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IGF-1 Gene Therapy : Future Strategy for Management of Laryngeal Paralysis

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Abstract : Current surgical strategies for the treatment of laryngeal paralysis are limited by the muscle atrophy associated with denervation. Moreover, attempts at reinnervation have not affected significant change in surgical outcome. To address this clinical problem, we have developed a rat laryngeal paralysis model to study novel gene transfer strategies. A muscle specific non-viral vector containing the α -actin promoter and hIGF-I gene formulated with polyvinyl polymers injected into denervated adult rat thyroarytenoid muscle has been shown to produce significant increase in muscle fiber diameter, significant decrease in motor endplate length and significant increase in percentage of endplates with nerve contact.

Applied to laryngeal paralysis, hIGF-I gene therapy provides opportunity for augmentation of surgical treatment modalities by prevention or reversal of muscle atrophy, enhanced nerve sprouting and reinnervation. If proven effective, gene therapy may be applied clinically to fine-tune current surgical procedures or even eliminate the need for surgical intervention.

Introduction

Surgical treatments for unilateral laryngeal paralysis, including vocal fold injection, thyroplasty and arytenoid adduction, achieve vocal fold medialization by static change to the vocal fold tissue or laryngeal framework¹⁾. Surgical outcome with these procedures may be limited by muscle atrophy associated with denervation. Laryngeal reinnervation procedures have had little impact on return of dynamic laryngeal function and are not widely accepted as treatment options. Failure of reinnervation procedures to achieve functional results reflects multiple factors including a decrease in motor fiber

density, central motoneuron loss, associated muscle atrophy, and inappropriate or misdirected innervation by antagonistic motoneurons^{2,3)}.

A multitude of neurotrophic factors have been shown to prevent central motoneuron loss, enhance nerve sprouting, preserve motor endplate morphology, and promote reinnervation^{4~6)}. Of these, insulin-like growth factor-I (IGF-I) is both neurotrophic and myotrophic and plays an important role in maintenance and regeneration of muscle and peripheral nerves^{6~8)}. The use of IGF-I and other trophic factors may prove beneficial for the treatment of peripheral nerve injuries. The clinical application of trophic factor therapy, however, is limited by poor

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bio-availability, the inability to achieve steady state levels, and toxicity associated with systemic delivery^{9,10}. Gene therapy provides a mechanism, whereby, infrequent administration achieves long-term, steady-state levels of gene product using common routes of delivery^{11,12}.

In prior investigations, a rat laryngeal paralysis model was established to evaluate the potential of muscle specific non-viral gene therapy based on an hIGF-I plasmid formulated with polyvinyl pyrrolidone (PVP)¹³⁻¹⁵. Single injection of this hIGF-I formulation produced comparable effects on muscle fiber diameter and motor endplate morphology to those achieved using a cumbersome model of daily recombinant IGF-I protein injections for 3-6 weeks^{16,17}. This report describes our initial experiences with gene transfer into adult rat laryngeal muscle using a muscle specific gene expression system containing the human IGF-I gene sequence.

Muscle specific Vector

Details and construction of the muscle specific α -actin/IGF-I hybrid gene have been previously described^{18,19}. The construct consists of a chicken skeletal α -actin promoter, hIGF-I coding sequence and human growth hormone (hGH) 3'UTR region.

The promoter region is skeletal muscle specific and up-regulated by the gene product hIGF-I. The hGH 3'UTR region enhances secretion of hIGF-I protein product, and thus, increases the paracrine effects of hIGF-I. Secretion of gene product will, theoretically, produce a more homogenous response within muscle tissue at effectively lower doses.

The construct was initially cloned into the pBlue-script II KS- backbone (Stratagene, La Jolla, CA), which contains the plasmid origin of replication and ampicillin resistance. The ampicillin resistance gene has been eliminated and replaced with a kanamycin resistance gene to avoid the use of lactams in production. The construct is used in formulation with polyvinyl pyrrolidone (PVP)²⁰ to enhance delivery into muscle cells.

Reporter gene transfer in rat laryngeal muscle

Gene transfer in rat laryngeal muscle has been demonstrated using a reporter gene, β -galactosidase, formulated in a polyvinyl-based delivery system¹³. In eight adult rats, the formulation containing β -galactosidase DNA was injected into denervated rat thyroarytenoid muscle. Production of β -galactosidase was detected one month after

Fig. 1 a : Photomicrograph of hemilarynx immunostained with antibodies directed against β -galactosidase. Immunoreactivity is seen within the TA/LCA muscle fibers of the rat larynx (Alkaline phosphatase detection system with Fast Red substrate and Mayer's hematoxylin counter stain ; magnification 100 $\times$), b : Photomicrograph of sequential section assayed for galactosidase enzyme activity using the chromagenic substrate Xgal. Chromagen deposition is seen only in areas corresponding to anti- β galactosidase immunoreactivity (Eosin counterstain ; magnification 100 $\times$)

injection by immunostaining and XGAL histochemical staining (Figure 1a,b). Using both methods, gene transfer with functional gene product was detected in 7 of 8 animals.

Human IGF-I gene transfer in denervated rat laryngeal muscle

A muscle specific non-viral vector containing the α -actin promoter and hIGF-I gene was used in formulation with a polyvinyl-based delivery system and injected into paralyzed adult rat laryngeal muscle¹³⁾. Eight specimens transfected with the

Fig. 2 High power photomicrograph demonstrating intracellular immunoreactivity to human IGF-1 (arrows) in rat laryngeal muscle transfected with human IGF-1 plasmid (magnification 400 $\times$)

hIGF-I plasmid were processed for immunostaining with antibodies directed against human IGF-I. Both intracellular and extracellular immunoreactions were observed in 3 of eight specimens (Figure 2). Immunostaining was absent in all control specimens.

Sequential sections from the eight IGF-I transfected specimens and controls were also immunostained with antibodies directed against GAP-43. Moderate to heavy staining localized to neural elements and motor end plates was observed in 7 of 8 IGF-I specimens. Similarly, moderate to heavy staining of neural elements and motor end plates was observed in the denervated muscle of all control specimens (n=8). However, in 2 of 3 IGF-I immuno-positive specimens, GAP-43 immunostaining was localized to the areas of IGF-I immunostaining (Figure 3a, b).

Motor endplate morphology

Motor endplate morphology and neural elements studied 28 days following single injection with hIGF-I formulation¹³⁾. In denervated control specimens (n=4), characteristic changes in motor endplates were observed in all animals. As seen in Figure 4a, denervated endplates are elongated, cholinesterase staining is diffuse, and silver staining neural elements are absent. Two of four IGF-I transfected animals were distinguished from controls by

Fig. 3 a : High power photomicrograph demonstrating immunoreactivity to human IGF-1 in rat laryngeal muscle transfected with human IGF-1 plasmid (200 $\times$), b : High power photomicrograph of sequential section 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's hematoxylin counter stain ; magnification 200 $\times$)

Fig. 4 a : Photomicrograph of untreated denervated muscle 4 weeks after nerve section demonstrating elongation of motor endplates and absence of neural filaments (cholinesterase/silver-gold staining technique), b : Photomicrograph of laryngeal muscle transfected with hIGF-I plasmid demonstrating nerve sprouting and motor endplate contact 4 weeks after nerve section and injection with plasmid formulation (400 $\times$)

Table 1 Percentage of positive hIGF-I treated animals detected using RT-PCR and PCR techniques.

Treatment	Number of animals	RT-PCR for hIGF-I mRNA		PCR for hIGF-I plasmid	
		positive rate	%	positive rate	%
Single Injection hIGF-I	16	13/16	81.3%	16/16	100%
Multiple Injection hIGF-I	14	14/14	100%	14/14	100%
Control	18	0/18	0%	0/18	0%

the presence of neural elements and nerve to motor endplate contact. In these hIGF-I treated animals, motor endplates appeared shorter and cholinesterase staining was more concentrated (Figure 4b).

Biologic effect of hIGF-I gene transfer

To assess biologic effect of hIGF-I gene transfer, muscle fiber size, motor endplate length and nerve to motor endplate contact were evaluated 28 days after single injection with the hIGF-I formulation¹⁴⁾. Gene transfer was detected in 100% of injected animals by PCR. Gene transfer with expression, as measured by RT-PCR for hIGF-I mRNA, was detected in 81.3% of injected animals. When compared to controls, hIGF-I transfected animals had significant increase in muscle fiber diameter (17.56 ± 0.97 SD μm vs 14.70 ± 1.43 SD μm , $P=0.0002$), significant decrease in motor endplate length (20.88 ± 1.42 SD μm vs 25.41 ± 3.19 SD μm , $P=0.0025$), and significant increase in percentage

of endplates with nerve contact ($20.3\% \pm 13.9$ SD vs $4.4\% \pm 4.2$ SD, $P=0.0079$).

These findings demonstrate direct benefit of hIGF-I gene therapy based on muscle fiber size, motor endplate length and nerve contact evaluation. These data not only support the benefit of local protein production or paracrine action of hIGF-I but also introduce a treatment regimen requiring only one therapeutic injection to achieve comparable effects to systemic or local injection of recombinant hIGF-I protein. Optimal delivery schedule and limitations in application of this gene transfer system are currently unknown. A multi-dose delivery strategy with more frequent injections will conceivably result in a corresponding increase in biologic effect. To test this hypothesis, gene transfer efficiency, muscle fiber size, motor endplate length and nerve to motor endplate contact were evaluated following single injection ($n=16$), multiple injection ($n=14$) and in control animals ($n=18$) twenty-eight days

Fig. 5 Comparison of mean lesser diameter of thyroarytenoid muscle fiber the denervated control group, hIGF-I gene single and multiple injection groups and normal control animals. Values are given as mean + standard deviation (SD). A significant increase in mean lesser diameter of thyroarytenoid muscle is seen in the hIGF-I gene single and multiple injection groups compared to the denervated control group. No statistical difference is seen between single and multiple injection groups.

Abbreviations Control : denervated control group, SIIGF : hIGF-I gene single injection group, MIIGF : hIGF-I gene multiple injection group, Normal : normal finding.

after the first injection¹⁵⁾. Although plasmid DNA was detected by PCR in 100% of single injection and multiple injection animals, gene expression, assessed by RT-PCR for hIGF-I mRNA, occurred in 81.3% of animals receiving single injections and 100% of multiple injection animals (Table 1). Compared to controls, hIGF-I transfected animals in both single and multiple injection groups had a significant increase in muscle fiber lesser diameter, a significant decrease in motor endplate length, and a significant increase in percentage of endplates with nerve contact (Figures 5~7). Although the

Fig. 6 Comparison of the motor endplate length in the thyroarytenoid muscles of the denervated control group, hIGF-I gene single and multiple injection groups and normal animals. Values are given as mean + standard deviation (SD). A significant decrease in motor endplate length is observed in the hIGF-I gene single and multiple injection group compared to the denervated control group. No statistical difference is seen between single and multiple injection groups.

percentage of denervated muscles demonstrating hIGF-I expression was increased following multiple injection, no difference was observed in biologic response when compared to the single treatment group.

Discussion

Laryngeal reinnervation has not been widely accepted as a treatment option for patients with laryngeal paralysis. Clinically, laryngeal reinnervation procedures have had little or no impact on dynamic function, and if any benefit is realized, it is due to a reversal of muscle atrophy, increase in muscle mass and improved vocal fold tension. Failure to achieve functional reinnervation may reflect a decrease in motor fiber density, either from cen-

Fig. 7 Comparison of percentage of endplates with nerve contact in the thyroarytenoid muscle of the denervated control group, hIGF-I gene single and multiple injection groups and normal animals. Values are given as mean + standard deviation (SD). A significant increase in percentage of endplates with nerve contact is observed in the hIGF-I gene single and multiple injection group compared to the denervated control group. No statistical difference is seen between single and multiple injection groups.

Abbreviations Control : denervated control group, SIIGF : hIGF-I gene single injection group, MIIGF : hIGF-I gene multiple injection group, Normal : normal finding.

tral loss of motoneurons or failure of reinnervating neurons to cross the anastomotic site, 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 delivery of neurotrophic factors either locally or systemically⁴⁻⁶). Key trophic factors affecting nerve regeneration include nerve growth factor (NGF), brain derived neurotrophic factor (BDNF),

ciliary neurotrophic factor (CNTF), and IGF-1&2. IGF-1 (formerly called somatomedin) is both neurotrophic and myotrophic⁶⁻⁸). IGF-1 is a critical factor 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 at target tissue are generally achieved at systemically toxic levels and steady state requires an unacceptable or impractical frequency of delivery^{9,10}). Direct, site-specific, gene therapy provides a steady state delivery of therapeutic proteins, such as growth factors, in target tissue over prolonged periods^{11,12}). Gene delivery vehicles can also be constructed to induce tissue specific gene expression. These two major advantages of gene therapy strategies diminish the rapid clearance of locally injected growth factors and avoids the toxicity of systemically delivered proteins¹⁰⁻¹²).

In this report, gene transfer into laryngeal muscle is described using a polyvinyl-based formulation and plasmid containing a reporter gene encoding β -galactosidase¹³). Therapeutic gene transfer is demonstrated using a muscle specific gene expression system containing the human IGF-I gene sequence applied experimentally in a rat model for laryngeal paralysis. Compared to controls, hIGF-I transfected animals in both single and multiple injection groups had a significant increase in muscle fiber lesser diameter, a significant decrease in motor endplate length and a significant increase in percentage of endplates with nerve contact¹⁴). Although the percentage of denervated muscles demonstrating hIGF-I expression was increased following multiple injection, no difference was observed in biologic response when compared to the single treatment group¹⁵). This observation suggests that single injection of the hIGF-I formulation results in equivalent biologic effect when compared to multiple injection strategies.

Gene therapy provides opportunity for enhancing outcome of current surgical approaches for the management of laryngeal paralysis by prevention or

reversal of muscle atrophy, enhanced nerve sprouting and reinnervation. Demonstration of increased muscle fiber size and reduced atrophy would itself be a clinically functional benefit of hIGF-I gene therapy for laryngeal paralysis. If proven effective, gene therapy may then be applied clinically to fine-tune current surgical procedures or even eliminate the need for surgical intervention. To achieve the latter, functional benefit with intact neural connections and normal laryngeal movement must be demonstrated. Long term benefits, physiologic responses, and limitations of this potentially valuable therapeutic strategy are currently being investigated.

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