IGF-1 Gene Transfer Into Denervated Rat Laryngeal Muscle

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Objectives: To demonstrate gene transfer in rat laryngeal muscle using a reporter gene, β-galactosidase, and a muscle-specific expression system containing the human IGF-1 (hIGF-1) gene sequence and to investigate the myotrophic and neurotrophic effects of hIGF-1 gene transfer in denervated rat laryngeal muscle.

Methods: In 8 adult rats, a polyvinyl-based formulation containing β-galactosidase DNA was injected into denervated thyroarytenoid muscle. Twelve animals were similarly administered a polyvinyl-based formulation containing a muscle-specific expression system and hIGF-1 DNA. Twelve animals were injected with isotonic sodium chloride solution, and all animals survived for 1 month. The production of β-galactosidase and hIGF-1 was detected using immunohistochemical techniques. The effects of hIGF-1 on motor endplates and nerve sprouting were assessed using cholinesterase or silver staining and immunostaining for growth-associated protein (GAP-43).

Results: β-Galactosidase was detected in 7 of 8 animals by immunostaining, X-gal histochemical staining, or both. In frozen section specimens, hIGF-1 immunoreactivity was positive in 3 of 8 animals. In sequential sections, GAP-43 was localized to areas of hIGF-1 expression in 2 of the 3 hIGF-1-positive specimens. Increased nerve sprouting and motor endplate contact occurred in 2 of 4 animals treated with hIGF-1.

Conclusions: Gene transfer into laryngeal muscle was demonstrated using a polyvinyl-based formulation containing a muscle-specific gene expression system. Preliminary findings indicate a positive effect on motor endplates, nerve sprouting, and the expression of GAP-43. Further studies are necessary to quantify myotrophic and neurotrophic effects and to establish therapeutic benefit.


Surgical Options: The management of unilateral laryngeal paralysis consists of medialization of the vocal fold by injection, thyroplasty, arytenoid adduction, and reinnervation. With the exception of reinnervation procedures, vocal fold medialization is achieved by static change to the vocal fold tissue or laryngeal framework, with no influence on dynamic function. Although improved laryngeal function can be demonstrated in 90% of patients surgically treated with medialization, physical limitations of muscle atrophy associated with denervation persist.

Reinnervation has not been widely accepted as a treatment option for patients with laryngeal paralysis. Clinically, laryngeal reinnervation procedures have had little or no effect on dynamic function, and if any benefit is realized, it is due to a reversal of muscle atrophy and an increase in muscle mass, tension, or both. The failure to achieve functional reinnervation may reflect a decrease in motor fiber density from a central loss of motoneurons, the failure of reinnervating neurons to cross the anastomotic site, the loss of viable motor endplates, or as a result of inappropriate innervation by antagonistic motoneurons.

Attempts to influence motoneuron viability and enhance the process of reinnervation have focused on the delivery of neurotrophic factors either locally or parenterally. Key trophic factors affecting nerve regeneration include nerve growth factor, brain-derived neurotrophic factor, ciliary neurotrophic factor, and insulinlike growth factors 1 and 2 (IGF-1 and -2). Formerly called somatomedin, IGF-1 is both neurotrophic and myotrophic. Insulinlike growth factor 1 is critical in muscle development, muscle growth, and nerve sprouting and has been shown to prevent or reverse atrophy and enhance nerve regeneration in denervated muscle.

A major problem in the delivery of growth factors is the rapid clearance of the factor in vivo and the failure to achieve steady-state levels. Therapeutic levels in tar-
MATERIALS AND METHODS

INJECTION TECHNIQUE

In anesthetized adult male rats, the larynx was exposed through a midline incision. The left recurrent laryngeal nerve and superior laryngeal nerve were divided and the ends suture ligated. A midline thyrotoomy was performed to expose the laryngeal musculature. The denervated thyroarytenoid and lateral cricoarytenoid muscles were injected with a reporter gene (β-galactosidase) or therapeutic gene (hIGF-1) using a Hamilton syringe and 30-gauge needle. In animals receiving the reporter gene and the therapeutic gene, a single injection containing 90 µg of DNA in 30 µL of a vector solution was delivered, in a similar manner, parallel to the long axis of the muscle body to maximize exposure to the vector. The thyrotoomy and incision were closed, and the animals survived for 1 month.

hIGF-1 GENE CONSTRUCT AND DELIVERY SYSTEM

A muscle-specific expression system and a polyvinyl-based formulation was developed to enhance intramuscular diffusion, gene transfer, and expression in muscle (Gene Medicine, Inc, Woodlands, Tex). The plasmid contains a muscle-specific α-actin promoter. The construct consists of an SkA (chicken skeletal α-actin) promoter, SkA 5′ untranslated region (3′UTR), including the SkA first intron, the hIGF-1 coding sequence, and the human growth hormone 3′UTR region and polyadenylated (poly A) tail. The tail region modification is designed to route the hIGF-1 gene product extracellularly and enhance the paracrine effect of hIGF-1. The construct was cloned into a plasmid backbone containing the origin of replication and kanamycin resistance gene. The plasmid was formulated with polyvinyl pyrrolidone for direct delivery into muscle by injection.

TRANSFECTION WITH β-GALACTOSIDASE (REPORTER GENE)

β-Galactosidase, a reporter or marker gene that encodes for a nontherapeutic protein or enzyme, is used for the identification of gene transfer with product expression. The plasmid containing the reporter gene sequence was also formulated with polyvinyl pyrrolidone.

In 8 anesthetized adult male rats, the denervated thyroarytenoid and lateral cricoarytenoid muscles were injected with the formulation containing the β-galactosidase gene sequence (90 µg of DNA in 30 µL of the vector solution). At 1 month, the animals were killed and the larynges excised and processed for frozen section. Coronal sections were cut at 10 µm and alternate sections mounted. In all 8 animals, sections were assayed for β-galactosidase enzyme activity using a chromogenic substrate (X-gal, Boehringer Mannheim Corporation, Indianapolis, Ind). Immunostaining with primary antibodies to β-galactosidase was performed on sequential sections from 5 animals using rabbit anti–β-galactosidase (Cappel, Durham, NC). Immunoreactivity was detected using a biotinylated secondary antibody (goat anti-rabbit immunoglobulin) and streptavidin-conjugated alkaline phosphatase (BioGenex Labs, San Ramon, Calif) with fast-red substrate (Vector Labs Inc, Burlingame, Calif). Alternate sections were also immunostained for growth-associated protein (GAP-43) using mouse anti–GAP-43 (Boehringer Mannheim Corporation) and biotinylated goat anti-mouse immunoglobulin (BioGenex Labs). The protein GAP-43 is up-regulated in the denervated condition and is a biologic marker for nerve sprouting and nerve regeneration. Specimens were examined under light microscopy and assessed for immunoreactivity and the X-gal chromagen.

TRANSFECTION WITH hIGF-1 (THERAPEUTIC GENE)

Twenty-four animals were anesthetized and prepared as described earlier. In 12 animals (treatment group), the thyroarytenoid and lateral cricoarytenoid muscles were injected with the formulation containing the hIGF-1 gene sequence (90 µg of DNA in 30 µL of the vector solution) and survived for 1 month. Similarly, 12 animals were injected with isotonic sodium chloride solution (control group) and survived for 1 month. Eight animals from each group were evaluated for hIGF-1 production and GAP-43, and the remaining 4 animals in each group were processed for motor endplate study.

For the immunodetection of hIGF-1 and GAP-43, laryngeal specimens were obtained and processed as described earlier. Sequential sections were immunostained with antibodies directed against IGF-1 (mouse anti-human IGF-1, Diagnostic Systems Laboratories, Webster, Tex) or GAP-43. Specimens were examined under light microscopy and assessed for immunoreactivity.

Motor endplate structure was assessed using the cholinesterase or silver-gold technique described by Pestronk and Drachman.1,11 Eight animals were anesthetized and prepared as described earlier. Laryngeal specimens were processed for frozen section, cut transversely in 35-µm sections, and mounted in 3% disodium EDTA. Tissue was then processed using bromoindoxyl acetate dye staining for cholinesterase in motor endplates and silver-gold impregnation for nerve terminals. Specimens were examined under light microscopy and assessed for changes in endplate structure and the presence of neural filaments.
plasmid containing a reporter gene encoding β-galactosidase. Therapeutic gene transfer is further explored using a muscle-specific gene expression system containing the human IGF-1 (or hIGF-1) gene sequence. Applied to laryngeal paralysis, hIGF-1 gene transfer provides new opportunities to improve laryngeal function by reducing or preventing muscle atrophy, supporting neural elements, and enhancing the process of reinnervation.

**RESULTS**

**TRANSFECTION WITH β-GALACTOSIDASE PLASMID**

Five of the 8 specimens were studied using antibodies directed against β-galactosidase. Focal areas of intracellular immunostaining for the marker gene were detected within thyroarytenoid and lateral cricoarytenoid muscle fibers in all 5 specimens (Figure 1, A). All 8 specimens were processed for β-galactosidase enzyme activity using the X-gal chromagen detection method. In 7 of 8 specimens, intracellular X-gal chromagen deposition was detected within thyroarytenoid and lateral cricoarytenoid muscle fibers (Figure 1, B). Intracellular chromagen deposition corresponded to intracellular immunostaining on sequential sections in 4 of the 5 specimens studied using both detection methods. Using both methods, gene transfer and expression were observed in 7 of 8 animals at 1 month. Immunostaining and X-gal chromagen deposition were absent in all controls.

**TRANSFECTION WITH hIGF-1 PLASMID**

Eight specimens transfected with the hIGF-1 plasmid were processed for immunostaining with antibodies directed against hIGF-1. Intracellular and extracellular immunoreactions were detected in 1 specimen, and intracellular immunostaining was detected in 2 specimens. Immunostaining was absent in all control specimens.

Sequential sections from the 8 hIGF-1–transfected specimens and controls were also immunostained with antibodies directed against GAP-43. Moderate to heavy staining localized to neural elements and motor endplates was observed in 7 of 8 hIGF-1–transfected specimens. Similarly, moderate to heavy staining of neural elements and motor endplates was observed in the denervated muscle of all 8 control specimens. In 2 of 3 hIGF-1–immunopositive specimens, however, GAP-43 immunostaining was localized to the areas of hIGF-1 immunostaining (Figure 2, A through D).

**MOTOR ENDPLATE STRUCTURE**

Motor endplate structure and neural elements observed in normal controls are demonstrated in Figure 3, A. In denervated control specimens (n = 4), characteristic changes in motor endplates were observed in all animals. As seen in Figure 3, B, denervated endplates are elongated, cholinesterase staining is diffuse, and silver-staining neural elements are absent. Two of 4 hIGF-1–transfected animals were distinguished from controls by the presence of neural elements and nerve-to-motor endplate contact. In these hIGF-1–treated animals, motor endplates appeared shorter and cholinesterase staining was more concentrated (Figure 3, C). Although localization of hIGF-1 was not performed in these specimens, the observed changes were homogeneously distributed through the muscle body.

**COMMENT**

Reinnervation is a rational therapeutic consideration for the treatment of patients with laryngeal paralysis, but numerous approaches to selectively innervate laryngeal muscle have ultimately failed because of uncontrolled factors, including the loss of central motoneurons, a decrease in nerve fiber density, and random or misdirected reinnervation.3,4 A variety of trophic factors, including nerve growth factor, brain-derived neurotrophic factor, ciliary neurotrophic factor, and IGF-1 and -2, have been used to prevent cell death centrally and enhance nerve sprouting and motor endplate contact peripherally.3 Insulinlike growth factor 1 is both neurotrophic and myotrophic and has been demonstrated to play a key role in normal muscle development, late-stage muscle growth, and muscle and nerve...
Insulin-like growth factor 1 effects the proliferation of muscle precursor cells and fusion into myotubes and subsequent differentiation. The induction of nerve sprouting is also, in part, IGF-1–dependent.

The clinical application of trophic factors, including IGF-1, is limited by bioavailability to the target organ and the inability to maintain steady-state levels. Therapeutic benefit is generally achieved at systemically toxic doses of intravenously administered recombinant proteins. Gene therapy allows for the prolonged delivery of specific growth factors in a high concentration at the target site with minimal systemic exposure. This important principle of gene therapy may provide the opportunity to facilitate and manipulate the process of reinnervation in the absence of systemic toxic effects.

Muscle is an excellent target organ for gene therapy for many reasons. Muscle cells have a large capacity for protein synthesis and have the ability to secrete proteins both locally and systemically. Direct needle injection of protein/DNA complexes containing the β-galactosidase marker gene leads to a steady-state local expression in vivo for several weeks to months. DNA plasmids injected into muscle remain episomal and are cleared by normal muscle cell mechanisms with no reported sequelae. Because the plasmid does not integrate into the muscle cell chromosomes, there is little risk of genetic alteration or damage. This property contrasts with the potential risks involved in retrovirus-mediated gene transfer, which results in “permanent” chromosomal integration. Furthermore, retroviral vectors have low transduction efficiency in general and are not ca-

Figure 2. A, Low-power photomicrograph of hemilarynx demonstrating immunoreactivity to hIGF-1 gene (arrows) in rat laryngeal muscle transfected with hIGF-1 plasmid (magnification ×25). B, Photomicrograph of sequential section to A, demonstrating immunoreactivity to GAP-43 (arrows) in hIGF-1–positive region (magnification ×25). C, High-power photomicrograph of A (lower arrow) demonstrating immunoreactivity to hIGF-1 in rat laryngeal muscle transfected with hIGF-1 plasmid (magnification ×200). D, High-power photomicrograph of B (lower arrow) demonstrating immunoreactivity to GAP-43 in hIGF-1–positive region. Immunostaining is localized to neural elements (alkaline phosphatase detection system with fast-red substrate and Mayer hematoxylin counterstain; magnification ×200).
pable of infecting nondividing cells. Adenoviral vectors represent a gene delivery system that is highly effective in replicating and nonreplicating cells. Adenoviral vectors have, however, substantial limitations when applied to muscle. Adenovirus does not effectively transduce muscle cells except in the perinatal period. A muscle-specific gene delivery system containing the actin gene promoter that produces more than 10-fold higher levels of muscle-specific gene expression in vitro and in vivo with no evidence of systemic secretion or adverse effects has been developed. This system is ideal for expressing hIGF-1 because the actin promoter incorporated in the gene construct is up-regulated by IGF-1, thus maximizing expression and enabling the delivery of lower quantities of the DNA vector.

The efficiency of this system has been optimized for the expression of hIGF-1 in muscle with the recent development of a polyvinyl-based delivery system. This system enhances the retention, stability, and dispersion of DNA after intramuscular injection and increases the level of expression 10- to 100-fold over protein/DNA complexes. The polymer used in this system, polyvinyl pyrrolidone, is approved by the United States Pharmacopeia for use in conventional pharmaceutical products and is generally regarded as safe.

Using the muscle-specific vector and polyvinyl-based delivery system, gene transfer into laryngeal muscle is demonstrated in this study, using both a reporter gene and a therapeutic gene, 1 month after delivery of the vector. Definitive hIGF-1 expression was observed in only 3 of 8 treated animals. This observation may reflect a true decrease in transfection with the hIGF-1 plasmid, a more rapid clearance of the hIGF-1 plasmid, a decrease in effective transcription, instability of the IGF-1 gene product, or simply the diffusion of gene product into the extracellular compartments. The gene delivery system used in these experiments was designed to route hIGF-1 extracellularly, thereby exposing surrounding nontransfected muscle and motor nerves to the therapeutic factor. Although this modification may augment the therapeutic response, it limits our ability to detect gene product using immunohistochemical staining techniques. The gene delivery system designed for β-galactosidase, where gene product remains intracellular, results in a greater rate of gene product detection (7 of 8 treated animals) and supports product diffusion as a factor limiting the detection of hIGF-1 by immunohistochemical techniques. The identification of the hIGF-1 gene may require more sensitive methods for detection, specifically, polymerase chain reaction and reverse transcriptase polymerase chain reaction. Limited gene expression may also reflect the single injection technique used for the delivery of plasmid vector in this study. The delivery technique to optimize gene transfer and therapeutic effect will be the focus of future studies.

The neurotrophic effect of hIGF-1 is suggested by the localization of GAP-43 immunoreactivity to regions immunostaining for hIGF-1. The nonspecific up-regulation of GAP-43 activity in denervated control muscle.

**Figure 3.** A, Photomicrograph of normal laryngeal muscle demonstrating motor endplates and nerve terminals (arrows) using cholinesterase or silver-gold staining technique. B, Untreated denervated muscle 4 weeks after nerve section demonstrating elongation of motor endplates and the absence of neural filaments. C, Photomicrograph of laryngeal muscle transfected with hIGF-1 plasmid demonstrating nerve sprouting and motor endplate contact (arrows) 4 weeks after nerve section and injection with plasmid formulation (magnification × 400).
The production of hIGF-1 and β-galactosidase, introduced into rat laryngeal muscle using a polyvinyl-based formulation containing plasmid DNA, was demonstrated using immunostaining and histochemical techniques.

Preliminary findings in laryngeal muscles indicate a positive effect on motor endplates, nerve sprouting, and the expression of GAP-43 in animals treated with hIGF-1. Further investigation is necessary to maximize dosing requirements of gene therapy and to quantify the pharmacological effects on nerve sprouting, the innervation of motor endplates, muscle fiber size, and myosin heavy-chain production.

Applied to laryngeal paralysis, gene therapy provides a tremendous opportunity for the augmentation of current surgical treatment modalities by preventing or reversing muscle atrophy. Manipulation by site- or function-specific growth factors to achieve selective reinnervation may ultimately enhance our ability to achieve a true functional recovery in patients with laryngeal paralysis.

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