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Salivary Gland Gene Therapy

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Why consider gene transfer to salivary glands? Two primary reasons have motivated us. First, no adequate treatment is available for irreversibly damaged salivary glands, such as found in patients receiving therapeutic irradiation (IR) for a head and neck cancer or in patients with the autoimmune exocrinopathy Sjögren's syndrome (SS). Second, salivary glands can produce and secrete large amounts of protein locally to the oral cavity and gastrointestinal (GI) tract or into the bloodstream systemically, making them attractive targets for gene therapeutics (ie, using genes as drugs). This article provides a general background in gene therapy and presents examples, primarily from the authors' laboratory, for clinical salivary gland applications shown feasible in animal models (Table 1). The authors believe that the transfer of genes to salivary glands will prove to be a valuable clinical tool within the next 10 to 20 years. However, and importantly, as of mid-2005, there have been no approved clinical trials involving salivary gland gene transfer.

General strategy

Because of their general organizational structure, salivary glands have some remarkable advantages as target sites for in vivo gene transfer. Almost all epithelial cells in these glands are accessible intraorally in a noninvasive manner. The orifices of the excretory ducts of the major salivary glands (parotid, submandibular/sublingual) exit into the mouth, and are visualized clinically. The delivery of gene transfer vectors in animal models by

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Table 1
Clinical applications of salivary gland gene transfer shown feasible in animal model experiments

Application	Animal model	Transgene	Reference
Irradiation damage	rat, minipig	hAQP1	[1,2]
Sjögren's syndrome	mouse	hIL10, hVIP	[3,4]
Oral-GI tract therapeutics	rat	histatin 3	[5]
Systemic therapeutics	rat, mouse	hGH, hEpo	[6–8]

Abbreviations: hAQP1, human aquaporin-1; hEpo, human erythropoietin; hGH, human growth hormone; hIL10, human interleukin-10; hVIP, human vasoactive intestinal peptide.

cannulation of these ducts, such as done during routine contrast radiography (sialography) of salivary glands, is fairly straightforward to accomplish [9,10]. This approach theoretically allows the vectors to reach the luminal membranes of almost all cells present. Additionally, salivary glands are not critical-for-life organs, and can be removed if an unanticipated adverse effect with considerably less morbidity than would occur with a liver or lung. Also, because human salivary glands have a fibrous capsule, undesirable extra-glandular vector dissemination following intraductal delivery can be minimized.

Genes are transferred into cells by way of vectors, which are critically important variables for success (Table 2). In general terms, two types of gene transfer vectors exist, viral and nonviral. Viral vectors are much more efficient at mediating gene transfer, whereas nonviral vectors pose less of a safety risk. For most of the authors' studies with salivary gland gene transfer one of two viral vectors was used, derived from a serotype 5 adenovirus (Ad5) or a serotype 2 adeno-associated virus (AAV2). Presently, no such thing as a perfect gene transfer vector exists and each of these vectors provides certain advantages [9,10].

Table 2
Key gene transfer vectors tested in salivary glands of animal models

Vector	Animal	Gene expression[b]	Clinical potential[c]	Reference
Plasmid[a]	rat	0.5 +[e]	+	[11,12]
Ad5	many[d]	+ + + +[e]	+ +	[13–16]
AAV2	mouse	+ +[e]	+ + +	[6,17]
FIV	mouse	+ +[e]	+	[18]

[a] Note that plasmids (also sometimes called "naked DNA") are a non-viral means of gene transfer and have been used to deliver genes to salivary glands with the help of cationic lipids.

[b] Relative expression of the transgene on a zero (none) to + + + + (excellent) scale.

[c] Assessment of current relative clinical potential on a zero (none) to + + + + (ideal) scale.

[d] Ad5 vectors have been shown effective for salivary gland gene transfer in mice, rats, minipigs, and non-human primates.

[e] Extent of gene expression is transient for plasmids (1–2 days) and Ad5 vectors (7–14 days), long-lived for AAV2 (>1 year; as long as tested) and FIV (>1 year; as long as tested).

Abbreviations: AAV2, serotype 2 adeno-associated virus; Ad5, serotype 5 adenovirus; FIV, feline immunodeficiency virus.

Ad5 vectors are extremely useful to prove a concept for potential clinical applications. Ad5 vectors lead to transduction of (gene transfer to) virtually all types of epithelial cells in salivary glands, and achieve high levels of gene transfer in vivo, (eg, 20%–35% of the cells) [1,19]. However, Ad5 vectors also can have a negative effect after delivery; they elicit a considerable immune response in salivary glands [13,20,21] as in other tissues. This response, overall, results in high level, but transient, expression of the delivered gene (7–14 days) [19]. Nonetheless, studies by Crystal and colleagues [22,23] have examined the safety of local delivery (to several sites) of low and intermediate doses ($\leq 10^{11}$ viral particles) of Ad5 vectors in humans and concluded that at such doses Ad5 vectors are tolerated well [22,23].

The other viral vector that the authors have used frequently for preclinical, animal model salivary gland gene transfer is derived from AAV2. AAV2 vectors are considerably more difficult to construct than Ad5 vectors, in large part because their biology is less understood than that of Ad5 vectors [9,10]. Use of AAV2 vectors, however, results in much longer transgene expression, with substantially less host immune reactivity, than seen with Ad5 vectors [6,17,24]. Importantly, wild type AAV2 is not associated with any known pathology in humans [25]. Consequently, the authors have decided to use Ad5 vectors routinely to demonstrate conceptual feasibility (though for certain short-term studies, Ad5 vectors may have actual clinical utility) and thereafter use an AAV2 (or in the authors' more recent studies other AAV serotypes) vector if long-term expression is required.

To accomplish actual gene transfer in salivary glands, after suspension in a diluent buffer, vector is infused in a retrograde direction through a cannula placed in Stensen's or Wharton's duct [9]. The volume in which the vector is suspended is optimized for the size and gland type being used [11,14]. This optimization seems to be critical for attaining maximal transgene (the transferred gene) expression and use of a suboptimal volume of suspension buffer leads to marked reductions in the level of transgene product (the encoded protein) produced [14,15]. The optimal volume distends the gland considerably but does not lead to loss of glandular integrity. For example, maximal transgene expression in the murine submandibular gland is achieved with a 50 μL infusate volume, whereas in the rat submandibular gland a 150 to 200 μL infusate is required. In the minipig parotid gland, maximal transgene expression occurs with an infusate volume of 4.0 mL. In the human parotid gland, based on the volume of contrast medium used in sialography, it is estimated that the desirable infusion volume is 1.0 mL.

Irradiation damage: using gene transfer to prevent and repair gland dysfunction

Oral and laryngeal cancer will affect 40,000 Americans in 2005 and more than 350,000 new cases will be diagnosed worldwide [26,27]. The current curative treatment modalities consist of surgery and IR. Salivary glands in the

IR field are damaged severely and, consequently, this results in marked salivary hypofunction in 80% of patients [28–31]. Patients experiencing reduced salivary flow suffer considerable morbidity, including dental caries, mucosal infections, dysphagia, and extensive discomfort. Importantly, the ability to optimize cancer treatments because of relative risks for normal tissue injury has significant implications in oncology, because higher doses of radiation might, in some cases, improve local control and survival [32].

The underlying mechanism of IR-induced injury to the salivary glands is still unknown and somewhat enigmatic. Typically, the most radiosensitive tissues, like hematopoietic progenitor cells, are primitive, undifferentiated cells with a high turnover rate. Conversely, salivary gland cells are highly differentiated epithelial cells and characterized by slow cellular turnover. Yet salivary cells show considerable radiosensitivity, including an acute response to IR resulting in changes in the quantity and composition of saliva within 7 days after beginning radiotherapy [33–37]. Whether direct effects of radiation on the salivary acinar or ductal cells cause radiation damage or if the damage is secondary to injury of adjacent tissue (eg, the fine vascular structures), leading to increased capillary permeability, interstitial edema, and inflammatory cell infiltration is unclear [28–31]. No accepted conventional regimen exists to prevent or correct IR-induced salivary gland damage, and, thus, gene therapy offers a potentially novel way to address this condition. Several genes exist that could be used for these purposes. One example of each is discussed below, for prevention and repair of IR-induced salivary hypofunction.

IR-induced reactive oxygen species (ROS) likely contribute in a significant way to the molecular damage mechanism involved in this condition. When IR interacts with a cell, the resultant transfer of energy increases the intracellular concentration of ROS. These molecules include superoxide ions and hydroxyl radicals. Although these highly reactive free radicals have an extremely short half-life, on the order of 10^{-6} seconds [38], they can have tremendously damaging oxidative effects in the cell. The essential mechanism involved in ROS-mediated damage is the redox (reduction–oxidation) reaction. This reaction involves the transfer of electrons between reactants. Reactants that gain electrons are reduced and those that lose electrons are oxidized. The cellular defense against superoxide ions is the enzyme superoxide dismutase (SOD). At least three forms of this enzyme exists in cells; one found in mitochondria (MnSOD), another in the cytoplasm and nucleus, and a third, an extracellular form [39].

In a series of key studies, involving epithelial tissues generally similar to salivary glands biologically, Greenberger and colleagues [40–47] have advocated a prophylactic role for MnSOD gene transfer in the prevention of IR-induced damage. These investigators demonstrated that pretreatment of mouse lung tissue with the MnSOD gene protected lungs from the acute and chronic sequelae of IR, including radiation pneumonitis and organizing alveolitis and fibrosis [40–43]. This group also has demonstrated that

MnSOD gene transfer could prevent IR-induced esophagitis and oral mucositis in mice in vivo [44–47]. These studies, although not conducted on salivary glands, were conducted on similar tissues and are thus instructive. The MnSOD gene transfer results in a down-regulation of several proinflammatory cytokines involved in IR-induced damage. This cytokine down-regulation importantly does not protect orthotopic carcinomas (ie, only normal tissue appears protected from IR damage) [48,49]. Although hydroxyl radicals are likely the primary mediators of oxidative damage to cells, these studies show ample evidence for the benefit of reducing the levels of the superoxide ions for prevention of IR damage to epithelial tissues [50].

The authors' studies have focused on a gene transfer strategy to repair damaged salivary glands following IR. Many surviving former head and neck cancer patients suffer daily from the absence of saliva. To appreciate the rationale behind these studies its important to keep in mind that acinar cells are the only fluid producing cells in the salivary glands, and they are sensitive to IR [51]. Ductal cells, which convey saliva into the mouth, although less sensitive to radiation, are impermeable to water and normally are not considered to be capable of generating salivary fluid flow [9]. Therefore, the loss of acinar cells in a gland will have profound consequences for a patient. Accordingly, the authors have developed a repair strategy designed to permit ductal cells to secrete fluid. The authors hypothesized that by increasing water permeability in surviving cells (presumably mostly ductal) increased fluid secretion would occur. For this purpose the authors and their colleagues constructed a recombinant Ad5 vector (AdhAQP1) encoding the human water channel protein aquaporin-1 (hAQP1) [1]. Water channels allow the rapid movement of water in response to an osmotic gradient across the hydrophobic cell membrane.

Initially, the authors examined rats whose submandibular glands were subjected to a single IR dose of 17.5 or 21 Gy. Three to 4 months after IR, rats were administered a single dose of AdhAQP1, or a control virus, by way of retrograde ductal instillation, and 3 days later, stimulated saliva was collected from all rats. A control virus had no effect on salivary flow and the irradiated rats exhibited marked salivary hypofunction (35% the flow of nonirradiated rats). Conversely, irradiated rats given AdhAQP1 displayed a two- to threefold increase in salivary output above that of irradiated rats given the control virus, approaching salivary flow rates for unirradiated animals treated with the control virus [1].

Subsequently, the atuhors examined the utility of AdhAQP1 for repairing IR damage in nonhuman primates [16]. In this study, one parotid gland of rhesus monkeys ($n = 5$) was irradiated with a single dose of 10 Gy. This IR dose significantly reduced salivary flow in all monkeys [16]. AdhAQP1 was administered intraductally at 19 weeks after IR and salivary secretion examined 3, 7, and 14 days later. The results, however, were inconsistent, and only two of the four AdhAQP1-treated monkeys displayed increased salivary flow rates compared with a single animal administered an irrelevant

virus [16]. Possible reasons for the disparity in results from the rat studies include too few monkeys to permit all desirable control experiments to be performed, an inadequate perfusion of the virus into the primate glands, potential differences between these two animal models in the distribution of viral receptor on the luminal surfaces of gland cells [19], and physiologic differences in the target cells of these two species. However, because of these results, it was unclear if the AdhAQP1 strategy of repairing IR-damaged salivary glands was useful in animals larger than rats.

Therefore, a different, more convenient, and less expensive large animal IR model, the miniature pig (minipig), was developed [15,52]. Using this model, the authors and their colleagues recently evaluated the AdhAQP1-mediated gene transfer strategy after parotid gland IR (20 Gy) [2]. Sixteen weeks following IR, salivation from the targeted gland was decreased by 80%. AdhAQP1 administration resulted in a dose-dependent increase in parotid salivary flow to 80% of pre-IR levels on day 3. A control virus had no significant effect on irradiated minipig parotid flow rates. The effective AdhAQP1 dose was one that leads to comparable transgene expression in murine and minipig salivary glands [15]. Furthermore, 3 days after AdhAQP1 administration little change was observed in clinical chemistry and hematology values in the treated minipigs. Together, these findings demonstrate localized delivery of AdhAQP1 to IR-damaged salivary glands can lead to increases of salivary secretion, without significant general adverse events, in a large animal model, and suggest that the AdhAQP1 strategy may be useful clinically. Based on these results the authors have proposed the use of AdhAQP1 to the FDA for a trial in patients who present with IR-induced parotid hypofunction, and the authors are now developing the clinical protocol for regulatory review.

Sjögren's syndrome

SS is an autoimmune disease, of unclear etiology, characterized by a focal and diffuse lymphoid cell infiltration into the salivary and lacrimal glands (autoimmune exocrinopathy) [53,54]. This chronic immune cell activation leads to reduced secretory function with resulting symptoms of xerostomia (dry mouth) and keratoconjunctivitis sicca (dry eyes). Although SS is characterized by the presence of chronic sialadenitis and dacryoadenitis, many other tissues (eg, the lungs and nervous system) may be involved [53,54]. SS may occur alone (termed primary SS) or develop in association with other autoimmune rheumatic diseases, such as rheumatoid arthritis and systemic lupus erythematosus (termed secondary SS). As in most autoimmune diseases, a sexual dimorphism exists in the prevalence of SS with women affected in greater frequency than men (9:1). One to 2 million persons in the United States are effected.

Although the pathogenesis of SS remains unclear, it has been proposed that a combination of immunologic, genetic, and environmental factors

may play important roles in the development of autoimmune reactivity. The glandular lymphocytic infiltrates consist of T cells (up to 80%) and less B cells and plasma cells (20%) [55]. SS is associated with the production of autoantibodies also and these likely reflect B-cell activation and a loss of immune tolerance in the B cell compartment. Several nonorgan specific autoantibodies are important in SS (eg, anti-Ro/SSA, anti-La/SSB, rheumatoid factor, and anti-α-fodrin). Antimuscarinic receptor antibodies seem to be organ-specific autoantibodies that may be important in understanding the pathogenesis of impaired glandular function in SS [56,57].

T helper cell type 1 (Th1) and T helper cell type 2 (Th2) cytokine profiles have been studied in blood, saliva, and salivary gland tissues from patients who have SS [58,59] and from autoimmune mice [60]. Th1 cells produce interleukin (IL)-2, interferon gamma (IFN-γ), and lymphotoxin, all of which are associated with cell-mediated immunity [61]. Th2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13, which stimulate humoral responses [62]. Cytokines are expressed by lymphocytes infiltrating the salivary glands of patients who have SS. Th2 cytokines are predominant in the early phase of SS, whereas a shift toward Th1 cytokines is associated with advanced lymphocytic infiltration at a later stage of the disease [63].

Apoptotic (cell death) pathways may play important roles for the pathogenesis of SS. For example, Kong and colleagues [64] and Matsumura and colleagues [65] have reported that ductal and acinar cells, and some lymphocytes, undergo apoptosis. In other studies, it has been suggested that perforin and granzyme B from cytotoxic T-lymphocytes are important for the apoptotic pathogenesis of SS [66]. Several generally expressed autoantigens, such as α-fodrin, and tissue-restricted autoantigens, such as the muscarinic receptor isoform 3, which are targeted in SS, are cleaved specifically by granzyme B [67]. Thus, apoptosis plays an important role in the pathogenesis of SS.

At present, treatment of SS is essentially palliative. Artificial salivas are used to improve oral dryness and to prevent dental disease, with minimal success. Pilocarpine and civemilene are muscarinic receptor agonists that are used to stimulate salivary secretions in patients with remaining parenchymal tissue. These treatments help to improve symptoms locally [68], but these are not useful for managing the immune features of SS. Develop new treatment strategies for SS is important. The authors and their colleagues have proposed using local gene transfer for managing the salivary component of SS [3,69].

AAV2 vectors have been used for in vivo gene transfer in various autoimmune diseases, including SS and rheumatoid arthritis. AAV2 vectors can infect dividing and nondividing cells and lead to stable transgene expression. Further, as noted earlier, AAV2 vectors result in a modest host immune response. In aggregate, AAV2 vectors seem to be useful for gene transfer in SS [3,69]. However, a single transgene is difficult to identify as useful for correcting the pathology in SS because the pathogenesis of SS is unclear at present. Based on several immunologic characteristics described earlier, there are immunomodulatory molecules that might be useful for

local gene transfer to salivary glands [69]—certain cytokines or factors affecting apoptosis, for example.

Although the roles of individual cytokines in the pathogenesis of SS still have not been established clearly, the proinflammatory cytokines probably stimulate cytotoxic T cell processes within the gland. Previously, the authors' laboratory reported that transfer of the human interleukin (IL)-10 gene into salivary glands using an AAV2 vector was effective in preserving salivary flow and reducing the autoimmune sialadenitis in the nonobese diabetic (NOD) mouse that is a model of SS [3]. IL-10 is a homodimeric cytokine with a wide spectrum of immunosuppressive activities. In these studies, NOD mice were treated with an AAV2 vector encoding human IL-10 or a control protein (β-galactosidase) by retrograde submandibular ductal administration at 8 weeks (early, before onset of sialadenitis), or at 16 weeks (late, after onset of sialadenitis). At 20 weeks, salivary flow rates of early and late hIL-10 gene-treated mice were significantly higher than salivary flow rates of control vector-treated mice. Importantly, inflammatory infiltrates (focus scores) in the submandibular glands were reduced significantly in hIL-10 treated NOD mice [3].

Many recognized inhibitors of apoptosis exist and some may be useful for gene transfer to salivary glands in SS. In the salivary glands of SS patients, Bcl-2 (B-cell leukemia/lymphoma-2) and Bcl-x (B-cell leukemia/lymphoma-x) are expressed preferentially in infiltrating mononuclear cells rather than in the acinar and ductal epithelial cells of minor salivary glands. In contrast, acinar and ductal epithelial cells from SS patients express the X-chromosome–linked inhibitor of apoptosis protein (XIAP), a member of the IAP family that inhibits caspase-7 and caspase-3 activation by blocking cytochrome c-induced activation of pro-caspase-9 [70]. Preventing apoptosis may be possible in salivary gland cells through transfer and overexpression of the XIAP (or some related) gene. The authors' laboratory has begun studies to test this hypothesis.

Because the pathogenesis of SS is not understood at present, these treatment strategies must be considered speculative. However, the results obtained in early studies [3] suggest that a local immunomodulatory strategy for SS may be beneficial, though considerably more animal model study is needed before commencing any clinical testing.

Protein secretion pathways in salivary cells

Although salivary glands are considered to be classic exocrine glands, they can secrete proteins in exocrine (to saliva) and endocrine (to the bloodstream) directions. This characteristic is valuable for the two specific gene therapeutics applications mentioned in earlier discussion (see Table 1) and described below: oral/GI tract and systemic. To appreciate how salivary glands can be employed to use genes encoding secreted proteins as drugs, it is important to understand how proteins are secreted from salivary cells. Studies in animal

models show that there are at least two general pathways by which protein secretion occurs in salivary cells: a *constitutive* pathway in which certain proteins are secreted continuously from cells at the rates at which they are synthesized and a *regulated* pathway in which secretory proteins are first stored in vesicles within the cells awaiting an extracellular signal for secretion [71–73]. Typically, in cells all over the body, constitutive pathway secretion occurs in a random manner directionally (ie, with an equal probability of the protein crossing all membrane surfaces in a cell), whereas regulated pathway secretion occurs in highly differentiated cells and in a directional manner [71]. In salivary glands, protein secretion by way of the regulated pathway goes across the apical membrane into the forming saliva, whereas most constitutive pathway protein secretion occurs across the basolateral membranes (the largest membrane surface in epithelial cells) in an endocrine manner toward the interstitium and bloodstream [72,74]. Proteins secreted by way of these two different pathways are sorted or segregated in the trans-Golgi network soon after they are synthesized. This sorting is based on specific amino acid sequences of the protein that in effect form a ''zip code'' directing the cell to deliver them in one or the other manner [75–77]. Classic cell biological studies by Kelly and colleagues [71,78] in the 1980s showed that regulated and constitutive pathway proteins from one cell type are handled in a similar manner when expressed in other cell types.

Because of these unique protein chemical and cell biological characteristics, the transfer of genes encoding proteins secreted by way of the regulated or constitutive pathway can lead to therapeutic proteins being secreted into saliva for delivery to the oral cavity and upper GI tract or into the bloodstream for systemic delivery. The notion of endocrine secretion by salivary glands was suggested as early as the 1950s [79–81], and subsequently described numerous times, eg, a parotid hormone in pigs [82] and glucagon in several species (rat, mouse, rabbit, human) [83]. However, despite accumulating evidence, a role for endocrine secretion in salivary gland physiology is neither widely recognized nor appreciated [84,85].

The authors' laboratory has shown numerous proofs of concept for the secretion of transgenic proteins by way of these two pathways in experimental animals. For example, growth hormone (GH), an endocrine protein normally secreted into the bloodstream by way of the regulated pathway in anterior pituitary somatotrophs, is secreted from salivary glands into saliva [7,72]. Conversely, erythropoietin (Epo), which is secreted by way of the constitutive pathway by kidney epithelial cells, is secreted by this same pathway from salivary cells leading to its' secretion primarily into the bloodstream [6,8]. The former presents a significant but not insurmountable problem for using salivary glands as a surrogate endocrine gland to correct a deficiency in GH or other regulated pathway proteins (ie, GH secreted into saliva is wasted therapeutically) [86]. However, salivary glands should be useful readily as a therapeutic site for correcting deficiencies in constitutive pathway secretory proteins, such as occur in patients with Epo-responsive

anemias as a result of chronic renal failure [6]. As is described below, salivary glands can serve as endogenous bioreactors making therapeutic proteins for exocrine (oral/GI tract) and endocrine (systemic) purposes while using classical pharmacologic principles.

Oral/gastrointestinal tract gene therapeutics

Salivary glands are particularly useful target sites for the delivery of therapeutic genes encoding exocrine proteins for use pharmacologically in the oral cavity and the upper GI tract. Saliva continuously covers these tissues and salivary glands normally secrete into saliva many physiologically beneficial proteins that help maintain tissue integrity [87]. Unfortunately, few studies have examined potential salivary gland exocrine gene-therapeutic applications. Early experiments from the authors' own laboratory showed that transgenic proteins could be secreted at significant levels into saliva for therapeutic purposes. Specifically, these studies showed that transfer of the gene for histatin 3, an anticandidal peptide that normally is found in the saliva of old-world primates and humans, could be expressed in rat salivary glands following gene transfer with an Ad5 vector [5]. Clinically, oral mucosal candidiasis is a common opportunistic infection seen in immunosuppresed patients, and its' management is increasingly difficult with the appearance of drug (eg, azole derivatives)-resistant candidal species. The transgenic histatin 3 produced experimentally in rat saliva was effective in killing azole-resistant Candida albicans [5].

Many other naturally occurring antimicrobial peptides exist that might be useful clinically against antibiotic resistant microorganisms, including the defensins and magainins [88,89]. Although these peptides seem useful therapeutically, concern exists because of their potential toxicity with systemic use [90]. However, this toxicity is unlikely a concern with oral/GI tract gene therapeutics because of the concentrated local bioavailability. This subject is ripe for investigation, particularly because of the morbidity from emerging antibiotic resistant bacteria in the oropharyngeal region [91].

A second potential application for local oral/GI tract gene therapeutics is to promote mucosal wound healing. Severe mucosal ulcerations (eg, in patients who have Bechet's syndrome) or in patients receiving cancer treatment (radiation or chemotherapy), are painful, and clinically difficult to manage [92]. In various protein-therapeutic studies, certain growth factors (eg, epidermal growth factor, keratinocyte growth factor) and cytokines (eg, interleukin-11) improve mucosal wound healing [93–95]. However, to be useful therapeutically, the proteins must be applied to mucosal tissues reasonably often and in fairly high concentrations [93]. Conversely, a gene transfer approach could provide continuous local expression of the protein after salivary gland delivery and theoretically be more effective and less expensive. Indeed, it seems that therapeutically necessary concentrations of transgenic

secreted proteins in saliva can be achieved following salivary gland gene transfer [5,96].

An important concern for all gene therapeutics (oral/GI tract and systemic) applications, however, is that almost all vectors used for preclinical studies, and all vectors thus far used clinically, lead to the continuous production of the encoded therapeutic proteins [97]. This production may be desirable for some situations, but it is unlikely to be suitable generally. Transgene expression ideally should be regulated, leading to expression of the therapeutic protein as clinically required [97,98]. Although many regulatory systems have been used in preclinical studies, none has yet been approved for clinical use. The rapamycin inducible system [97,98] has been used to control the expression level of a model exocrine protein secreted from salivary glands [96]. By varying the time and dose of rapamycin, tight control of protein expression can be obtained in saliva, with no detectable transgenic protein expression in the absence of the drug. Furthermore, regulation occurred repeatedly over a 2-week period, consistent with the time-course required in typical conventional therapy for oral infections and ulcers. Certainly, some type of regulation controlling the production of the transgenic protein is essential for the wide general use of salivary glands, or other tissues, for gene therapeutics.

Systemic gene therapeutics

Salivary glands at present are considered potentially excellent targets for gene therapeutics applications in many monogenetic, single endocrine protein deficiency disorders [6,8,84]. Because salivary glands exhibit the constitutive and regulated secretory pathways, they can be used for conveying both types of expressed transgenic secretory proteins [9,72]. As discussed earlier, transgene products in salivary glands continue to use their normal physiologic secretion routes. Proteins that are secreted constitutively in their primary site of production continue to use this route when expressed as a transgene product in salivary glands, and such proteins are predominantly secreted into the bloodstream (ie, in an endocrine manner). Thus, salivary glands may be useful especially as a surrogate endocrine gland for certain diseases in which the deficient protein is normally secreted by way of the constitutive pathway [8].

In 1996, the authors' laboratory reported the first unequivocal demonstration of the secretion of a transgene product from salivary glands into the bloodstream [99]. Human α1-antitrypsin (hα1AT), which is normally secreted in the liver by way of the constitutive pathway, was encoded in an Ad5 vector and delivered to rat submandibular glands. The levels of hα1AT in the bloodstream were four- to fivefold greater than in the saliva [99]. More recently, the authors' laboratory has studied endocrine secretion from salivary glands using a transgene encoding human Epo (hEpo) [6,8].

Physiologically, as discussed above, hEpo is secreted by way of the constitutive pathway. Using an AAV2 vector encoding hEpo, the authors observed stable production of hEpo from mouse salivary glands and its secretion into the bloodstream for more than 1 year, along with associated elevations in hematocrit values [6,8]. The salivary hEpo levels in these studies were 10% of those in the serum. Using various transgenes encoding proteins secreted by the constitutive pathway, it seems that salivary glands readily can achieve circulating levels of transgene products up to 5 ng/mL. Such levels are therapeutically adequate for treating an Epo-responsive anemia but not for treating emphysema caused by a deficiency of α1AT, where levels of greater than 100 μg/ml are required.

As discussed above, administration of vectors encoding GH to salivary glands leads to secretion of GH mainly into saliva; 10 to 20 fold higher levels than that found in the bloodstream. Nonetheless, the GH levels in serum can be sufficient therapeutically [7,8,86]. High levels of GH are produced by salivary cells, saturating entry into the regulated pathway with the excess GH being secreted by way of a constitutive route into the bloodstream. However, because most of the GH produced is being secreted into saliva, it is therapeutically inefficient. In order for salivary glands to be useful as a gene transfer target site for systemically required regulated pathway proteins, such as GH, or other neuroendocrine hormones, some redirection of this type of secretory protein is needed. The authors' laboratory has addressed this concern by using two strategies. With the first approach, studies have tried to alter the specific sorting signal for human GH (hGH) entry into the regulated pathway, so that instead most hGH would exit the salivary cells by the constitutive pathway [100]. This strategy has yielded modest success, likely because the exact sorting signal for hGH is not fully known [100]. With the other strategy, studies have used hydroxychloroquine (HCQ; Plaquenil), a commonly used antimalarial and antirheumatic drug. HCQ disrupts regulated pathway sorting by alkalinizing transport vesicles in the trans-Golgi network, resulting in a mis-sorting of hGH [86]. HCQ treatment in rats leads to a considerable shift in hGH secretion from salivary glands by way of the constitutive pathway, yielding hGH serum levels $\sim$30 times those required therapeutically. Although both strategies have shown utility in animal studies, considerably more research is required before any human clinical testing.

Summary

Salivary glands have proven to be unusual but valuable target sites for multiple clinical gene transfer applications. Access to salivary glands for gene transfer is easy, and multiple studies in animal models have yielded proofs of concept for novel treatments for damaged salivary glands following therapeutic IR, in SS, and for gene therapeutics systemically by way of the bloodstream and locally in the oral cavity and upper GI tract.

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