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Towards Gene Therapy for Growth Hormone Deficiency via Salivary Gland Expression of Growth Hormone

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Abstract

Objectives—Salivary glands are useful targets for gene therapeutics. After gene transfer into salivary glands, regulated secretory pathway proteins, such as human growth hormone, are secreted into saliva, whereas constitutive secretory pathway proteins, such as erythropoietin, are secreted into the bloodstream. Secretion of human growth hormone into the saliva is not therapeutically useful. In this study, we attempted to re-direct the secretion of transgenic human growth hormone from the saliva to the serum by site-directed mutagenesis.

Materials and Methods—We tested human growth hormone mutants first in vitro with AtT20 cells, a model endocrine cell line that exhibits polarized secretion of regulated secretory pathway proteins. Selected mutants were further studied in vivo using adenoviral-mediated gene transfer to rat submandibular glands.

Results—We identified two mutants with differences in secretion behaviour compared to wild type human growth hormone. One mutant, N₁₋₆, was detected in the serum of transduced rats demonstrating that expression of this mutant in the salivary gland resulted in its secretion through the constitutive secretory pathway.

Conclusion—This study demonstrates that mutagenesis of therapeutic proteins normally destined for the regulated secretory pathway may result in their secretion via the constitutive secretory pathway into the circulation for potential therapeutic benefit.

Keywords

gene therapy; gene engineering; endocrinology; cell biology

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Declarations

The Authors declare no conflict of interest and that the work described herein was carried out in accordance with *EC Directive 86/609/EEC for animal experiments* and the *Uniform Requirements for manuscripts submitted to Biomedical journals*.

Introduction

Salivary glands (SGs) are promising target organs for gene therapeutics (Baum et al., 1999, Kagami et al., 1996, O'Connell et al., 1996, Perez et al., Wang et al., 2005, Zheng et al., 2011, Baum et al., 2012, Monami et al., 2012, Rowzee et al., 2013, Rocha et al., 2013). They can secrete proteins in large amounts apically to the saliva (Baum et al., 1999, O'Connell et al., 1996) or basolaterally to the circulation (Kagami et al., 1996, Perez et al., Wang et al., 2005), the latter of which can be used for systemic delivery of therapeutic proteins. The route of secretion for the transgenic proteins is determined by their sorting and trafficking behaviours within the transduced SG cells. SG acinar cells exhibit at least two regulated secretory pathways (RSPs) (Gorr et al., 2005). Proteins secreted through the major RSP are stored in dense-core granules, which are discharged in response to high doses of β -adrenergic agonists such as isoproterenol (Castle & Castle, 1998). In addition, a minor RSP originates in immature secretory granules (Castle & Castle, 1996), whereby smaller transport vesicles are secreted in response to muscarinic or weak β -adrenergic stimulation. These secretory pathways lead to polarized protein secretion into saliva. In addition to the RSPs, SG acinar cells can secrete proteins through secretory pathways that do not depend on extracellular stimulation (Gorr et al., 2005), i.e., the constitutive secretory pathway (CSP). The CSP transports non-granule proteins from the trans-Golgi network (TGN) (Castle & Castle, 1998), whereas the constitutive-like pathway originates in maturing secretory granules and carries proteins that are inefficiently retained in dense-core granules during maturation (Castle & Castle, 1998, Gorr et al., 2005). Proteins of the CSP or the constitutive-like pathway are expected to be secreted mostly into the circulation from SGs.

Human growth hormone (hGH) is normally secreted into the circulation from somatotrophs of the anterior pituitary in a regulated manner, i.e. through the RSP. When expressed in rat SGs, hGH follows the exocrine RSP route as expected, which delivers it into the saliva (Wang et al., 2005), and thus is not therapeutically useful. Therefore, to utilize SGs as a surrogate endocrine organ for systemic hGH gene therapy, the trafficking of hGH has to be diverted from the RSP to the CSP. This requires an understanding of the molecular determinants of hGH sorting to the RSP.

Two models have been proposed to explain the targeting of proteins to secretory granules (Arvan & Castle, 1998). In the *sorting-for-entry* hypothesis, sorting signals in RSP proteins are recognized by intracellular sorting receptors. Proteins not binding to sorting receptors are excluded from the granules and are secreted constitutively. This sorting process operates in the TGN. In the *sorting-by-retention* model, secreted proteins can freely enter the forming secretory granules. RSP proteins are then retained, while other proteins are progressively removed from the maturing granules via the constitutive-like secretory pathway. Theoretically, protein sequence mutations, either by altering a sorting signal or by changing the physicochemical properties of a protein, could divert its sorting from the RSP (e.g., (Burgess & Kelly, 1987)), as shown for several proteins (Cool et al., 1995, Creemers et al., 1996, Dhanvantari et al., 2003). Previously we attempted to redirect the sorting of hGH from the RSP into the CSP (Samuni et al., 2008b, Wang et al., 2005). Our results identified the C-terminal region as an important determinant in hGH trafficking to the RSP granules of

AtT20 cells. Also, point mutations and a C-terminal extension arising from a cloning artifact, demonstrated partial redirection of hGH to the CSP in SG cells in vivo (Wang et al., 2005). In another study (Samuni et al., 2008b), the N-terminal region of hGH was also shown to influence its sorting into the RSP. Our aim in this study was to use site-directed mutagenesis in the N- or C-terminus of hGH to divert its sorting from the RSP to the CSP.

Materials and Methods

Mutagenesis of human growth hormone

The pACCMVpLpA Ad5 shuttle plasmids, containing hGH cDNA (Baum et al., 1999) and gene (He et al., 1998), have been described previously. Mutants of the hGH (Table 1) were created by PCR as shown in Supplementary Tables 1, 2 and 3, respectively, verified by sequencing and used for transient transfection or viral vector production.

Cell culture and transfection

AtT-20/D16v-F2 cells were cultured as described earlier (Wang et al., 2005). hGH constructs were transiently transfected to AtT20 cells using Polyfect reagent (Qiagen, Valencia, CA) according to the manufacturer's guidelines.

In vitro hGH secretion assay

In vitro hGH secretion assays were performed two days post transfection as described previously (Wang et al., 2005). Conventional cell culture medium (Dulbecco's modified Eagle's medium, DMEM) was replaced with 1 ml of fresh serum-free DMEM containing 0.01% bovine serum albumin (BSA; basal medium). The cells were then incubated at 37°C for 30 min after which the basal medium was collected and replaced with 1 ml of high-potassium (55 mM) DMEM (Biosource, Camarillo, CA), containing 1 mM BaCl₂ and 0.01% BSA (stimulation medium). The cells were incubated for a further 10 min after which the stimulation medium was also collected. The cells were rinsed once with ice cold PBS and then lysed in 1 ml of M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL). A soluble cell extract was generated after centrifugation at 13,000 × g for 10 min. An ELISA (Anogen, Mississauga, Canada) was used to measure hGH in the samples. The wild type (WT) hGH construct was included in each experiment as a positive control for the transfection and hGH assay procedures and as a baseline for normal secretion of WT hGH in these cells. The levels of expression of each mutant were normalized to that of the WT levels so that relative comparisons could be made between the mutants and the WT hGH. For each mutant, total hGH made (basal media plus stimulated media plus cell lysate) was calculated and expressed relative to total WT hGH made. The total amount of mutant hGH secreted (basal media plus stimulated media) was calculated and expressed as a percentage of total mutant hGH made. This was then normalized to the percentage of WT hGH secreted calculated in the same way. Finally, the amounts of mutant hGH secreted under basal and stimulated conditions were expressed separately as a percentage of total mutant made and normalized to that of the basal or stimulated secretion of WT hGH, respectively.

Recombinant serotype 5 (Ad5) vectors

Recombinant Ad5 vectors encoding two N-terminus hGH mutants (N₁₋₆, #42) were created as previously described (He et al., 1998). The Ad5 vector encoding WT hGH, was described earlier (He et al., 1998). Physical viral particle titers were determined by real-time PCR as described previously (Zheng et al., 2008).

In vivo experiments

All animal studies were performed with the approval of the Animal Care and Use Committee, NIDCR, NIH and the NIH Biosafety Committee. In vivo experiments were conducted as described previously (Samuni et al., 2008a, Zheng et al., 2008). In brief, rats were anesthetized by intramuscular (IM) injection of ketamine (60 mg/kg; Phoenix Scientific, St. Joseph, MO) and xylazine (6 mg/kg; Phoenix Scientific). Atropine (0.5 mg/kg, Sigma-Aldrich, St. Louis, MO) was given IM 10 min prior to vector delivery to decrease salivation. The submandibular glands were cannulated and 10⁹ viral particles were administered to each gland by retrograde infusion in 200 µl virus dilution buffer (10 mM TrisHCl pH7.4, 0.1 mM MgCl₂, 10% glycerol). After 48 h, the animals were re-anesthetized, 0.5 mg/kg pilocarpine was given subcutaneously and whole saliva, blood and the submandibular glands were collected. hGH was measured in serum, saliva and gland extracts using ELISAs.

Statistics

In vitro data were analyzed by ANOVA and Dunnett's post hoc test or unpaired t test, as appropriate. In vivo data were analyzed by the Kruskal-Wallis test and Dunn's post hoc test or by Mann-Whitney test, as appropriate. Differences were considered significant at $p < 0.05$.

Results

Site-directed mutagenesis of hGH

Multiple hGH mutants were created with amino acid substitutions at the C- or N-terminus (Table 1), or with the deletion of the first six (N₁₋₆) or the first 39 (N₁₋₃₉) amino acids of WT hGH. A deletion mutant lacking the last 11 residues of mature WT hGH (C) was described previously (Wang et al., 2005). See Supplemental Tables 1-3 for primer and PCR conditions for the generation of the mutants.

In vitro synthesis, secretion and sorting of hGH mutants in AtT20 cells

Plasmids encoding hGH constructs were transiently transfected into AtT20 cells and hGH secretion assays were performed. Three hGH mutants were not produced (#21, #43, N₁₋₃₉), presumably due to defects in their structure that targeted them for degradation, while several others (#2, #10, #18, #19, #30, #32, #33, #41, #42) were synthesized but at levels significantly lower than WT (Table 2). Mutant N₁₋₆ was produced significantly higher than WT hGH (>20-fold higher). However, its basal secretion, normalized to WT hGH, was only 0.48 ± 0.05 (Table 2) indicating that in spite of being efficiently made this mutant was less

efficiently secreted from the AtT20 cells. Nevertheless, due to its high levels in the cells, the absolute levels of N₁₋₆ being secreted basally were considerably higher than for WT hGH.

The hGH mutant devoid of the conserved C-terminal loop, C, was also secreted less efficiently than WT ($p < 0.01$), recapitulating our previous data with this mutant (Wang et al., 2005). The percent secretion for the rest of the hGH mutants was not significantly different from that of the WT, with the exception of a small, but significant, increase in the basal secretion of mutant #42 (Table 2). The relative stimulated secretion for most mutants was not different from that of WT hGH, however, for both N₁₋₆ and C mutants, a reduction in this parameter was observed (0.29 ± 0.02 and 0.34 ± 0.01 , respectively), reflecting reduced trafficking to the granules of the RSP for these mutants (Table 2).

In vivo production and secretion of hGH proteins in rat submandibular glands

WT hGH and mutants N₁₋₆ and #42 were examined for their total production and secretion routes in rats following SG gene transfer. We administered vectors as explained in the legend to Fig. 1. In all cases, hGH was detected in the glands demonstrating accuracy of the cannulation procedure and functionality of the Ad5 vectors. The amounts of hGH produced per gland after delivery showed significant differences between WT and the mutants, similar to that seen when expressed in AtT20 cells (Table 2), i.e. N₁₋₆ > WT $\gg$ mutant #42 (Fig. 1A). For secretion, both mutants were found in saliva, albeit at a reduced level compared to WT hGH. However, only the N₁₋₆ was readily detected in the serum. Hence the saliva to serum ratio for WT hGH (Fig. 1C) of over 100 demonstrates that most of the WT preferred secretion through the RSP to the saliva as reported previously (He et al., 1998). In both cases for the mutants, the saliva:serum ratio were reduced to below 10 (Fig. 1C) indicating a significant shift from secretion into the saliva to secretion into the serum.

Discussion

Isolated Growth Hormone Deficiency (IGHD) can be divided into 3 groups; autosomal recessive (Type I), autosomal dominant (Type II) and X-linked (Type III) (Hernandez et al., 2007). In many cases, the defects result in hGH being absent or unable to function normally due to an inability to be made or to be secreted properly into the circulation. For instance, a mutation of R183H or R178H at the C-terminus of hGH allows the protein to be trafficked to the granules of the RSP but somehow interferes with WT hGH secretion when co-expressed (Deladoey et al., 2001, Petkovic et al., 2010), presumably due to aberrant aggregation properties within the granules. Similarly, missplicing to cause a deletion of exon 3 leading to del32-71-GH, a protein that cannot fold normally, results in an N-terminal deletion mutant that rapidly gets degraded and in doing so causes the degradation of endogenous WT hGH (Lee et al., 2000, Moseley et al., 2002).

While a great deal has been discovered about hGH and its pathophysiology when mutated (for review see (Dannies, 2012)), treatment of IGHD is still by injection of recombinant hGH on a daily basis (Pawlikowska-Haddal, 2013). To provide an alternative to injection, gene therapy using SGs appears promising. However, once expressed in SGs, the issue of where WT hGH is secreted is a real problem; in this case predominantly through the RSP into the saliva where it would not be therapeutically useful (He et al., 1998). Hence, efforts

to identify and generate functional mutants of hGH that are synthesized and can be trafficked and secreted from polarized cells through either the non-regulated or CSP could yield promising proteins for their use in treating IGHD after expressing them in the parenchymal cells of the salivary gland with subsequent secretion into the circulation as recently done for GLP-1, proinsulin and erythropoietin (Monami et al., 2012, Rocha et al., 2013, Rowzee et al., 2013).

To this end we tested the production and secretion of WT hGH and a battery of hGH mutants first in vitro in AtT20 cells. We chose AtT20 cells because they are a well studied model cell line with a RSP and CSP that can be analyzed for protein secretion behaviour i.e. basal versus regulated secretion. Previously we reported efforts to identify a sorting signal in hGH (Wang et al., 2005). The C terminus of hGH contains a highly conserved disulfide-stabilized loop structure that is similar to an N-terminal loop in pro-opiomelanocortin (Cool et al., 1995) and chromogranin B (Chanat et al., 1993). The loops in both of these proteins are sufficient and necessary for sorting to the granules of the RSP. Hence removal of the C-terminal loop of hGH was hypothesized to result in poor trafficking to the granules.

Indeed, the C hGH mutant was previously found to be retained in the Golgi apparatus in AtT20 cells and poorly secreted (Wang et al., 2005), suggesting that the C-terminal loop contained structural information necessary for trafficking to the granules. To study this further, we created C-terminal mutants that either disrupted the disulfide-stabilized loop or introduced substitutions to change the polarity and/or the charge of specific residues. We also made small C-terminus deletions in two mutants (Table 1). We did this in an attempt to obtain mutants that were intermediate in phenotype between C-hGH and WT hGH, such that trafficking to the granules would be perturbed, but that the physicochemical properties not be sufficiently changed so as to prevent secretion due to presumed aberrant aggregation in the Golgi as observed for C-hGH previously (Wang et al., 2005). Unfortunately, these mutants were all poorly made (Table 2) and not affected in their secretion behaviour. We therefore did not continue with these in the current study.

We also focused on the N-terminus of hGH based on the original findings of Moore and Kelly (Moore & Kelly, 1986) using a hGH-VSVG fusion protein and on the reported results with an hGH-erythropoietin fusion protein (Samuni et al., 2008b), suggesting the N-terminus of hGH may encode sorting properties for the RSP. We created three N-terminal mutants, with substitutions that changed polarity and charge (Table 1), and two N-terminus deletions. In the in vitro AtT20 secretion experiments, two of the mutants showed a difference with respect to WT hGH; mutant #42 was poorly made but showed a small but significant increase in its percent basal secretion whereas mutant N₁₋₆ was very well expressed (>20 fold compared to WT) but showed a reduction in its percent basal secretion. Although the reduction in percent basal secretion of this mutant was not what we expected, the quantal basal release of the mutant exceeded that of WT due to its expression levels. We extended the in vitro studies with these two mutants through in vivo studies and found that mutant #42 was produced poorly in the SG (< 1 % versus WT, Fig. 1A). However, while it may have shown a change towards preferential CSP secretion from the gland, mutant #42 is unlikely to be useful for any clinical applications due to its low expression in the gland.

Conversely, the N₁₋₆ protein was efficiently produced in vivo (>2-fold higher than WT levels, Fig. 1A, $p < 0.01$), but more importantly, it was also found in the serum, which led to a saliva/serum hGH ratio significantly reduced compared to that of WT hGH (Fig. 1C). Thus, this mutant, while still favoring the RSP (saliva), was being secreted into the circulation by the CSP more readily than WT hGH. This suggests that the 6 amino acids at the N-terminus contain structural information necessary for efficient trafficking of hGH through the RSP in SGs. The fact that hGH N₁₋₆ was detected in the basal medium of AtT20 cells (Table 2) and the serum of rats (Fig. 1) demonstrates that the protein is structurally intact and not recognized as an aberrant protein marked for degradation within the cells (as were some of the other mutants). Therefore, hGH N₁₋₆ may have GH bioactivity, a hypothesis that requires future testing. Since the ELISA used to quantify this mutant recognizes the native structure of hGH, it suggests structural identities between the WT and N₁₋₆ proteins and that the above hypothesis is worthy of direct testing. To do this future studies will include long term and regulated expression of hGH N₁₋₆ via adeno-associated viral vector –mediated transduction of SGs in an animal model of IGHD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Ad5	serotype 5 adenovirus
ANOVA	analysis of variance
CSP	constitutive secretory pathway
ELISA	enzyme-linked immunosorbent assay
hGH	human growth hormone
PCR	polymerase chain reaction
POMC	pro-opiomelanocortin
RSP	regulated secretory pathway
SG	salivary gland
TGN	trans-Golgi network
vp	vector particle
WT	wild-type

Figure 1. Production and secretion of WT and mutant hGH in vivo in rat submandibular glands
 Ad5 vectors encoding WT or mutant (N₁₋₆ and #42) hGH were generated and used to transduce single rat submandibular glands (10 rats per group) at 10⁹ vp/gland as described in Materials and Methods. **A.** Total amount of immunoreactive hGH protein made (saliva, serum and gland extract). **B.** Percentage of each mutant or WT hGH secreted (saliva plus serum). **C.** Saliva to serum ratio of hGH after administration of 10⁹ vp into single glands. The ratio value is not shown for a rat when the hGH concentration was 0 both in serum and in saliva (one rat in the WT group and three in the mutant 42 group). The result of each individual animal is represented by a circle. There have been identical values for several mutants and therefore some circles overlap. Medians are indicated by horizontal bars. *: p < 0.05 compared to WT. Differences are not significant unless otherwise indicated.

Table 1

Human growth hormone mutants.

C terminal mutants																		
residue	181	182	183	184	185	186	187	188	189	190	191	stop						
WT sequence	Q	C	R	S	V	E	G	S	C	G	F							
mutant #2	S			P					S			stop						
mutant #10	S				P	R			S			stop						
mutant #18	A			P	P			A	A			stop						
mutant #19	S			P	P	A			S			stop						
mutant #21	S		A	P					S			stop						
mutant #30	A								stop									
mutant #32	A						stop											
mutant #33	S		A						S			stop						

N terminal mutants																		
residue	8	...	16	...	19	...	22	23	...	26	...	30	31	32	33			
WT sequence	R	...	R	...	R	...	Q	L	...	D	...	E	F	E	E			
mutant #41	A	...	A	...	A	...	G	S	...									
mutant #42	E	...	E	...	E	...	G	S	...									
mutant #43	...	...	...	...	...	...	G	S	...	A	...	A		A	A			

Amino acid sequence of human growth hormone (hGH) and mutants. Shown are the wild type (WT) hGH amino acid sequence for the relevant portions of the protein and the amino acid substitutions for each mutant generated. Mutations were created as described in Materials and Methods.

Table 2

In vitro synthesis and secretion of human growth hormone mutants.

mutant	2	10	18	19	21	30	32	33	41	42	43	N 1-39	N 1-6	C
amount synthesized: normalized to wild type as fold amount, mean $\pm$ SEM	0.41 $\pm$ 0.03	0.74 $\pm$ 0.05	0.20 $\pm$ 0.02	0.19 $\pm$ 0.02	n.m.	0.32 $\pm$ 0.01	0.33 $\pm$ 0.03	0.34 $\pm$ 0.06	0.43 $\pm$ 0.15	0.31 $\pm$ 0.13	n.m.	n.m.	21.80 $\pm$ 1.30	0.54 $\pm$ 0.03
significance compared to wild type	**	**	**	**	***	***	**	**	*	**			**	ns
% secreted: normalized to wild type as fold amount, mean $\pm$ SEM	1.00 $\pm$ 0.06	0.98 $\pm$ 0.04	1.15 $\pm$ 0.14	1.08 $\pm$ 0.13	0.94 $\pm$ 0.08	0.96 $\pm$ 0.17	1.00 $\pm$ 0.20	1.14 $\pm$ 0.23	1.24 $\pm$ 0.27	1.24 $\pm$ 0.27			0.33 $\pm$ 0.03	0.36 $\pm$ 0.01
significance compared to wild type	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns			**	**
basal secretion normalized to wild type as fold amount, mean $\pm$ SEM	1.06 $\pm$ 0.08	0.88 $\pm$ 0.05	1.22 $\pm$ 0.15	1.20 $\pm$ 0.15	1.12 $\pm$ 0.11	0.98 $\pm$ 0.14	0.98 $\pm$ 0.17	1.06 $\pm$ 0.07	1.31 $\pm$ 0.09	1.31 $\pm$ 0.09			0.48 $\pm$ 0.05	0.41 $\pm$ 0.03
significance compared to wild type	ns	ns	ns	ns	ns	ns	ns	ns	ns	*			**	**
stimulated secretion normalized to wild type as fold amount, mean $\pm$ SEM	0.92 $\pm$ 0.06	1.05 $\pm$ 0.07	1.11 $\pm$ 0.13	1.02 $\pm$ 0.12	0.87 $\pm$ 0.06	0.96 $\pm$ 0.17	1.01 $\pm$ 0.21	1.24 $\pm$ 0.32	1.27 $\pm$ 0.35	1.27 $\pm$ 0.35			0.29 $\pm$ 0.02	0.34 $\pm$ 0.01
significance compared to wild type	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns			**	**

Shown are relative amounts (mean $\pm$ SEM) of total mutant hGH synthesized, levels secreted (basal plus stimulated), levels secreted constitutively (basal secretion), and stimulated secretion, for each mutant, all normalized to WT hGH, as described in Materials and Methods. Statistical calculations were performed as described in Materials and Methods.

n.m. not made.
 * : $p < 0.05$;
 : $p < 0.01$;
 *** : $p < 0.001$;
 ns: not significant.