Salivary glands as a potential gene transfer target for gene therapeutics of some monogenetic endocrine disorders

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Abstract

Salivary glands (SGs) exhibit several important features which are also common to endocrine glands: self-containment due to a surrounding capsule, highly efficient protein production and the ability to secrete proteins into the bloodstream. We have hypothesized that SGs are potentially useful as gene transfer targets for the correction of inherited monogenetic endocrine disorders. In the present communication, we extend our studies and attempt to test our hypothesis by comparing the efficacy of two commonly used viral vectors and the resulting serum and salivary distribution of transgene encoded hormones.

A low dose (5×10^9 particles) of either an adenoviral serotype 5 (Ad5) vector encoding the human erythropoietin (hEPO) cDNA or an adeno-associated virus serotype 2 (AAV2) vector encoding either the hEPO or human growth hormone (hGH) cDNA was administered to individual submandibular SGs of Balb/c mice. Serum and salivary hEPO and hGH levels were determined at defined time points. Two additional recombinant viruses encoding enhanced green fluorescence protein (GFP)

were also used (AdGFP and AAVGFP) in order to perform immunohistochemical analyses of transgenic protein localization in SG sections post-administration.

AAV2 vectors led to stable gene transfer unlike the results with the Ad5 vectors. Indeed, in one mouse we observed hEPO production for a period of two years after administration of AAVhEPO to SGs. hEPO, which is a constitutive pathway secretory protein, was readily secreted into the bloodstream from the SGs, yielding therapeutically adequate serum levels. Conversely, hGH, a regulated secretory pathway protein, was preferentially secreted into saliva.

SGs may be an attractive candidate target tissue for gene therapeutics of some monogenetic endocrine deficiency disorders. At present, AAV2 vectors seem particularly useful for such applications, and transgenes encoding constitutive secretory pathway hormones are more suitable for this application with SGs than those encoding regulated secretory pathway hormones.

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Introduction

Inherited monogenetic endocrine disorders are attractive candidates for gene therapy. The optimal gene therapy target is the physiological production site of the deficient hormone (e.g. pituitary gland somatotropes for growth hormone) to allow for regulated hormone secretion (Dannies 2002). However, the delicate nature and life-sustaining role of these highly differentiated organs along with current biotechnology limitations render such approaches unappealing, especially with respect to non-life threatening hormone deficiencies. Various tissues such as muscle, liver or lung have thus far been primarily targeted

to overcome this obstacle (Barzon *et al.* 2000, Auricchio *et al.* 2002, Goldspink 2003, Harding *et al.* 2004).

We have considered the salivary glands (SGs) to be an unusual, yet promising target tissue for gene therapeutics (i.e. use of the gene as a drug: Crystal 1995, Voutetakis *et al.* 2004a,b, Zufferey & Aebischer 2004). The classic physiological role of SGs is to produce an exocrine secretion, saliva. In addition to water and electrolytes, saliva contains digestive enzymes, anti-microbial proteins, calcium binding phosphoproteins, growth factors and mucoproteins suggesting that SGs exhibit very efficient protein production mechanisms (Amerongen & Veerman 2002). These salivary exocrine proteins are stored in

granules and exit into saliva following a predominant regulated pathway (Castle & Castle 1998). SGs also exhibit a constitutive secretory pathway leading towards the interstitium and bloodstream (Castle & Castle 1998). Convincing evidence has confirmed the endocrine secretion of digestive enzymes by SGs in the serum through this constitutive pathway and proved their presence in the bloodstream to be neither inadvertent nor due to pathology (Isenman *et al.* 1999). Importantly, both secretory pathways (constitutive and regulated) operative in SGs have proven readily available for conveying expressed transgenic secretory proteins (Baum *et al.* 1999, 2002).

SGs thus exhibit several important features of the various endocrine glands: highly efficient protein production and ability to secrete proteins into the bloodstream, as well as self-containment due to the surrounding capsule. Consequently, we have hypothesized that SGs may be an attractive candidate for gene therapeutics for monogenetic endocrine deficiency disorders (i.e. to act as a surrogate endocrine gland: Voutetakis *et al.* 2004b). In the present report, we have extended our studies and directly compared the efficacy of two commonly used viral vectors to mediate gene transfer to SGs, as well as the resulting serum and salivary distribution of two transgene-encoded hormones, human growth hormone (hGH) and human erythropoietin (hEPO). These hormones exit through different secretory pathways (the regulated and constitutive respectively) when synthesized in their primary production sites (anterior pituitary gland and kidney respectively) and therefore are useful as model proteins for 'proof of concept' experiments (Mujais *et al.* 1999, Dannies 2002).

We constructed an adenoviral serotype 5 (Ad5) vector encoding the hEPO cDNA and two adeno-associated virus serotype 2 (AAV2) vectors encoding the hGH and hEPO cDNA respectively. A low dose (5×10^9 particles/animal) of each recombinant virus was administered to individual submandibular SGs of Balb/c mice via cannulation of the main excretory ducts (Baum *et al.* 2002). Serum and salivary concentrations of both hGH and hEPO were measured at distinct time-points post administration. Target cell localization was assessed by immunohistochemistry after transfer of the cDNA for green fluorescence protein (GFP) using either an Ad5 or an AAV2 vector.

Materials and Methods

Construction of AdhEPO, AdGFP, AAVhEPO, AAVhGH and AAVGFP

Generation of the E1⁻, replication deficient Ad5 vectors was performed as previously described (He *et al.* 1998). Briefly, 293 cells were co-transfected with the shuttle plasmid pAC-CMV-pLpA containing either the hEPO cDNA or the GFP cDNA, and the adenoviral plasmid

pJM17 using a calcium phosphate transfection system (Life Technologies, Gaithersburg, MD, USA) to generate the recombinant vectors AdhEPO and AdGFP. The cytomegalovirus (CMV) promoter was employed to drive both hEPO and GFP expression. Dr Y Terada (Tokyo Medical and Dental University, Tokyo, Japan) generously provided the hEPO cDNA. Recombinant viruses were plaque screened, propagated in 293 cells, and purified by CsCl gradient centrifugation, as described (He *et al.* 1998, Baum *et al.* 1999). After purification, recombinant viruses were dialyzed against 4 liters dialysis buffer containing 10% glycerol, 0.1 M Tris (pH 7.4), 5 mM MgCl₂, for 4 h at 4 °C and stored in aliquots at -80 °C for later use. Vector titers were initially determined by measuring the optical density at 260 nm as described (Mittereder *et al.* 1996), and then by real-time quantitative PCR (QPCR) using transgene-specific primers. Vector concentrations used herein were calculated based on the QPCR assay using the ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA).

Generation of the replication deficient AAV2 vectors was performed as previously described (Chiorini *et al.* 1995, Kaludov *et al.* 2002, Voutetakis *et al.* 2004b). Briefly, 293T cells were co-transfected by calcium phosphate precipitation with the *trans* plasmids pMMTV2.1 (provides the AAV serotype 2 *Rep* and *Cap* genes), pAd12 (provides adenoviral helper genes) and one of the *cis* plasmids pAAVhEPO, pAAVhGH or pAAVGFP, containing the hEPO, the hGH or GFP cDNA respectively, flanked by the AAV2 ITRs to generate the recombinant viral vectors AAVhEPO, AAVhGH, or AAVGFP respectively. The Rous sarcoma virus (RSV) promoter was employed to drive transgene expression in these AAV2 vectors. The cells were harvested 48 h post-transfection and a crude viral lysate was obtained after three freeze-thaw cycles. The lysate was treated with benzonase, adjusted to a refractive index of 1.372 by addition of CsCl and centrifuged at 38 000 r.p.m. (SW-41 rotor) for 65 h at 20 °C. Equilibrium density gradients were fractionated and fractions with a refractive index of 1.369–1.375 were collected and stored at 4 °C and assayed for infectious activity. Immediately before experiments, viral fractions were dialyzed against 0.9% NaCl. The number of AAV genomes (particles) was estimated using a QPCR assay, amplifying an RSV sequence using the ABI Prism 7700 Sequence Detector (Applied Biosystems). Vector titers and concentrations presented are based on the QPCR assay.

Mice, gene transfer and salivary and serum collections

Animal studies were approved by the NIDCR Animal Care and Use Committee and the NIH Biosafety Committee. All procedures were conducted in accordance with the International Association for the Study of Pain standards. Male Balb/c mice were obtained from the Division of Cancer Treatment, NCI, Bethesda, MD,

USA. Three groups of mice ($n=5$ each) received 5×10^9 particles/animal (suspended in 50 μ l of 0.9% NaCl) of AdhEPO, AAVhEPO or AAVhGH respectively by retrograde ductal delivery to their submandibular salivary glands (Baum *et al.* 2002, Yamano *et al.* 2002). One additional group of Balb/c mice ($n=5$) was given 50 μ l 0.9% NaCl, thus serving as the control group. Mild anesthesia was induced with 1 μ l/g body weight of a 60 mg/ml ketamine (Phoenix Scientific, St Joseph, MO, USA) and 8 mg/ml xylazine (Phoenix Scientific) solution given intramuscularly (i.m.). Mice given AdhEPO also received an i.m. injection of 100 μ g dexamethasone at the time of viral delivery and 24 h later to diminish virus-mediated acute inflammation (Baum *et al.* 2002). Blood samples were obtained by retro-orbital plexus bleeding at distinct time-points: (i) prior to, and at weeks 1, 2, 3 and 4 after administration of the AdhEPO vector and (ii) prior to, and at weeks 2, 4, 8, 12 and 16 after administration of AAVhGH, AAVhEPO and 0.9% NaCl. For one mouse, originally reported in Voutetakis *et al.* (2004b), blood samples were obtained up to 2 years after AAVhEPO administration (see Fig. 5). Whole saliva was also collected at week 4 of the experiment from the AAVhGH, AAVhEPO and control groups as described (Yamano *et al.* 2002), after stimulation of secretion using 0.5 mg pilocarpine/kg body weight administered subcutaneously. Hematocrits (Hcts) were determined using micro-hematocrit capillary tubes (Fisher Scientific, Pittsburgh, PA, USA) throughout the experiment.

In addition, four mice were given 10^{10} particles of either AdGFP or AAVGFP ($n=2$ per group; one gland each). The AdGFP-treated animals were killed at day 2 or week 6 of the experiment. The AAVGFP-treated animals were killed at week 6. Both SGs (i.e. transduced and not transduced) from all 4 animals were removed at the time of death, fixed in formalin and embedded in paraffin for histological analyses. Sections were cut at 5 μ m thickness. Transduced cells were detected by immunohistochemistry (GFP positive staining) using the streptavidin-biotin peroxidase complex method. Briefly, sections were deparaffinized, endogenous peroxidase was blocked, and sections were incubated for 1 h with a polyclonal primary antibody against GFP (1/200 dilution; ab290; Abcam, Cambridge, MA, USA) raised in rabbits. Staining was developed by using a biotinylated goat antibody (Vector Laboratories) directed against the primary antibody, followed by 3, 3'-diaminobenzidine (SK-4100; Vector Laboratories, Burlingame, CA, USA). Sections were lightly counterstained with hematoxylin. Sections from the opposite, non-transduced SGs from the same mice served as controls.

Quantification of hGH and hEPO

Serum was obtained by centrifugation of the blood samples at $5000 \times g$ for 2 min at room temperature. Levels of hGH in mouse serum and saliva were detected with a

chemiluminescence immunoassay kit (Nichols Institute Diagnostics, San Juan Capistrano, CA, USA) according to the manufacturer's instructions. The lower limit of detection was 20 pg/ml. Levels of hEPO in mouse serum and saliva were determined by an ELISA using commercial assay kits (R&D Systems, Minneapolis, MN, USA). The lower limit of detection was 0.6 mU/ml. Assays were performed according to the manufacturer's instructions.

Anti-hGH antibody detection

The presence of anti-hGH antibodies in mouse sera was evaluated indirectly. For this, 98 μ l of an hGH-containing solution (2 ng/ml) and 2 μ l mouse serum obtained 12 weeks post AAVhGH administration were mixed. The mouse serum was either undiluted or diluted using the hGH assay dilution buffer in a 1:1 to 1:512 ratio. As a result, the final mouse serum dilutions in the hGH solution ranged from 1:50 to 1:25 600. The (hGH+mouse serum) samples were incubated at 37 °C for 30 min. Two control samples containing 98 μ l of the same hGH solution (2 ng/ml) and 2 μ l mouse serum obtained either from a control animal or an AAVhEPO-treated animal were also included. The above-described chemiluminescence immunoassay hGH kit was used to measure all the samples. Mouse anti-hGH antibodies contained in the mouse serum bind to hGH, resulting in interference of detection (i.e. inhibit detection).

Data analysis

Data are reported as mean values $\pm$ S.E. Statistical analyses employed repeated measures ANOVA for the long term comparison of the AdhEPO- and AAVhEPO-treated groups, with Fisher's protected least significant difference (PLSD) for within time point comparison. A paired *t*-test was used for the comparison of the saliva and serum distribution within the AAVhEPO- and AAVhGH-treated groups (Statview Version 5.0.1; SAS Institute, Cary, NC, USA).

Results

For all animals used in our experiments, mean serum hEPO and hGH levels were non-detectable and mean Hct levels ($\pm$ S.E.) were $59.6 \pm 2.5\%$ prior to vector administration (week 0). Mean serum hEPO levels from the AdhEPO- and AAVhEPO-treated groups are depicted in Fig. 1 (16 week period).

In the AdhEPO-treated group (5×10^9 particles/animal; $n=5$), serum hEPO levels were increased one week post administration (mean $\pm$ S.E.: 94 ± 27 mU/ml; $n=4$, one animal died). Hct levels at week 1 were also increased ($72.2 \pm 1\%$). At week 2 and thereafter, serum hEPO levels were undetectable. Hct levels at week 2 fell

Figure 1 Serum hEPO levels at various time points after vector delivery to mouse submandibular glands. Data are the mean ($\pm$ S.E.) values after 5×10^9 particles were administered to animals ($n=5$; each group). In the AdhEPO-treated group ($\square$), hEPO levels were significantly increased one week post administration ($*P<0.01$). The mean Hct level at week 1 was 72%. At week 2 and thereafter, hEPO levels became undetectable and Hct levels at week 4 were back to pre-administration levels (61%). In the AAVhEPO-treated group ($\blacktriangle$), hEPO levels remained elevated throughout the experiment (16 weeks; F: 11.5, $P<0.01$). The hEPO values were significantly increased at week 4 and 8 when compared with the previous available measurement respectively ($*P<0.05$). Transgene expression was relatively stable thereafter since hEPO levels at 8, 12 and 16 weeks were not different from one another ($\#P>0.05$). Hct levels increased in parallel with serum hEPO levels (see text; mean Hct value at week 16 was 86%). Comparison between serum and salivary hEPO secretion at week 4 is depicted in Fig. 2. Both serum and salivary hEPO secretion at week 4 is depicted in Fig. 2. Both serum and salivary hEPO levels were undetectable in the AAVhGH and control (naïve) groups ($n=5$ each; controls receiving 0.9% NaCl), in which Hct levels remained $\sim 60\%$ until the end of the experiment. †, one animal died prior to this timepoint.

towards baseline ($68 \pm 1.2\%$) and at week 4 were back to pre-administration levels ($61 \pm 2\%$). The elevation of serum hEPO levels at week 1 was significant (F: 11.7, $P<0.01$).

In the AAVhEPO-treated group (5×10^9 particles/animal; $n=5$), serum hEPO levels gradually increased to the 12 week measurement (69.4 ± 26.4 mU/ml). The overall elevation of serum hEPO levels was significant (F: 11.5, $P<0.001$). The hEPO levels significantly increased between weeks 0, 4 and 8 ($P<0.05$). There was no statistical difference in the serum hEPO levels when comparing results between weeks 8, 12 and 16. The mouse with the highest serum hEPO levels at week 12 (168 mU/ml) died at week 14. Consequently, although serum hEPO levels remained relatively stable at week 16 (the longest time studied) with respect to each of the remaining mice ($n=4$), the mean serum hEPO levels appear slightly decreased albeit not significantly (42.5 ± 12.2 mU/ml). Hct levels increased significantly ($P<0.01$ when compared with both pre-administration levels of the AAVhEPO-treated group and the control group (naïve mice) at the same time point) and occurred in parallel with serum hEPO levels ($77.2 \pm 4.2\%$, $82.6 \pm 3.9\%$ and $86.2 \pm 2\%$ at weeks 4, 8 and 16 respectively). Mean

Figure 2 Transgene encoded hEPO and hGH continue to follow the same secretory pathway (constitutive and regulated respectively) as in their primary site of production. (A) Mean serum and salivary hEPO levels ($\pm$ S.E.) at week 4 after AAVhEPO administration in mouse submandibular glands (long-term data are depicted in Fig. 1). hEPO produced in SGs is predominantly secreted into the bloodstream (constitutive pathway); salivary levels are $\sim 10\%$ of the serum levels ($*P<0.05$ with paired *t*-test). Both serum and salivary hEPO levels were undetectable in the AAVhGH group. (B) Mean serum and salivary hGH levels ($\pm$ S.E.) at week 4 after AAVhGH administration in mouse SGs. hGH produced in SGs is predominantly secreted into saliva (regulated pathway); serum levels are $\sim 15\%$ of salivary levels ($*P<0.05$ with paired *t*-test). Both serum and salivary hGH levels were undetectable in the AAVhEPO group. Data shown for hEPO and hGH levels are based on experiments with 5 and 4 mice respectively.

salivary hEPO levels at week 4 (Fig. 2A) were 3.8 ± 1.1 mU/ml, markedly less ($P<0.05$) than mean serum hEPO levels at the same time point (39.2 ± 13.6 mU/ml). Both serum and salivary hEPO levels were undetectable in the AAVhGH and control groups ($n=5$ each; controls receiving 0.9% NaCl), in which Hct levels remained $\sim 60\%$ until the end of the experiment.

In the AAVhGH-treated group (5×10^9 particles/animal; $n=5$) mean salivary hGH levels were significantly increased at week 4 post administration (Fig. 2B; 1084 ± 102 pg/ml). Although mean serum hGH levels also increased (151.5 ± 17.3 pg/ml), the difference between salivary and serum distribution of hGH was significant ($P<0.05$). By week 8 and thereafter, however, both serum and salivary hGH levels were undetectable (not shown). A

Figure 3 Inhibition of hGH detection due to the presence of anti-hGH antibodies in mouse serum 12 weeks after AAVhGH administration. Serial dilutions (1:50–1:25 600) of mouse serum (serum from AAVhGH-treated, as well as from naïve and AAVhEPO-treated mice as controls) were mixed together with an hGH-containing solution (2 ng/ml). A chemiluminescence immunoassay kit was used to measure hGH levels after incubation at 37 °C for 30 min. Marked inhibition of detection of hGH was observed with respect to the hGH control (no mouse serum used; considered as 100% of detection) when serum from AAVhGH-treated animals was used (♦). The 50% inhibiting concentration of the mouse serum was between 1:3200 and 1:6400. No inhibition of hGH detection was observed when the same hGH solution was incubated with serial dilutions of serum from either naïve animals (given 0.9% NaCl; data not shown) or AAVhEPO-treated animals (dotted line). All assays were performed in duplicate (mean values are shown).

high amount of mouse anti-hGH antibodies was found in serum obtained from the AAVhGH-treated group at week 12 after administration. The 50% inhibiting concentration of the mouse serum was between 1:3200 and 1:6400 (Fig. 3). No inhibition of hGH detection was observed when serum from both control (naïve) animals or AAVhEPO-treated animals was used. Both serum and salivary hGH levels were undetectable in the AAVhEPO and naïve mouse control groups ($n=5$; controls receiving 0.9% NaCl).

In the AdGFP-treated animals GFP was only detected in sections obtained two days after viral delivery. Both acinar and ductal cells were positively stained (Fig. 4). Infiltration of inflammatory cells and a disruption of the normal morphology of SGs was also observed (Fig. 4B). No GFP-positive staining was observed at week 6 after AdGFP administration (data not shown). In contrast, in the AAVGFP-treated animals GFP was detected at week 6 after administration. Only ductal cells were positively stained and no infiltration of inflammatory cells was observed (Fig. 4C). No staining was detected in the control sections obtained from the opposite (i.e. not cannulated) SG from each mouse (Fig. 4D).

Discussion

Recent advances in gene transfer to replace therapeutic proteins, although significant are far from defining a

universal vector or delivery site. At present, vectors and gene therapy approaches are best evaluated in a disease- and/or system-specific manner. Our efforts have focused on the study of a potentially useful target site for some endocrine disorders, the SGs.

Human SGs are not-critical-for-life organs with considerable secretory abilities, able to produce 0.75–1.5 liters of saliva daily, and to secrete protein into both the gastrointestinal tract and the bloodstream (Isenman *et al.* 1999). These unique features motivated us to suggest SGs as potential targets for gene therapeutics. Efficient production and secretion of transgene-encoded proteins can occur with vector doses 10 to 100 times lower than required at many commonly used tissue target sites (e.g. muscle, liver, lung: Snyder *et al.* 1997, Almazan *et al.* 2000, Bohl *et al.* 2000, Chao *et al.* 2001, Auricchio *et al.* 2002, Samakoglu *et al.* 2002, Johnston *et al.* 2003, Voutetakis *et al.* 2004a,b, Zufferey & Aebischer 2004). The use of lower vector doses likely reduces the potential danger of a viral vector-related adverse event.

Moreover, the SGs are well encapsulated (i.e. self-confined) organs (Baum *et al.* 2002). Under our experimental conditions, we have previously shown that dissemination of the vector beyond the SGs, and subsequent stable infection of other tissues post-administration with such low viral doses, is below the QPCR assay sensitivity levels (Kok *et al.* 2003, Voutetakis *et al.* 2004b). To further ensure safety in clinical studies, use of SG tissue-specific promoters will likely be beneficial (Zheng *et al.* 2001).

Importantly, the number of viral copies present in the SGs and the transgene-encoded protein levels synthesized are strongly correlated, facilitating a crude yet useful control over protein production (Voutetakis *et al.* 2004b). Tighter regulation of transgene expression in SGs can be achieved

through small molecule drug-mediated activation of transcription (Wang *et al.* 2004).

Gene transfer to SGs can be accomplished in a relatively non-invasive manner by intra-oral cannulation of the main excretory ducts. Vector is infused in a retrograde

direction through the cannula, after suspension in a diluent buffer (Fig. 4A; Baum *et al.* 2002). The procedure is similar to the one performed clinically without anesthesia for contrast radiographs of the SGs. SGs consist of two general types of well differentiated and slowly dividing epithelial cells, acinar (in humans >80% of the parenchymal cells) and ductal (Cook *et al.* 1994). Ductal cells form an extensive tubular network conducting saliva to the mouth, while acinar cells are found at the distal end of the ductal system (Fig. 4A). Most SG epithelial cells exist as a monolayer, lining up along the luminal system and therefore are a potential target after vector administration (Fig. 4A).

SG cells exhibit at least two distinct secretory pathways: a predominant regulated one leading to exocrine protein secretion into saliva via zymogen granules, and a constitutive one mainly leading to endocrine secretion into the bloodstream (Fig. 4A; Castle & Castle 1998, Baum *et al.* 1999, Isenman *et al.* 1999). Secretory proteins produced as transgene products in SGs, after viral-mediated gene transfer, continue to follow the same general secretory pathway as in their primary site of production (Baum *et al.* 1999). Physiologically, hGH is synthesized in somatotropes residing in the anterior pituitary gland and is secreted via the regulated pathway, and hEPO is synthesized in kidney tubular epithelial cells and is secreted via the constitutive pathway (Mujais *et al.* 1999, Dannies 2002). The pathway followed for secretion is not affected by general protein structure, since EPO and GH proteins are of a similar size and tertiary structure (Bazan 1989).

Ad5 and AAV2 vectors are able to infect both non-dividing and slowly dividing cells (Lai *et al.* 2002). Consequently, these vectors can be used for transferring genes to SGs *in vivo* (Baum *et al.* 2002). Data herein presented constitute the first direct comparison between AAV2 and Ad5 vectors in salivary glands in any species. In the AdhEPO-treated group, mean serum hEPO levels were significantly elevated one week after administration, but became undetectable thereafter (Fig. 1).

Ad5 vectors elicit a potent host cellular and humoral immune response in SGs as in other tissues (Fig. 4B; Adesanya *et al.* 1996, Kagami *et al.* 1998). As a consequence, vector DNA and transduced epithelial cells are rapidly destroyed and transgene-encoded protein production ceases. Thus, first-generation Ad5 vectors do not seem suitable for gene transfer to SGs with respect to management of monogenetic endocrine deficiencies, as such applications require long-term expression of the transgene.

AAV2 is a relatively small (<5 kb), naturally replication-incompetent member of the parvovirus family. There is no evidence that AAV2 is an etiological agent for human disease (Kay *et al.* 2001). Since AAV2 is a small vector, there is relatively limited space available for the transgene cassette (i.e. requiring at least the promoter, transgene, and polyadenylation sequence). Importantly, *in vivo* recombinant AAV2 vectors can mediate long-term transgene expression in salivary glands and other tissues (Yamano *et al.* 2002, Voutetakis *et al.* 2004b).

After AAVhGH administration to the SGs, hGH was predominantly secreted into saliva through the selective and multistep regulated secretory pathway. Entry into the regulated pathway can be saturated by the overexpression of the transgene product (Marmorstein *et al.* 2000, Hoque *et al.* 2001). The 'overflow' can then exit salivary epithelial cells through the constitutive pathway and be secreted into the bloodstream inefficiently (He *et al.* 1998, Hoque *et al.* 2001). Accordingly, serum concentrations of hGH shown herein were relatively low compared with those in saliva (Fig. 2B). We were unable to follow hGH production after week 4 of the experiment probably due to a substantial anti-hGH humoral response (Fig. 3). However, our results from the week 4 time-point clearly show that although possible, it is inefficient to deliver a regulated secretory pathway (RSP) transgenic hormone into the bloodstream from mammalian SGs. Most of the produced peptide will be physiologically wasted by being secreted via saliva into the gastrointestinal tract.

Figure 4 (A) Schematic representation of the structure of murine salivary glands *in situ*. Most SG epithelial cells exist as a monolayer, lining up along the luminal system. SGs principally consist of two general types of epithelial cells, acinar and ductal. SG cells exhibit a predominant regulated secretory pathway leading to saliva (through dense core secretory granules across the apical membrane; white arrow) and a constitutive pathway leading into the interstitium and the bloodstream (across the basolateral membrane; black arrow). Ductal cells form an extensive tubular lumen conducting saliva, via the excretory duct (ED), to the mouth. Acinar cells are found at the distal end of the ductal system, and form the acini. Tight junctions (TJ) between SG cells are also depicted (black connections). Gene transfer to SGs can be accomplished in a relatively non-invasive manner by intra-oral cannulation of the main excretory ducts. After vector infusion in a retrograde direction (dotted arrow) through the cannula, almost the entire SG cell population is a potential target. (B, C, D) Immunohistochemical detection of green fluorescence protein (GFP) expression in mouse submandibular glands after administration of 10^{10} particles of either adenoviral (AdGFP) or adeno-associated viral (AAVGFP) vectors. Sections were prepared at various time points after vector delivery, and GFP expression detected using an anti-GFP antibody. Sections were counterstained with hematoxylin. Representative examples of acini (a) and ducts (d) are depicted. (B) Two days after administration, AdGFP led to GFP production in both acinar and ductal cells (brown color). Infiltration of inflammatory cells and disruption of the SGs normal morphology was also observed. No GFP producing cells were observed at week 6 after AdGFP delivery, probably due to rapid immune-mediated destruction of infected cells (Adesanya *et al.* 1996, Kagami *et al.* 1998). SGs at week 6 appeared to have a normal morphology with no inflammatory infiltrates present (data not shown). (C) At week 6 after administration, AAVGFP-mediated transgene expression (brown color) is clearly evident. Only ductal cells are infected and no infiltration of immune cells is observed. (D) No GFP immunostaining was detected in the control tissue sections obtained from the contralateral SG.

There are a number of reports suggesting that RSP sorting is signal dependent (i.e. amino acid sequence dependent; e.g. Kelly 1985, Gerdes & Glombik 1999, Loh *et al.* 2002). The example of pro-insulin mis-sorting both in humans and in transgenic mice due to point mutations in the insulin gene is well described (Chan *et al.* 1987, Carroll *et al.* 1988). Consequently, one strategy to enhance hGH (and other regulated pathway proteins) secretion into the constitutive pathway and therefore into the bloodstream could involve manipulation of putative sorting signals (Wang *et al.* 2005). An alternative strategy is to induce mis-sorting of RSP transgenic hormones by using intracellular alkalinizing drugs, such as hydroxychloroquine (Hoque *et al.* 2001).

After AAVhEPO administration to the SGs, hEPO was secreted preferentially into the bloodstream following the constitutive pathway. Salivary concentrations of hEPO were $\sim 10\%$ serum levels (Fig. 2A). SGs were able to sustain serum hEPO levels well above the normal range (10–30 mU/ml; Kendall 2001) throughout the 16 weeks of the current experiment, and longer, as we have reported (Voutetakis *et al.* 2004b). Erythropoietin targets erythroid progenitor cells in the bone marrow and increases red blood cell production and therefore Hct levels (Kendall 2001). Circulating hEPO proved to be not only therapeutically adequate but also functional in the present and in earlier studies: Hct levels in both AdhEPO- and AAVhEPO-treated groups generally followed serum hEPO levels (see Results). This indicates that SGs are capable of the necessary post-transcriptional modifications required for the secretion and biological activity of this transgene product (Dube *et al.* 1988).

In previously published experiments we have shown AAV2-mediated transgene expression for up to 54 weeks in mice (Voutetakis *et al.* 2004b). Interestingly, a mouse included in that original study (receiving 5×10^9 particles of AAVhEPO), has survived for more than two years after vector administration, and despite the sustained elevated Hct levels ($\sim 70\%$). Serum hEPO levels in this mouse were quite stable throughout this two-year period (Fig. 5). No inflammatory infiltrates or structural abnormalities have been observed in gland sections after AAV2 vector administration, suggesting that retrograde ductal delivery *per se* and AAV2 transduction do not cause any irreversible damage to the SG tissue (Fig. 4C; Yamano *et al.* 2002, Voutetakis *et al.* 2004b).

Our data suggest that SGs could be an alternative gene delivery target for some monogenetic endocrine disorders, potentially offering a safer way of vector administration without limiting the efficiency of hormone production. Using the SGs for such applications is consistent with the primary role of these cells, i.e. it takes advantage of already existing normal physiological functions. AAV2 vectors currently appear as the gene transfer vector of choice for such applications, since they appear safe and mediate stable, uneventful and long-term transgene expression.

Figure 5 Serum hEPO levels at various time points after retrograde ductal delivery to a single mouse (5×10^9 AAVhEPO viral particles to one submandibular gland). This mouse has survived from an original group ($n=5$) transduced with AAVhEPO and reported in Voutetakis *et al.* (2004b). This mouse was able to produce relatively stable serum hEPO levels for two years after vector administration. The Hct level at week 104 was 70%.

With respect to transgene products, constitutive pathway secretory proteins currently appear to be more suitable for SG applications, since RSP proteins are preferentially secreted into saliva. Efficient strategies for re-direction of the latter must be achieved before useful clinical applications with RSP proteins can be realized.

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