in the supernatant (ELISA assay); and (3) the morphological change in L6 cells. (1) In the L6 cells infected with hIGF-I/SeV with a multiplicity of infectivity (MOI) of 0.1, a polypeptide with an approximate molecular mass of 12–13 kDa reactive to the anti hIGF-I antibody was observed in the culture supernatant at 3 to 4 days after infection (Figure 1a, lanes 3 and 4). This peptide is expected to be the hIGF-I precursor, which has an additional peptide of 35 amino acid residues at its carboxyl terminus.¹⁷ This protein species was absent in the *LacZ/SeV*-infected samples (Figure 1a, lane 2). These results demonstrated that substantial

Figure 1 The SeV vector-mediated expression of hIGF-I in vitro. (a) The expression in L6 cells infected with SeV vectors at an MOI of 0.1. The 10-fold concentrated culture supernatants (10 μ l) were electrophoresed on SDS-PAGE and analyzed with Western blotting using anti-hIGF-I antibody (1, no infection; 2, *LacZ/SeV* (3 days after infection); 3, hIGF-I/SeV (3 days); 4, hIGF-I/SeV (4 days)). (b) Time- and MOI-dependent expression of hIGF-I by L6 cells infected with hIGF-I/SeV. The supernatants at each incubation time and MOI were assayed with the ELISA kit.

amounts of hIGF-I were produced by the recombinant SeV vector and were secreted into the culture supernatant. (2) Figure 1b shows the kinetics of the hIGF-I secretion in the supernatant of L6 cells infected with hIGF-I/SeV under single-cycle growth conditions. Under the infectious conditions with an MOI of 0.05, the hIGF-I concentration of the supernatant reached 203 ng/ml at 48 h after infection without any induction of the cytopathogenicity to the cells and thereafter increased up to 435 ng/ml at 96 h. At an MOI of 0.5, hIGF-I of 566 ng/ml and 935 ng/ml were secreted into the medium at 48 and 96 h, respectively. When an MOI was 2.5, hIGF-I secretion was detectable even at 12 h (39 ng/ml) and reached 463 ng/ml at 24 h. No hIGF-I was observed in the *LacZ/SeV*-infected fluid. These results show the noteworthy enrichment of the products via SeV vector-mediated gene transfer. These levels of hIGF-I protein production by the L6 cell line infected with the hIGF-I/SeV were over 100-fold higher than those reported for the C2C12 cell line transfected with a naked plasmid.¹⁸ (3) The L6 cell line is known to not express IGF-I but present IGF-I receptor.¹⁹ Therefore, the cell line is responsive to an exogenous IGF-I, thus resulting in the induction of hypertrophy.²⁰ To examine the biological functions of the hIGF-I derived from hIGF-I/SeV on such myogenic properties, we differentiated confluent L6 myoblast cultures in the media containing the hIGF-I derived from the cells infected with the virus. L6 cells were first grown in a proliferating medium containing 20% FCS, and when 80% confluent, the cells were transferred to differentiation conditions, such as in a serum-free medium, a serum-free medium with 10 ng/ml of authentic hIGF-I protein or in a serum-free medium containing the virus-derived hIGF-I. All samples contained 500 μ g/ml of bovine serum albumin to suppress the loss of hIGF-I by adsorption to vessel surfaces.²¹ Four days after the virus infection clear differences in cell morphology were observed depending on these conditions (Figure 2). The cells were treated with the monoclonal antibody against the embryonic subunit of the myosin heavy chain (MyHC), BF-45.^{22,23} The bound antibody was detected by Alexa Fluor 568 goat anti-mouse IgG(H+L) conjugate. The nuclei of myogenic cells were visualized by propidium iodide staining (PI). The cells infected with an MOI of 0.05 (Figure 2a, e) or 0.2 (Figure 2b, f) of hIGF-I/SeV formed larger myotubes, and exhibited a multiple nuclear organization in the middle of the myofibers (Figure 2e, f). The induction of hypertrophy is apparent from the increased larger myotube size and width (Figure 2a, b), than those seen in the serum-free control conditions lacking hIGF-I (Figure 2d, h). The morphological change induced by the viral infection seems to be consistent with that induced by hIGF-I protein (Figure 2c and g). These results indicated that the recombinant hIGF-I derived from the SeV vector was biologically functional *in vitro* and could induce myogenesis for the neonatal rat myoblast L6 cells.

LacZ reporter gene expression in vivo

To test the efficiency of SeV vector-mediated gene delivery to skeletal muscle *in vivo*, *LacZ/SeV* (5×10^7 p.f.u. in 200 μ l) was injected into the anterior tibialis muscle of adult Sprague-Dawley rats. Transgene expression was detected by X-gal staining for the whole anterior tibialis muscle, followed by transverse sections. In some rats,

Figure 2 Enhancement of myogenic differentiation and hypertrophy of L6 cells by hIGF-I/SeV. The control cells (no infection, d and h) and cells infected with SeV vectors (MOI 0.05, a and e, or 0.2, b and f) were cultured for 4 days. The cells were also cultured in the presence of hIGF-I protein (10 ng/ml, c, g) for 4 days. All cells were cultured in serum-free media. After fixation, the myotubes were treated with monoclonal antibody against the myosin heavy chain embryonic subunit (BF-45) or were subjected to nuclear staining with propidium iodine. The virus-infected cells show moderate and much more pronounced myotube hypertrophy at an MOI of 0.05 and 0.2, respectively, when compared with the cells cultured in the conditions of serum withdrawal only. Nuclear organization grouped in the middle of the myotubes was observed in the vector-infected cells.

bupivacaine, which is known to enhance the transgene expression by provoking a regeneration of the muscle, was injected into the anterior tibialis muscle 3 days before LacZ/SeV injection. Seven days after the LacZ/SeV injection, the muscle tissue pretreated by the intramuscular injection of bupivacaine showed a high level of SeV transduction (Figure 3a and b). In the bupivacaine pretreated muscle (Figure 3b), a considerable number of X-gal positive fibers were observed in the small myofibers around necrotic fibers, infiltrates of polymorphonuclear leukocytes. The small myofibers are considered to be regenerated immature skeletal fibers induced by the intramuscular injection of bupivacaine as reported for the anterior tibialis muscle of adult rats²⁴ and/or by the vector injection. Therefore, the small myofibers to which SeV can introduce the *LacZ* gene are regarded to be mitotically active myoblasts or immature myofibers that appear during skeletal muscle maturation. In normal, untreated muscle, the necrotic myofiber area probably induced by inflammatory responses against the vector infection was much smaller than that with the bupivacaine treatment

Figure 3 Transducibility of SeV vector to skeletal muscle *in vivo*. The adult rat anterior tibialis muscle was pre-treated with/without a myonecrotic agent (bupivacaine). Seven days (a, b, g, h), 14 days (c, d, i, j) and 30 days (e, f, k, l) after the injection of LacZ/SeV (5×10^7 p.f.u. in 200 μ l), the whole muscle was excised and stained for β -galactosidase activity (a, c, e, g, i, k), and were cut 5 μ m thick (b, d, f, h, j, l).

(Figure 3b and h). X-gal-labeled myofibers were demonstrated in the normal, untreated muscle (Figure 3h), although the number of positive fibers was less than that in the bupivacaine-treated muscle. The cells around the transduced myofibers shown in Figure 3h had no central nucleus, were uniform in size and therefore were regarded as mature or non-dividing myofibers. Taken together, the X-gal-positive myofibers in the normal, untreated animals were expected to be mature myofibers, thus indicating that the SeV vector can introduce genes into mature myofibers. The transgene expression was well observed 14 days after the vector injection (Figure 3c, d, i, j). The profile of X-gal-positive fibers in bupivacaine-treated/untreated muscles is consistent with those of the 7-day samples, though the number of X-gal-positive fibers decreased (Figure 3c, d, i, j). Thirty days after

the vector injection X-gal-positive fibers were observed in the bupivacaine-treated muscle (Figure 3e, f), but not in the normal, untreated muscle (Figure 3k, l). In the latter case, only interstitial cells were found to show positive staining (Figure 3k, l). These results indicate that favorable gene delivery to the muscle can be achieved by the SeV vector for the mitotic myoblasts, as well as for post-mitotic immature and mature myofibers, and that SeV vectors carrying genes of clinical interest might be of particular use for muscle gene therapy.

IGF-I gene transfer *in vivo*

After confirming the high level expression for hIGF-I gene *in vitro* and that for LacZ gene *in vivo* for the regenerated and mature myofibers, we examined whether SeV vector-mediated hIGF-I gene transfer to rat skeletal muscle promotes the regeneration of adult rat skeletal muscle, such as an increase in the number of myofibers and myotube hypertrophy. The expression of hIGF-I in the anterior tibialis muscle was determined by a Western blotting analysis. Two hundred microliters of allantoic fluid, the fluid containing hIGF-I/SeV (2×10^8 p.f.u.) or LacZ/SeV (2×10^8 p.f.u.), respectively, were injected into the muscle of adult Sprague-Dawley rats. Seven days after the injection, acid-extracted samples from the muscle treated with hIGF-I/SeV demonstrated a polypeptide with a molecular mass of approximately 8–9 kDa reactive to the anti hIGF-I antibody (Figure 4A, lanes 4 and 5). This molecular mass was not consistent with that observed in the culture supernatant (12–13 kDa, Figure 1a), thus indicating that the hIGF-I was truncated *in vivo*, thus transforming into the mature form of 70 amino acid residues.¹⁷ No band reactive to the anti hIGF-I antibody was observed in the concentrated serum of the animals infected by LacZ/SeV (Figure 4a, lanes 2 and 3).

Detecting the high level expression of hIGF-I in the muscle tissues, we investigated the effects of the transgene expression on muscle regeneration in adult muscle tissues. In the study, four animal groups were set as follows: 12 adult rats (6 weeks) received an injection of hIGF-I/SeV (2×10^8 p.f.u.) into the left anterior tibialis muscle and LacZ/SeV (2×10^8 p.f.u.) into the right anterior tibialis muscle simultaneously (group 1), the same number of rats received an injection of hIGF-I/SeV (2×10^8 p.f.u.) only into the left anterior tibialis muscle (group 2), the same number of rats received an injection of LacZ/SeV (2×10^8 p.f.u.) only into the left anterior tibialis muscle (group 3) and three rats received only allantoic fluid into both anterior tibialis muscles (group 4).

Seven days after the vector injection, the myofibers at the injection site of both hIGF-I/SeV and LacZ/SeV underwent fiber resolution due to massive necrosis with edema formation of the perimysium and infiltration of numerous mononuclear phagocytotic macrophages, lymphocytes and some polymorphonuclear leukocytes in the extracellular space (Figure 4B, d and g). The remaining normal sized myofibers in the necrotic area were invaded by numerous acid phosphatase-positive macrophages (Figure 4B, e and h, open arrowheads). No damage with slight appearance of macrophages was observed in the muscle treated with the allantoic fluid (Figure 4B, a and b). Taken together, SeV introduction causes damage and induces necrosis in the infected muscle, followed by the infiltration of macrophages, lymphocytes and so on,

which are thought to phagocytose and remove the necrotic muscle. Phagocytosis, however, is a very important event preceding effective muscle regeneration. Once phagocytosis occurs, muscle precursor cells or satellite cells are known to be activated and thereafter begin to proliferate.^{25,26} It is interesting to note that very small round myofibers with central nuclei were scattered in foci of infiltrating macrophages along the normal-sized fibers without any morphological alterations. A significantly larger number of small myofibers reactive to the anti-embryonic MyHC monoclonal antibody BF-45, which were indicative of newly formed myofibers during regeneration,^{22,23} was observed in the left anterior tibialis muscle which received hIGF-I/SeV injection (Figure 4B, f), in comparison to the right muscles which received the LacZ/SeV injection (Figure 4B, i). As shown in Figure 4C, the average number of fibers immunoreactive to BF-45 was 446 ($n = 4$) and 1722 ($n = 4$) for LacZ/SeV and IGF-1/SeV in group 1 animals, respectively. No myofibers were immunoreactive to BF-45 in the animals treated with allantoic fluid (group 4, Figure 4B, c).

Fourteen days after the vector injection, the number of infiltrating macrophages decreased dramatically in both hIGF-1/SeV- and LacZ/SeV-treated muscle in groups 1, 2 and 3, as well as in the control muscle in group 4 (Figure 5). In the LacZ/SeV-treated muscle, medium- and uniform-sized myofibers with internuclei replaced the necrotic muscle (Figure 5c). Importantly, in the hIGF-1/SeV-treated muscle, a few necrotic fibers remained with scattered macrophages (data not shown) and various sized myofibers including small-sized fibers were observed (Figure 5b). This might be an indication of the continuous formation of new fibers by the recombinant hIGF-I. No infiltration of fibroblasts and consequent collagenization was observed in either the hIGF-1/SeV- or the LacZ/SeV-treated muscle. The space that had been occupied by such interstitial cells as macrophages at 7 days following the vector injection was found to be almost completely replaced by the regenerated myofibers (Figure 5b and c).

Thirty days after the vector injection, the size of the myofibers returned to nearly normal size and had also become uniform in size in the treated muscle in the group 1 animals (Figure 6A, a, c). In the muscle treated with hIGF-1/SeV in the group 1 animals, a small cluster of medium-sized myofibers was interspersed among the normal sized myofibers (data not shown). The total number of myofibers of the cross sections of the anterior tibialis muscles in the group 1 increased by 17% in the hIGF-1/SeV-treated muscle compared with that in the LacZ/SeV-treated muscle ($P < 0.03$, $n = 4$) (Figure 6B). In group 3, there was no statistically significant difference in the number of fibers between the right and left muscle (data not shown), thus indicating that LacZ/SeV has no potential to increase the number of regenerated myofibers. In addition, the numbers of split fibers, which is a common result of hypertrophy,^{27,28} increased after hIGF-I/SeV treatment (Figure 6A, a, b). The average number of split fibers of hIGF-1/SeV-treated muscle areas was 6.1 times larger than those treated with LacZ/SeV in group 1 animals (Figure 6B, closed column). These data indicate that such splitting stimulated by the treatment with hIGF-1/SeV might be another factor for an increase in the total number of myofibers.

Previous reports have investigated the effect of IGF-I

Figure 4 The expression of hIGF-I and the morphological change of myofibers after the hIGF-I/SeV injection to adult rat anterior tibialis muscle. (A) Western blot analysis with an anti-hIGF-I antibody. Seven days after the injection of either LacZ/SeV (2×10^8 p.f.u. in 200 μ l) or hIGF-I/SeV (2×10^8 p.f.u. in 200 μ l), 300 μ l of the tissue extraction solutions were obtained from 100 μ g of each of the excised frozen muscle tissues. Proteins in 50 μ l (16.7 μ g tissue equivalent) were precipitated with cold acetone and then were subjected to Western blot analysis with anti-hIGF-I antibody (M, marker proteins; 1, no treatment; 2 and 3, LacZ/SeV; 4 and 5, hIGF-I/SeV). (B) Regeneration of the anterior tibialis muscles of adult rats. Each of 200 μ l of LacZ/SeV (2×10^8 p.f.u.) and hIGF-I/SeV (2×10^8 p.f.u.) was injected in the right or left anterior tibialis muscles, respectively. The transverse sections of the excised muscle were treated with hematoxylin and eosin (HE) (a, d, g), the acidic phosphatase activity to detect macrophages (b, e, h) and the antibody BF-45 (c, f, i). The open and the closed arrowheads show cells such as macrophages and the BF-45 positive cells, respectively. Scale bars, 50 μ m. (C) The number of myofibers reactive to the antibody BF-45. The total number of the reactive cells observed in the blue arrowheads (B f, i) was counted ($n = 4$) and results are expressed as the mean $\pm$ s.d. Asterisk denotes a $P < 0.01$ for paired comparisons (Student's *t* test).

gene transfer on hypertrophy in skeletal muscles.^{10,11} Barton-Davis *et al*¹¹ transferred the IGF-I gene into aged rats using an AAV vector. Although fiber regeneration and muscle hypertrophy were observed, they could not see any increase in the total number of myofibers. In our study, a significant increase in the number of regenerated fibers, hypertrophied fibers, and total fibers was observed in the normal adult muscle due to the effect of overex-

pressed hIGF-I by SeV vector. The new myofiber formation observed in our study may be due to the high expression level of hIGF-I achieved by the SeV vector.

We conclude that muscle-targeted gene therapy using SeV vector may thus have a great therapeutic potential to treat not only neuromuscular disorders, but also systemic diseases. SeV vector-mediated hIGF-I gene transfer to skeletal muscle may therefore effectively augment cur-

Figure 5 Regeneration of the anterior tibialis muscles of adult rat observed 14 days after the injection of the vectors. Each of 200 μ l of LacZ/SeV (2×10^8 p.f.u.) and hIGF-I/SeV (2×10^8 p.f.u.) was injected in the right or left anterior tibialis muscle, respectively. The transverse sections of the excised muscle were stained with hematoxylin and eosin (a, control allantoic fluid; b, hIGF-I/SeV to left anterior tibialis muscle; c, LacZ/SeV to right anterior tibialis muscle).

rent treatment modalities for the treatment of muscle atrophy and the degeneration of myofibers in neuromuscular disorders.

Materials and methods

Construction of SeV vector.

The hIGF-I open reading frame was amplified by PCR from a human cDNA library (Gibco BRL, Rockville, MD, USA) with the primers 5'-ATCCGAATTCGCAATGGAAAAATCAGCAGTC-3' and 5'-ATCCGAATTCCTACATCTGTAGT TCTTGTTTCCTGC-3' based on the published DNA sequence.¹⁷ The resulting PCR product was cloned at the *Eco*RI site of pBluescript II and then was sequenced. To generate phIGF-I/SeV, the product with the correct sequence of hIGF-I gene was reamplified with primers containing SeV-specific transcriptional regulation signal sequences, 5'-ATCCGCGGCCGCCAAAGTTCAGCAATGGGAAAAATCAGCAGTCTTC-3' and 5'-ATCCGCGGCCGCGATGAACCTTCAACCTAAGTTTTCTTACTACGGCTACATCCTGTAGT TCTTGTTTCCTGC-3' and was cloned in the *Not*I site of pSeV18+b(+) which had been constructed to produce the exact SeV full-length antigenomic plus sense RNA of 15402 nucleotides. The phIGF-I/SeV was transfected to LLCMK2 cells previously infected with vaccinia virus vTF7-3, expressing T7 polymerase. The T7-driven full-length recombinant hIGF-I/SeV RNA genomes were encapsulated with N, P and L proteins, which were driven from the respective cotransfected plasmids. Forty hours later, the transfected cells were injected into embryonated chicken eggs to amplify the recovered virus.

In vitro study

L6 cells were maintained as myoblasts by culturing in DMEM medium with 20% FCS and penicillin/streptomycin. The cells were differentiated in the serum-free DMEM or in the virus-infected conditions with the indicated multiplicity. For immunoblotting, the 100 μ l of the culture supernatant was concentrated to 10 μ l with two volumes of cold-acetone, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 15–25% resolving gel and then was transferred to polyvinylidene-difluoride membranes (Daiichi Pure Chemicals, Tokyo, Japan). The blot was probed with the anti-hIGF-I monoclonal antibody (Diagnostic Systems Laboratories, Webster, TX, USA) and incubated with the horseradish peroxidase-labeled antibody (Amersham, Amersham, UK). The immunocomplexes were visualized

using enhanced chemiluminescence (ECL, Amersham). A standard ELISA assay to measure the serum level of anti hIGF-I antibodies was performed basically according to the manufacturer's recommendations (R&D Systems, Minneapolis, MN, USA).

LacZ reporter gene expression in vivo

Sprague-Dawley rats (male, 6-week-old, 160–180 g) were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg). The hind limbs were shaved, scrubbed with ethanol, and 1.5-cm skin incisions were made to expose the anterior tibialis muscle. For LacZ reporter gene transfer study, 200 μ l of LacZ/SeV (5×10^7 p.f.u.) were injected into the mid-belly of the left anterior tibialis muscle. In bupivacaine-treated animals, 200 μ l of 0.5% bupivacaine solution was injected into the anterior tibialis muscle 3 days before the vector injection.

X-gal staining for β -galactosidase

For an evaluation of the LacZ expression, the excised mid-belly portion of anterior tibialis muscle was fixed in cold 4% paraformaldehyde in phosphate-buffered saline (PBS) for 3 h, washed in PBS for 1 h, and then was stained in X-gal (5-bromo-4-chloro-3-indol- β -D-galactopyranoside) solution (β -gal staining kit, Invitrogen, Carlsbad, CA, USA) at 37°C overnight. After taking photographs of whole muscle, stained muscle was snap-frozen with an optimal cutting temperature compound (OCT) (Miles, Elkhart, IL, USA) in liquid nitrogen, cut in 5- μ m-thick axial sections and counterstained with eosin.

IGF-I/SeV gene expression in vivo

For the study to evaluate the effect of hIGF-I gene transfer, 200 μ l of hIGF-I/SeV (2×10^8 p.f.u.) was injected into the mid-belly of the left anterior tibialis muscle. As a control, LacZ/SeV (2×10^8 p.f.u. in 200 μ l) was injected into the mid-belly of the right anterior tibialis muscle, using 1-ml syringe with 30-gauge, 0.5 inch needle. The skin was closed using a 4-0 nylon suture. After surviving for 7 days, 14 days and 30 days, the animals were killed by a lethal dose of pentobarbital sodium. The anterior tibialis muscles were excised and processed for histological staining and Western blotting. hIGF-I in the rat muscle was assayed by Western blotting. One hundred micrograms of muscle tissue were frozen using liquid nitrogen, and then were pulverized to powder with a pestle and mortar followed by adding 500 μ l of chilled 1 M acetic acid. The mixture was homogenized, allowed to stand on ice for 2 h, and centrifuged at 3000 g for 15 min to collect the supernatant. The residual precipitate was

Figure 6 Regeneration of muscle fibers in the anterior tibialis muscle of adult rats 30 days after the vector injection. (A) Split fiber formation by hIGF-I/SeV. Inflammatory reaction was almost not seen in the section. hIGF-I/SeV-treated muscle showed the splitting of myofibers shown by the arrowheads (a, b (enlargement)). The myofibers regenerated and returned almost to normal (c).

subjected again to extraction with fresh 1 M acetic acid. The two supernatants were combined, frozen at -70°C , lyophilized to dryness and reconstituted with 50 mM Tris-HCl buffer (pH 7.8) in a ratio of 300 μl of buffer to 100 μg of the original tissue. After concentrating the solution from 50 μl to 10 μl , the solution equivalent to 16.5 μg of the original tissue underwent Western blotting.

Figure 6 (B) Effect of hIGF-I on the total number of myofibers (open columns) and the split fibers (closed columns). The total number of fibers of the LacZ/SeV-treated anterior tibialis muscle of each animal in group 1 served as the control (defined as a value of 1.0) for hIGF-I/SeV-treated ones (expressed relative to the control value). The average relative value ($n = 4$) is plotted. Single asterisk denotes a $P < 0.03$ for paired comparisons (Student's *t* test). The number of split fibers was counted and results are expressed as the mean $\pm$ s.d. ($n = 4$). Double asterisks denote a $P < 0.01$ for paired comparisons (Student's *t* test).

Immunohistochemical staining for regenerating myofibers

Excised mid-belly portion of anterior tibialis muscle was immediately frozen in isopentane cooled in liquid nitrogen. Frozen at -20°C , serial axial sections cut 10 μm -thick were placed on lysine-coated slides and air dried. In order to detect regenerating myofibers, the sections were incubated with primary mouse anti-embryonic myosin heavy chain monoclonal antibody (BF-45, American Type Culture Collection, Manassas, VA, USA). After washing with PBS, the sections were incubated with secondary biotinylated anti-mouse IgG (1:100; Vector, Burlingame, CA, USA) for 1 h, then with streptavidin-biotin complex (1:200 dilution; Vector) for 1 h at room temperature. The avidin-biotin complex was visualized with 0.05 M Tris-HCl buffer (pH 7.6) containing 0.05% 3, 3'-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide. The sections were thereafter counterstained with hematoxylin. To assess the hypertrophic effect of hIGF-1 on the affected muscle, the percentage of immunopositive fibers against BF-45 at 7 days after injection was determined by counting their numbers under light microscopy. In addition, the number of split fibers of muscles at 30 days was also counted to evaluate the hypertrophic effect of hIGF-1.

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