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Salivary epithelial cells: an unassuming target site for gene therapeutics

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Abstract

Salivary glands are classical exocrine glands whose external secretions result in the production of saliva. However, in addition to the secretion of exocrine proteins, salivary epithelial cells are also capable of secreting proteins internally, into the bloodstream. This brief review examines the potential for using salivary epithelial cells as a target site for in situ gene transfer, with an ultimate goal of producing therapeutic proteins for treating both systemic and upper gastrointestinal tract disorders. The review discusses the protein secretory pathways reported to be present in salivary epithelial cells, the viral gene transfer vectors shown useful for transducing these cells, model transgenic secretory proteins examined, and some clinical conditions that might benefit from such salivary gland gene transfer.

Keywords

salivary glands; gene therapeutics; viral vectors; protein sorting

1. Introduction

Salivary glands are classically considered to be exocrine glands whose secretions protect and facilitate the function of all oral and upper gastrointestinal tract tissues (Amerongen and Veerman, 2002). The vast majority of cells in these glands are epithelial and of two broad types: acinar and duct (Turner and Sugiyama, 2002). Acinar cells are secretory and the only site of fluid secretion in the glands. Acinar cells secrete a primary fluid that is isotonic and contains ~85% of the secreted proteins found in saliva. Salivary ducts constitute an absorptive epithelium. While duct cells secrete the remaining ~15% of salivary proteins, their primary physiological role is to absorb NaCl. By the time the forming saliva exits the duct and enters the mouth, the NaCl concentration has been reduced from ~150 mEq/L to ~25 mEq/L.

While studies of protein production by and secretion from salivary glands most often focus on the proteins found in saliva (e.g., Helmerhorst and Oppenheim, 2007), there is a long history recognizing protein secretion into the bloodstream by salivary glands (e.g., Leonora et al, 1987; Isenman et al, 1999). Due to this duocrine (Figure 1; both exocrine and endocrine) nature of salivary epithelial cell protein secretion, we began to study the potential applications of in situ

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gene transfer to salivary glands (Baum et al, 1999) for gene therapeutics. Although not typically considered a target tissue for gene therapeutics, and in particular not for systemic applications, salivary gland epithelial cells present multiple advantages as a gene transfer target site (Baum et al, 2004). They are: (i) easily accessible through the main excretory duct, which opens into the mouth; (ii) well-encapsulated limiting any spread of the gene transfer vector; (iii) well-differentiated, providing a relatively stable target site for non-integrating vectors; (iv) capable of producing significant amounts of protein for export; and (v) not-critical for life in case of the occurrence of a severe adverse event.

Our aggregate studies demonstrated two key findings. First, it is possible to deliver transgenes encoding various secretory proteins to salivary epithelial cells and find transgenic proteins in both saliva and the bloodstream (e.g., see O'Connell et al, 1996; Baum et al, 1999). Secondly, for several transgenic secretory proteins, there is no simple way to predict the direction of secretion (e.g., Adriaansen et al, 2008; Voutetakis et al, 2008; see below). Finding a way to circumvent this latter situation is essential if salivary gene therapeutics is to be clinically useful.

2. Cellular origins

Salivary glands develop from a thickening of embryonic oral epithelium, which then protrudes into the underlying mesenchyme (Tucker, 2007). This initial bud of epithelium undergoes branching morphogenesis in response to signals from the mesenchyme and extracellular matrix, so that by E14 a highly branched gland exists, with an elongated duct and formed lumen (Patel et al, 2006; mouse submandibular gland). Proacinar cells appear after E15, with acinar differentiation occurring postnatally and granular convoluted ducts becoming fully differentiated only at puberty (Patel et al, 2006; Tucker, 2007).

3. Viral vectors for use in salivary gland gene transfer

Gene transfer delivers a gene of interest into target cells or tissues using a carrier, termed a vector. Successful gene transfer requires efficient, nontoxic vectors (viral or non-viral) that provide adequate transgene expression for an appropriate duration. The simplest and least intrusive way to deliver a gene is with plasmid DNA. This method, however, is generally inefficient, especially in vivo. Viral vectors currently are important tools for in vivo gene transfer, because viruses have evolved efficient mechanisms to introduce their DNA into recipient cells.

Viral vector selection depends on the intended purpose (e.g., long or short-term gene expression), the target cell or tissue, and the method of delivery (e.g., in vivo, ex vivo). It follows that no single virus is suitable for all gene transfer applications. There are two general classifications of viral vectors, DNA and RNA, and only the former appear useful with salivary epithelial cells. The two most often used are serotype 2 adeno-associated viral (AAV2) vectors, which are single-stranded, and serotype 5 adenoviral (Ad5) vectors, which are double-stranded (Table 1; Baum et al, 2002). Typically, recombinant viral vectors are replication incompetent with several viral genes deleted. The gene of interest and associated regulatory elements replace the deleted viral genes.

Vector delivery to salivary glands is achieved through cannulation of the main excretory ducts whose orifices are accessible in the mouth (Baum et al, 2002). Ad5 vectors possess broad cell and tissue tropism, and, following intraductal delivery, transduce both duct and acinar cells efficiently (Mastrangeli et al, 1994). However, they can provoke vigorous immune responses resulting in relatively short-term transgene expression (2–4 weeks; Kagami et al, 1998). AAV2 vectors also possess fairly broad cell and tissue tropism, but after intraductal delivery only transduce duct cells. AAV2 vectors elicit minimal immune reactivity and yield long-term transgene expression in salivary glands (Voutetakis et al, 2007).

A significant issue general for gene transfer is controlling transgene expression levels. High and uncontrolled transgene expression could impair cell function and confound biological studies, as well as endanger patients in clinical studies. There are multiple levels at which control of transgene expression can be addressed. Of primary importance is the selection of key elements in the transgene cassette, e.g., the promoter, use of enhancers, introns, 5' and 3' untranslated regions and different poly-adenylation signals. We have tested multiple promoters in rodent salivary glands, e.g., cell type specific (AMY, from human amylase, acinar cell specific; Kall, from human kallikrein, duct cell specific) and non-specific (e.g., CMV, cytomegalovirus; EF1 α , human elongation factor 1 α) that lead to a broad range of transgene expression (Zheng and Baum, 2005). Control of transgene expression, in an off/on manner, also occurs using a small molecule (drug) inducible promoter system. One such system, which we use, employs rapamycin or its non-immunosuppressive analogues (Wang et al, 2006) and is quite effective regulating transgene expression following gene transfer to rodent submandibular glands with both Ad5 and AAV2 vectors.

4. Secretory pathways used by transgenic proteins in salivary cells

Physiological exocrine protein secretion in salivary glands is primarily triggered by the sympathetic nervous system, and secondarily by the parasympathetic system (Turner and Sugiyama, 2002). While protein secretion pathways can roughly be divided into exocrine and endocrine (Figure 1), three specific pathways have been well described in salivary cells: major regulated, minor regulated and constitutive-like (Castle, 1998; Castle et al, 2002; Gorr et al, 2005). The first two are external stimulus-dependent, with proteins stored in secretory granules until stimulation and secretion is directed apically, i.e., into saliva. Both the constitutive and constitutive-like pathways are non-directional and result in continuous secretion of protein at roughly the rate of translation, i.e., not modulated by external stimuli. They account for a small proportion of protein secretion from acini (Castle et al, 2002; Gorr et al, 2005), but, presumably, all endocrine secretion from salivary cells, although the molecular details of these secretory routes are essentially non-existent.

In general, the mechanisms responsible for sorting soluble secretory proteins are not well known. The notion emerged that signals lie within the protein's secondary or tertiary structure, e.g., pro-opiomelanocortin (Cool et al, 1995), though such signals are clearly not simple to identify (Wang et al, 2005) and no universal signals have been identified. When produced as a transgenic protein following gene transfer, it is assumed the protein will maintain its secretory behavior in other tissues. However, studies with several transgenic secretory proteins in salivary epithelial cells of multiple species demonstrate this assumption is not true (e.g., Adriaansen et al, 2008; Voutetakis et al, 2008; see below). Overall, these studies suggest that the mechanisms responsible for secretory protein sorting are cell-type specific and may correspond to the presence of different ligand or receptor proteins in the secretory pathways of individual cell types. To appreciate salivary epithelial cell type sorting differences, we are trying to map out these pathways for model proteins by co-localization with known markers for different intracellular compartments (Figure 2; Tables 2–4).

As described above, the behavior of some transgenic proteins can be different between species (Table 2). Two useful model human (h) secretory proteins demonstrating this are erythropoietin (hEpo) and parathyroid hormone (hPTH). Epo is a constitutive pathway protein, normally secreted by kidney peritubular fibroblasts, which promotes red blood cell development and survival. If expressed in murine submandibular glands, biologically active transgenic hEpo is secreted almost exclusively into the bloodstream (Table 3; Voutetakis et al, 2004). In rat submandibular glands hEpo is also primarily secreted via this endocrine route, but significant amounts are secreted into saliva (Table 3; Voutetakis et al, 2008). Additional species variations are observed in miniature pigs and rhesus macaques (Yan et al, 2007; Voutetakis et al, 2008).

Recently, we discovered a comparable species-specific difference in the sorting of hPTH, a regulated secretory pathway protein essential for calcium homeostasis. When hPTH is expressed in murine submandibular glands, biologically active hPTH is secreted primarily into the bloodstream (Table 3). Conversely, in rat submandibular glands, transgenic hPTH predominantly sorts into saliva (Adriaansen et al, 2008). Clearly, significant species-specific differences must exist in one or more key intracellular molecules used in sorting hEpo and hPTH to produce these results (Figure 2).

Proteins that consistently sort to the same compartment in multiple species can also provide important information, and we found growth hormone (hGH) a useful model protein for this purpose (Table 2). Physiologically, hGH is synthesized, stored and secreted via the regulated pathway in pituitary somatotrophs, and stimulates multiple anabolic responses. When expressed in salivary glands of rats (Baum et al, 1999), mice (Voutetakis et al, 2005), miniature pigs (Yan et al, 2007) and rhesus macaques (Voutetakis et al, 2008), transgenic hGH is reproducibly secreted into saliva. We anticipate that using such model proteins should help define different sorting mechanisms in salivary epithelial cells in vivo and facilitate a more general understanding of soluble protein sorting in polarized epithelial cells.

5. Clinical diseases that might be targeted

Given the ability of salivary glands to secrete proteins in both exocrine and endocrine directions, transferring genes encoding secretory proteins has the potential to treat both local (upper gastrointestinal tract) and systemic disorders. The key to clinical application is being able to produce therapeutically effective levels of the transgenic protein and secrete it in the appropriate direction. After Ad5 transduction peak levels of transgenic proteins vary widely, with salivary or serum concentrations ranging from pg to μ g levels (Table 4). The reason for this variation currently is not understood.

Secretion of a transgenic therapeutic protein in an exocrine direction augments saliva's natural protective features. Normally, human salivary glands secrete many antimicrobial proteins to control bacterial, viral and fungal populations and prevent overt oral infections (Amerongen and Veerman, 2002; Helmerhorst and Oppenheim, 2007). A group of cationic salivary proteins, termed histatins, are important to the human oral innate response and have potent anti-Candidal activity. As a potential therapy for patients with azole-resistant oral Candidiasis, we developed an Ad5 vector encoding histatin 3 and administered it to rat submandibular glands. The rats secreted on average $\sim 300 \mu\text{g/ml}$ histatin 3 into saliva and it was able to kill fluconazole-resistant *Candida* in vitro (O'Connell et al 1996). Another example of salivary augmentation uses a model of radiation-induced mucositis (Zheng et al, 2009). An Ad5 vector encoding human keratinocyte growth factor (hKGF) to mice receiving fractionated irradiation completely prevented the occurrence of severe oral mucositis. Peak salivary hKGF levels were $\sim 1 \text{ ng/ml}$.

The ability of salivary epithelial cells to produce bioactive transgenic proteins and secrete them into the bloodstream could be used to correct single protein deficiency disorders (Baum et al, 2004), e.g., for mono-endocrinopathies in which a peptide hormone or growth factor is absent or non-functioning. We have demonstrated proof of concept for this premise with several human genes including hEpo, hGH, and hPTH. For example, transfer of the hEpo gene to submandibular glands of healthy mice via an AAV2 vector resulted in stable, therapeutic serum hEpo levels of 10–30 mU/ml with dramatically increased hematocrits, suggesting potential for treating Epo-responsive anemias (Voutetakis et al 2004). A second example is targeted hGH deficiency. Ad5-mediated hGH expression in rat submandibular glands led to serum hGH levels $\sim 16 \text{ ng/ml}$, an amount well above therapeutic requirements and one which led to anabolic effects in healthy animals (He et al, 1998). Finally, delivery of an Ad5 vector encoding hPTH

to mouse submandibular glands suggests a potential therapy for hypoparathyroidism, leading to high levels (on average ~3 µg/ml) of bioactive hPTH in serum (Adriaansen et al, 2008).

However, as noted earlier, we cannot predict the sorting pathway to be followed by each of these transgenic proteins. Thus, the secretion of hEpo (from miniature pig parotid glands, Yan et al, 2007), hGH (rat submandibular gland, Baum et al, 1999) and hPTH (rat submandibular gland, Adriaansen et al, 2008) is overwhelmingly into saliva and therapeutically inefficient. Clearly, further understanding of the cellular biology of sorting pathways in these glands is necessary to realize the promise of salivary gene therapeutics.

Cell facts

- Salivary epithelial cells are of two general types: acinar and duct.
- Salivary epithelial cells have a primary function of producing saliva, a fluid with essential digestive and protective functions.
- Salivary epithelial cells can secrete protein both into saliva and into the bloodstream

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Figure 1.

General depiction of protein secretory pathways operative in salivary epithelial cells. The regulated pathway leads to exocrine protein secretion via secretory granules (circles at the apical pole of the cell). Endocrine secretion presumably occurs via a constitutive or constitutive-like pathway. TJ, tight junctions.

Figure 2.

Locations of marker proteins in or relevant to the protein secretory pathway in salivary epithelial cells. Secretory granules (SG); immature secretory granules (ISG); trans-golgi network (TGN). Markers that can be used to characterize the location of a secretory protein in specific salivary gland organelles are indicated. These include aquaporin -5 (AQP-5); early Endosome antigen 1 (EEA-1); RAB GTPases 4/5/11 (RAB-4/5/11); endoplasmic reticulum-associated protein disulfide isomerase (PDI); Na-K-Cl co-transporter 1 (NKCC1); 36-kD vesicular integral membrane protein (VIP-36); glutamic acid/glutamine-rich protein (GRP); vesicle-associated membrane protein 4/8 (VAMP-4/8); type I integral membrane protein from the *trans*-Golgi network (TGN-38); lysosomal-associated membrane protein (LAMP1); Golgi matrix protein (GM-130).

Table 1

Key characteristics of Ad5 and AAV2 vectors

Characteristics	Ad5	AAV2
Genome	37 kb	4.7 kb
DNA	double-stranded	single-stranded
Salivary cell targets	acinar, duct	duct
Transgene levels	high	modest
Stability of expression	low	high
Titers achieved	high	modest
Immune response	significant	modest
Production	easy	laborious

Table 2

General characteristics of several model proteins in mammalian salivary glands¹

Model protein	Size (kD)	Glycosylated	Type	Sorting	Biologically active	Entrez #
hEpo	34	yes	CSP	Depending on species	yes	2056
hGH	22	no	RSP	Apical	yes	2688 (GH1) 2689 (GH2)
hαLAT	52	yes	CSP	Primarily basolateral	yes	5265
hPTH	11	no	RSP	Depending on species	yes	5741

¹ hEpo, human erythropoietin; hGH, human growth hormone; hαLAT, human α1-antitrypsin; hPTH, human parathyroid hormone; CSP, constitutive (or constitutive-like) secretory pathway; RSP, regulated secretory pathway.

Table 3

Distribution ratios¹ of hPTH and hEpo after transduction of rat and mouse submandibular glands

	Mouse	Rat
hPTH	613	0.93
hEpo	180	11.5

¹ Distribution ratios represent the ratio of the total transgenic protein found in serum to that found in saliva (For details see Baum et al, 1999; Adriaansen et al). hPTH, human parathyroid hormone; hEpo, human erythropoietin

Table 4

Peak levels of transgenic secretory proteins produced after Ad5 transduction of rodent submandibular glands ¹

Protein ²	Total amount in saliva ³	Total amount in serum ⁴	Ad5 vector dose (vp) ⁵	Species	Reference
hKGF	6 pg	200 pg	10 ¹⁰	mouse	Zheng et al, 2009
hPTH	16 pg	5 ng	5×10 ¹⁰	mouse	Adriaansen et al, 2008
hEpo	0.01 ng	1 ng	5×10 ⁹	mouse	Voutetakis et al, 2008
hPTH	1 ng	0 ng	5×10 ⁹	rat	Adriaansen et al, 2008
hGH	1.7 µg	90 ng	10 ¹¹	rat	Baum et al, 1999
hα1AT	31 ng	90 ng	~1×10 ¹¹	rat	Baum et al, 1999
hH3	45 µg	ND	10 ¹¹	rat	O'Connell et al, 1998
hEpo	0.27 ng	4.1 ng	10 ¹⁰	rat	Voutetakis et al, 2008

¹Total amounts shown are approximate from those reported in the original references. Note that for hEpo the amounts originally reported were in mU, which have been converted using 100 mU = 1 ng.

²The abbreviations for the transgenic secretory proteins listed are as follows: hKGF, human keratinocyte growth factor; hPTH, human parathyroid hormone; hEpo, human erythropoietin; hGH, human growth hormone; hα1AT, human α1 antitrypsin; hH3, human histatin 3.

³The total amount of protein secreted into saliva was calculated as the concentration × 0.1 ml for mice and 0.15 ml for rats.

⁴The total amount of protein secreted into serum was calculated as the concentration × 2 ml for mice and 4 ml for rats.

⁵The Ad5 vector doses are shown as vector particles (vp). For studies with hGH and hH3 dosing was by infectious units and the vp shown are approximate values. All vectors used the cytomegalovirus promoter except that for hKGF, which used the human elongation factor 1α promoter (~10% as strong). All vector cassettes used the simian virus 40 polyadenylation signal.