Brief Report

Sorting of Transgenic Secretory Proteins in Rhesus Macaque Parotid Glands After Adenovirus-Mediated Gene Transfer

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Abstract

We have previously used viral vectors encoding either human growth hormone (hGH) or erythropoietin (hEPO) to study the sorting of transgenic proteins in mouse and minipig salivary glands. Whereas hGH (a regulated secretory pathway [RSP] protein) is secreted predominantly into saliva in both species, hEPO (a constitutive secretory pathway [CSP] protein) is found primarily in the bloodstream with mice, but overwhelmingly in saliva with minipigs. In view of the hEPO sorting difference, we have conducted a similar study in nonhuman primates. Specifically, we examined hGH and hEPO sorting after adenoviral (Ad) vector-mediated gene transfer to parotid glands of rhesus macaques, another large and important animal model. Two groups ($n = 2$ per dose group; total $n = 8$) of male macaques received either 10^{10} particles per gland (low-dose group) or 10^{11} particles per gland (high-dose group) of adenoviral (Ad) vectors encoding either hGH (AdhGH) or hEPO (AdhEPO) via intraoral cannulation of both parotid glands. All macaques tolerated administration of Ad vectors well, with no clinically significant changes observed in any hematological and serum chemistry parameters. In AdhGH-treated animals, hGH was secreted exclusively into saliva. In contrast, after AdhEPO delivery, hEPO was secreted both in serum and saliva, at levels intermediate between mice and minipigs. We conclude that RSP proteins are faithfully secreted into saliva in all model species tested, whereas patterns of CSP protein secretion are variable.

Introduction

GENE THERAPEUTICS is a broad application of gene transfer with considerable clinical potential (Felgner *et al.*, 1991). A variety of tissues, including muscle, liver, and lung, have been targeted to produce and secrete transgene products and, in the case of systemic protein deficiencies, provide physiological serum levels of recombinant proteins for extended periods (Barzon *et al.*, 2000; Auricchio *et al.*, 2002; Goldspink, 2003). Previously published studies have demonstrated that salivary glands (SGs) can also serve as promising target sites for applications of gene therapeutics (Baum *et al.*, 2002). Indeed, SGs present some distinct advantages compared with other tissues (Baum *et al.*, 2004). SGs are able to secrete proteins in both an exocrine and endocrine direc-

tion, using a well-studied regulated secretory pathway (RSP) leading to saliva, as well as a constitutive secretory pathway (CSP), primarily directed toward the interstitium and bloodstream (Castle and Castle, 1998).

To test the sorting behavior of transgene-encoded proteins in SGs we have previously used human growth hormone (hGH) and human erythropoietin (hEPO) as model secretory proteins. hGH is physiologically synthesized in somatotrophs residing in the anterior pituitary gland and is secreted via the RSP, whereas hEPO is synthesized in kidney tubular epithelial cells and is secreted by the CSP (Mujais *et al.*, 1999; Dannies, 2002). Administration of vectors encoding hGH and hEPO to the submandibular SGs of BALB/c mice showed that transgene-encoded proteins continue to follow the same general secretory pathway as in their primary site

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of production (Voutetakis *et al.*, 2005), that is, hGH was secreted predominantly into saliva (serum-to-saliva hGH concentration ratio, ~1:5), whereas hEPO was secreted preferentially into the bloodstream (serum-to-saliva hEPO concentration ratio, ~10:1). We next conducted similar studies in minipigs, using serotype 5 adenoviral (Ad5) vectors to deliver hGH and hEPO transgenes to parotid glands (Yan *et al.*, 2007). Whereas the pattern of hGH secretion was identical to that found in mice (essentially all hGH secreted into saliva), hEPO was secreted in a dramatically different manner, mainly into saliva. This result raised questions about the potential efficiency of SGs as target sites for general systemic gene therapeutics applications.

Accordingly, the current study was designed to examine the sorting and secretion of these same two model proteins in nonhuman primates, another important large animal pre-clinical gene transfer model. Specifically, we directly administered Ad5 vectors to the parotid glands of rhesus macaques and monitored the secretion of transgene-encoded hGH and hEPO into saliva and the bloodstream. In addition, we evaluated the effects of Ad5 vector delivery on multiple hematological and serum chemistry parameters, adding important general information about the safety of SG delivery by Ad5 vectors in nonhuman primates.

Materials and Methods

Construction of AdhEPO and AdhGH

Generation of the E1⁻, replication-deficient Ad5 vectors containing the cytomegalovirus (CMV) promoter and either the hEPO cDNA (AdhEPO) or the hGH gene (AdhGH) was performed as previously described (He *et al.*, 1998; Baum *et al.*, 1999). Y. Terada (Tokyo Medical and Dental University, Tokyo, Japan) generously provided the hEPO cDNA. Vector titers and concentrations used herein were determined on the basis of the real-time quantitative polymerase chain reaction (qPCR) with CMV-specific primers (ABI PRISM 7700 sequence detector; Applied Biosystems, Foster City, CA).

Macaque gene transfer, saliva and serum collection, and determination of viral dissemination after vector administration

The protocol for conducting the nonhuman primate experiments was approved by, and conducted under the guidelines of, the National Heart, Lung, and Blood Institute (NHLBI, National Institutes of Health [NIH], Bethesda, MD) Animal Care and Use Committee (ACUC) and the NIH Institutional Biosafety Committee. Macaques were housed, either singly or in compatible pairs, provided free access to water, and received 10–15 biscuits of monkey chow (Purina Lab-Diet 5038; PMI Nutrition International, St. Louis, MO) twice daily.

A total of two groups ($n = 2$ per dose group) of male macaques received either 10^{10} particles per gland (low-dose group) or 10^{11} particles per gland (high-dose group) of either AdhGH or AdhEPO (total $n = 8$). These vectors were the same as used in our study of minipigs (Yan *et al.*, 2007). The infusion volume was 500 μ l/gland. The higher dose used was comparable on a particle per kilogram basis to the dosing used in our previous murine experiments (macaque weight range, 4–6 kg; Voutetakis *et al.*, 2005). Vectors were administered via intraoral cannulation of both parotid

glands (O'Connell *et al.*, 1999; Baccaglini *et al.*, 2001; Baum *et al.*, 2002). For all procedures, macaques were anesthetized intramuscularly with tiletamine–zolazepam (Telazol; Fort Dodge Animal Health, Overland Park, KS) and ketamine, each at a dose of 3 mg/kg. As in previous rhesus Ad5 vector studies (O'Connell *et al.*, 1999), we administered 20 mg of Depo-Medrol (methylprednisolone) 1 day before vector delivery to decrease likely vector-induced inflammatory responses.

Blood (from the femoral vein) and whole saliva samples were obtained both before and after vector administration on days –7, 0, 3, 8, and 15 for the AdhEPO group and on days –4, 0, 3, 9, and 16 for the AdhGH group. For saliva collections (10 min) the animals were sedated with Telazol–ketamine and given 1 mg of pilocarpine intramuscularly. Levels of both hGH and hEPO in macaque serum and saliva, and of insulin-like growth factor-1 (IGF-I) serum levels in the AdhGH-treated animals, were determined with commercially available hGH, hEPO, and hIGF enzyme-linked immunosorbent assay (ELISA) kits (Anogen [Mississauga, ON, Canada], StemCell Technologies [Vancouver, BC, Canada], and R&D Systems [Minneapolis, MN], respectively) (100 mU of hEPO corresponds to ~1 ng of the protein; Bohl *et al.*, 1998).

In the AdhEPO-treated group, additional blood samples were obtained immediately before as well as 10 min, 2 hr, and 24 hr after vector administration in order to determine vector particle number in serum as a measure of vector dissemination beyond the targeted glands, using the TaqMan universal PCR master mix (Applied Biosystems; primers and probe targeting the hEPO cDNA). No inhibitory effects of serum on the assay were observed in samples spiked with AdhEPO.

At each designated time point, a complete blood count and multiple serum chemistry analyses were performed on all participating animals, as described (Voutetakis *et al.*, 2007).

Rodent experiments

Transgenic hEPO sorting was also evaluated in male mice (10^9 particles per gland) and rats (10^{10} particles per gland), using the same AdhEPO vector under protocols approved by the ACUC of the National Institute of Dental and Craniofacial Research (NIDCR, NIH) and the NIH Institutional Biosafety Committee.

Results and Discussion

Previously published experiments in rodents, miniature pigs, and nonhuman primates using Ad5 and adeno-associated viral (AAV) vectors have demonstrated the potential clinical utility of SGs for gene therapeutics applications (e.g., Hoque *et al.*, 2001; Baum *et al.*, 2004; Voutetakis *et al.*, 2004b; Yan *et al.*, 2007). SGs can secrete transgene products both into saliva and the bloodstream (Baum *et al.*, 2002), which can be applied to treat both oral and upper gastrointestinal tract disorders, as well as systemic monopeptide deficiencies. SGs use two general secretory pathways: a predominant RSP leading to exocrine (apical) protein secretion into saliva via zymogen granules and a CSP, leading mainly to endocrine (basolateral) secretion into the interstitium and bloodstream (Castle and Castle, 1998; Baum *et al.*, 1999; Isenman *et al.*, 1999; Gorr *et al.*, 2005). Previously, we have extensively studied transgenic secretory protein sorting in SGs of male mice

and minipigs, using Ad5 vectors encoding hGH and hEPO cDNAs. Whereas in both species hGH continues to follow the same general secretory pathway (RSP) as in their primary site of production, hEPO (a CSP protein) is handled in a dramatically different manner by male mouse (see Table 1 herein; Voutetakis *et al.*, 2004a) and minipig SGs (Yan *et al.*, 2007). The present study was conducted in part to address this remarkable dissimilarity.

Male macaques received either 10^{10} or 10^{11} particles per gland of either AdhGH or AdhEPO via intraoral cannulation of both parotid glands. All animals tolerated vector administration well. Saliva was of normal consistency with absence of purulence. Food consumption and weight gain were normal, suggesting parotid gland transduction by the Ad5 vectors caused little to no ill effects. There were no significant changes in hematological values, but there were a few minor, clinically insignificant and dose-independent changes in serum chemistry values, generally similar for both vectors (e.g., amylase, creatine phosphokinase, and lactate dehydrogenase levels transiently increased and serum Fe^{2+} decreased; data not shown). Because no experimental animals were killed, no tissue biodistribution data are available. However, we performed qPCR studies to assess vector presence in blood samples 10 min, 2 hr, and 1 day after admin-

FIG. 1. Serum and salivary human growth hormone (hGH) levels in macaques. Two groups ($n = 2$ per dose group) of male macaques received either 10^{11} (high dose; **A**) or 10^{10} (low dose; **B**) particles of AdhGH via intraoral cannulation of the parotid glands on day 0. Both serum (solid squares) and salivary (solid triangles) hGH levels were measured at distinct time points before (day -4) and after (days 3, 9, and 16) vector administration, using an hGH ELISA kit (Anogen, Mississauga, ON, Canada).

FIG. 2. Serum and salivary human erythropoietin (hEPO) levels in macaques. Two groups ($n = 2$ per dose group) of male macaques received either 10^{11} (high dose; **A**) or 10^{10} (low dose; **B**) particles of AdhEPO via intraoral cannulation of the parotid glands on day 0. Both serum (solid squares) and salivary (solid triangles) hEPO levels were measured at distinct time points before (day -7) and after (days 3, 8, and 15) vector administration, using an hEPO ELISA kit (Stem-Cell Technologies, Vancouver, BC, Canada).

istration. Importantly, the absence of vector in all of these samples suggests that little if any dissemination occurred beyond the targeted glands (data not shown; and Zheng *et al.*, 2006; Yan *et al.*, 2007). Furthermore, previously published detailed functional and safety studies with administration of similar doses of an Ad5 vector encoding human aquaporin-1 (AdhAQP1) to macaque parotid glands (O'Connell *et al.*, 1999) showed no untoward local or systemic effects.

The pattern of transgene production herein (i.e., the rapid elevation followed by rapid decline within 2 weeks; Figs. 1 and 2) is identical to what we have previously seen in rodent (Kagami *et al.*, 1998), miniature pig (Li *et al.*, 2004), and macaque (O'Connell *et al.*, 1999) parotid glands, that is, it is kinetically reproducible in all species. Further, it seems to be Ad5 vector specific because long-term SG secretion of hEPO and rhesus EPO has been observed with AAV2 vectors (Voutetakis *et al.*, 2004b, 2007). As observed in our previous mouse, rat, and minipig experiments, macaque serum hGH levels were below the detection limit at almost all time points for both dose groups. Accordingly, when we measured IGF-I levels in the AdhGH-treated macaques no significant changes were observed (data not shown).

TABLE 1. AVERAGE ERYTHROPOIETIN LEVELS IN SERUM AND SALIVA OF MALE MICE, RATS, MINIATURE PIGS, AND RHESUS MACAQUES AFTER AdhEPO GENE TRANSFER^a

Species	Serum EPO (mU/ml)	Salivary EPO (mU/ml)	Ratio	Serum EPO, total amount (mU) ^b	Salivary EPO, total amount ^c	Ratio
Mouse	~54	6	9:1	108	0.6	180:1
Rat	103	179	0.6:1	412	36	11.5:1
Miniature pig	10	139,000	~10 ⁻⁵ :1	7,280	278,000	~3 × 10 ⁻²
Rhesus macaque	35	450	~0.1:1	13,514	2,461	5.5

^aThe same AdhEPO vector, encoding human EPO, was used in all experiments. Calculations are based on data obtained on day 2 or 3 after transduction. For mice (10⁹ PU/gland in 50 μ l; MOI, 2 × 10⁷ PU/ μ l) and rats (10¹⁰ PU/gland in 200 μ l; MOI, 5 × 10⁷ PU/ μ l), submandibular glands were transduced and whole saliva was collected. For miniature pigs (10¹¹ PU/gland in 4000 μ l; MOI, 2.5 × 10⁷ PU/ μ l), parotid glands were transduced and parotid saliva was collected (data originally reported in Yen *et al.*, 2007). For macaques, the results shown were found after administration of 10¹⁰ PU/gland in 500 μ l (MOI, 2 × 10⁷ PU/ μ l) to the parotid glands and whole saliva was collected.

^bTotal serum EPO was calculated as follows: EPO concentration × volume (mice, 2 ml; rats, 4 ml; miniature pigs and macaques, based on weight and hematocrits).

^cTotal salivary EPO was calculated as follows: salivary EPO concentration × volume of collected saliva in milliliters (mice, 0.1; rats, 0.2; miniature pigs, ~2–3; macaques, ~5–10).

After AdhEPO administration to the high-dose group, day 3 salivary hEPO concentrations were ~7-fold higher than those in serum, but the total amount of secreted hEPO was ~15-fold higher in serum (not shown; see also low dose group in Table 1). These hEPO sorting results in macaques after Ad5 vector-mediated gene transfer are intermediate to those observed in mice and minipigs and somewhat similar to those found for rats (Table 1). Furthermore, the pattern of hEPO secretion is similar to that previously observed with rAAV2RhEPO administration to nonhuman primates (Voutetakis *et al.*, 2007), suggesting the sorting results are independent of the vector type used for transduction (Ad5 vs. AAV2), or of the cell types targeted (Ad5 targets both acinar and duct cells, whereas AAV2 targets primarily duct cells; O'Connell *et al.*, 1999; Voutetakis *et al.*, 2005).

Interestingly, the transient rise in serum hEPO levels of these nonanemic animals did not result in an increase in relevant hematologic parameters (e.g., red blood cell count, hemoglobin, and hematocrit percent; Kendall, 2001). We know that the transgene product, hEPO protein, is biologically active, as in minipigs treated with the same AdhEPO vector much lower serum hEPO levels led to significant elevations in hematocrit (Yan *et al.*, 2007). The reason for the lack of demonstrable biological function in the macaques herein is unclear, but may be attributable to tighter physiological controls on red cell production in this species compared with minipigs.

Gene transfer to SGs for use in treating both systemic and upper gastrointestinal tract diseases shows considerable potential. However, as pointed out by the results presented herein, (1) clear differences in the sorting of the model transgenic CSP protein hEPO exist between the four animal models studied (mouse, rat, minipig, and macaque) and (2) it is difficult to predict exactly how transgenic hEPO protein would be sorted by human SGs. Although the RSP is considered a more complex and tightly controlled secretion pathway than the CSP (Loh *et al.*, 2002), our current and previous results clearly show that in all studied species, the model RSP protein hGH is rigorously sorted via an exocrine pathway into saliva. In contrast, transgenic hEPO can be found both in saliva and serum in various proportions depending on the species studied.

The mechanism(s) responsible for the differences in hEPO-sorting patterns in SGs shown in Table 1 are not yet clear. We hypothesize that our hEPO results reflect important differences in the expression and/or metabolic fate of key molecules essential to the recognition and sorting of the protein within SGs of mice, rats, minipigs, and macaques. Since the description of the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) hypothesis by Rothman (1994), it is clear that the intracellular pathways followed by membrane and secretory proteins in epithelial cells, including SGs (e.g., Fölsch *et al.*, 1999; Gaisano, 2000), are directed by a huge repertoire of finely regulated molecules (e.g., Nakatsu and Ohno, 2003; Rodriguez-Boulan *et al.*, 2005). Thus, there are many intracellular steps at which a metabolic or genetic change can lead to a dramatic difference in the final destination of a membrane or secretory protein.

In conclusion, for SGs to be generally useful for systemic gene therapeutics sorting differences such as presented herein must be understood so that human applications can be performed efficiently and safely. On the basis of our studies, we hypothesize that mouse and rat submandibular glands, which are in general structurally and physiologically similar, have significant inherent differences in one or more key intracellular molecule(s) used in sorting hEPO for secretion. Identification of those differences may lead to useful strategies to employ for potential clinical applications of SG gene transfer.

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Author Disclosure Statement

No competing financial interests exist.

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