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Review

Transfer of the AQP1 cDNA for the correction of radiation-induced salivary hypofunction

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Abstract

The treatment of most patients with head and neck cancer includes ionizing radiation (IR). Salivary glands in the IR field suffer significant and irreversible damage, leading to considerable morbidity. Previously, we reported that adenoviral (Ad)-mediated transfer of the human aquaporin-1 (hAQP1) cDNA to rat [C. Delporte, B.C. O'Connell, X. He, H.E. Lancaster, A.C. O'Connell, P. Agre, B.J. Baum, Increased fluid secretion after adenoviral-mediated transfer of the aquaporin-1 cDNA to irradiated rat salivary glands. Proc. Natl. Acad. Sci. U S A. 94 (1997) 3268-3273] and miniature pig [Z. Shan, J. Li, C. Zheng, X. Liu, Z. Fan, C. Zhang, C.M. Goldsmith, R.B. Wellner, B.J Baum, S. Wang. Increased fluid secretion after adenoviral-mediated transfer of the human aquaporin-1 cDNA to irradiated miniature pig parotid glands. Mol. Ther. 11 (2005) 444-451] salivary glands ~16 weeks following IR resulted in a dose-dependent increase in salivary flow to ≥80% control levels on day 3. A control Ad vector was without any significant effect on salivary flow. Additionally, after administration of Ad vectors to salivary glands, no significant lasting effects were observed in multiple measured clinical chemistry and hematology values. Taken together, the findings show that localized delivery of AdhAQP1 to IR-damaged salivary glands is useful in transiently increasing salivary secretion in both small and large animal models, without significant general adverse events. Based on these results, we are developing a clinical trial to test if the hAQP1 cDNA transfer strategy will be clinically effective in restoring salivary flow in patients with IR-induced parotid hypofunction.
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Keywords: Gene therapy; Adenoviral vector; Aquaporin-1; Salivary gland; Radiation damage; Animal model; Clinical trial

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1. Introduction

Each year ~30,000–40,000 patients develop head and neck cancer in the USA, with ~400,000 cases worldwide [1,2]. The treatment for the vast majority of such patients in industrialized countries includes ionizing radiation (IR) [3,4]. A significant side effect of IR treatment is damage to normal tissues in the IR field [5], which can lead to reduced quality of life and significant disability in head and neck cancer patients [4,6]. In particular, salivary glands in the IR field can be severely damaged, resulting in dramatic reductions in saliva production and considerable morbidity [3,7–11]. Although the exact mechanism for IR-induced salivary gland damage remains somewhat enigmatic [12], fluid-secreting acinar cells are lost, and relatively water-impermeable duct cells survive [7–9]. Saliva is critical for the physiology and protection of upper gastrointestinal (GI) tract tissues, providing critical antimicrobial, lubricatory and reparative functions [13]. Following IR treatment, patients experience oral infections, such as candidiasis and rampant dental caries, mucositis, dysphagia and frank discomfort leading to a marked decline in quality of life [3,4,10]. There is no adequate conventional therapy available to correct IR-induced salivary hypofunction and most treatment options are essentially palliative in nature [8,9,14]. It was the absence of a conventional therapy for IR-induced salivary hypofunction that initially prompted our efforts to utilize gene transfer [15,16].

Salivary glands are an unusual but appealing target site for in vivo gene transfer [17–19]. The duct orifice of each major salivary gland (parotid, submandibular–sublingual) is readily visible in the mouth. This orifice is directly contiguous with virtually every epithelial cell in the gland via the duct lumen [17,19]. Additionally, salivary glands are a site of considerable protein synthesis, albeit for exocrine purposes normally. Transgene expression in these glands after administration of a first-generation recombinant serotype 5 adenoviral (rAd5) vector generally follows the distribution of α_v, $\beta_{3/5}$ integrins along acinar and duct cell luminal membranes, and almost all cell types can be transduced [20]. Many different types of transgene products have been expressed in various cell lines, including a salivary epithelial cell line; all of which were correctly targeted to their appropriate subcellular location [21]. This suggests that appropriate targeting likely will occur in native tissues. Our aggregate studies clearly show that first generation rAd5 vectors can readily infect and direct transgene expression in mammalian salivary glands [17,19].

To address IR-induced salivary hypofunction, we hypothesized that the critical element needed to provide upper GI tract protection and lubrication is water, reasoning that adequate water secretion by surviving epithelial cells within the gland would dissolve the protective exocrine proteins produced by undamaged duct cells and remaining acinar cells, and deliver them to the upper GI tract [22]. Although the physiology of normal salivary duct cells is not well understood, based on available evidence, we postulated that after IR duct cells could generate an osmotic gradient (lumen>interstitium) allowing fluid secretion if a facilitated water permeability pathway was introduced [22]. We therefore hypothesized that increasing the water permeability of surviving salivary duct cells via transfer of the human aquaporin-1 (hAQP1) cDNA could accomplish this [22–24]. To achieve gene transfer, we constructed a rAd5 vector encoding hAQP1. This brief review describes our efforts to move AdhAQP1 (Fig. 1) from the laboratory bench to clinical trials with patients suffering from IR-induced parotid gland hypofunction.

2. Vector construction and in vitro efficacy studies

To construct AdhAQP1 (Fig. 1, [22]), the hAQP1 cDNA [23] was cloned directly into the Ad5 shuttle vector pACCMV-pLpA (a gift from Dr. C. Newgard, University of Texas, Southwestern Medical Center [25]). This plasmid contains part of the Ad5 genome (0–1.2 and 9.2–17 μm) with the human cytomegalovirus immediate early promoter/enhancer substituted between map units 1.3 and 9.1 (Ad5 E1 region; Fig. 1). PCR amplification of the hAQP1 cDNA (~3 kb) was performed using specific primers containing *Kpn*I and *Bam*HI restriction sites for cloning [22]. The resultant plasmid, termed pCMV-hAQP1, was co-transfected with the Ad5 plasmid pJM17 into 293 cells as described [25], to yield AdhAQP1. This vector, with subsequent plaque-purifications, has been used in all studies described herein, including in vitro cell culture and in vivo animal model experiments. This vector will also be produced at a clinical grade for the planned clinical studies described below.

Initial in vitro characterization of AdhAQP1 used three epithelial cell lines that do not endogenously express hAQP1;

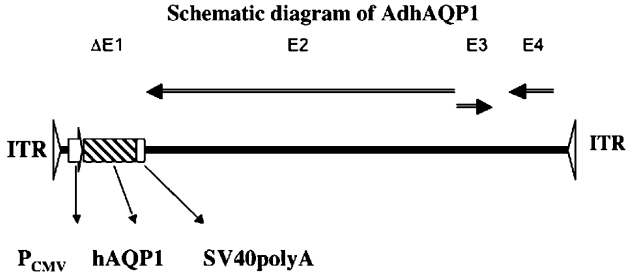

Fig. 1. Schematic diagram of the structure of AdhAQP1. The following abbreviations are used: ITR, inverted terminal repeat; P_{CMV}, cytomegalovirus immediate early promoter/enhancer; hAQP1, human aquaporin-1 cDNA; SV40 polyA, Simian Virus 40 polyadenylation signal; ΔE1, deletion of adenoviral E1 sequences; E2, adenoviral E2 genes; E3, adenoviral E3 genes; E4, adenoviral E4 genes.

293, MDCK and SMIE cells [22]. Following infection with AdhAQP1, Western blot analyses of crude membranes prepared from all cell lines were positive for the presence of hAQP1. Thereafter, we evaluated the cellular distribution and function of the transgenic hAQP1 by infection of a polarized monolayer of MDCK cells with AdhAQP1. The transgenic hAQP1 could be detected uniformly distributed around the basolateral and apical cell membranes of MDCK cells by confocal microscopy and mediated a 4-fold increase in osmotically-obliged net fluid secretion across both MDCK and SMIE monolayers (e.g., see Fig. 2; [21,22]).

In order to study transgenic hAQP1 physiological function, we used the SMIE cell line, which provides an in vitro model of a polarized salivary epithelium, and has been characterized in detail [21]. We extensively tested the relationship between AdhAQP1 vector- mediated expression of hAQP1 and fluid movement across this model epithelium [24]. To do this, we infected polarized SMIE cell monolayers grown in Transwell-Col (polycarbonate filters coated with collagen) chambers with AdhAQP1 at different multiplicities of infection (MOIs; defined for in vitro studies as plaque-forming units [pfu]/cell) and subsequently examined fluid movement in response to an osmotic gradient (440 to 340 mosm; apical to basal). Osmotically obliged fluid movement occurred rapidly in response to imposition of the gradient 24-h after infection with the vector at a MOI of 5. Fluid movement was linear for 15–30 min [24], with an initial rate of ~2–3 μL/min, which was much greater than that of uninfected cells (~0.1 μL/min), and also was proportional to the osmotic gradient applied [24].

Furthermore, both the expression of hAQP1 in SMIE cell membranes and fluid movement were AdhAQP1 dose-dependent (0–10 MOI). A MOI of as little as 0.1 led to detectable expression by Western blotting, which increased ~80–100 fold at the highest MOIs (5–10) [24]. Importantly, at relatively low vector doses (and hAQP1 expression levels) fluid movement was disproportionately high, being linear between 0–1.0 MOI, while thereafter it began to plateau and changed little (<5%) between a MOI of 5 and 10. An implication of these latter results is that it could be unnecessary to achieve high hAQP1 protein expression levels in transduced salivary epithelial cells in vivo for correction of IR-induced salivary gland hypofunction.

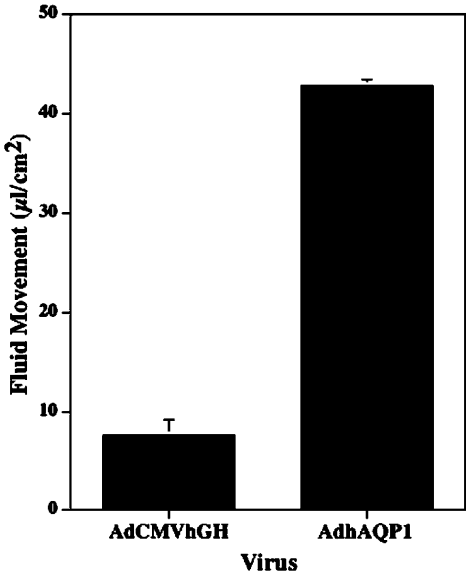

Fig. 2. Effect of AdhAQP1 infection on net fluid movement across SMIE cells. Data shown are the mean±S.E.M. of experiments reported in He et al. [21]. SMIE cell monolayers were either infected at a MOI=5 with AdhAQP1 or AdCMVhGH (encoding human growth hormone), and 24 h later transepithelial fluid movement was measured for 60 min.

3. In vivo efficacy studies

Initially, we studied rats whose submandibular glands were subjected to a single IR dose of 21 Gy [22]. Four months following IR rats exhibited a dramatic reduction in salivary flow and were administered either a single dose (5×10^9 pfu/gland; ~100 vector particles:pfu; in 200 μL, i.e., 2.5×10^7 pfu/μL) of AdhAQP1, or a control virus (Addl312; no encoded transgene), via retrograde submandibular duct instillation. Three days later, stimulated saliva was collected from all rats following a pilocarpine (parasympathomimetic) stimulus. The control virus had no beneficial effect on salivary flow (Table 1) and the control virus-treated irradiated rats exhibited marked salivary hypofunction (~35% of the salivary flow of sham-irradiated rats; not shown). Conversely, rats given AdhAQP1 (±IR) displayed salivary flow rates approaching those of sham-irradiated animals treated with a control virus [22].

We next examined the utility of AdhAQP1 for repairing IR damage in non-human primates [26]. One parotid gland of five 8–10 kg rhesus monkeys was irradiated with a single dose of 10 Gy. This dose significantly reduced salivary flow in all monkeys (~50–90%; [26]). AdhAQP1 (either 2×10^9 or 1×10^8 pfu/gland in 0.5 mL) was administered intraductally at 19 weeks post-IR and salivary secretion examined 3, 7 and 14 days later. Note that the choice of a 19-week time point between IR and vector delivery, versus the similar ~16-week intervals used in the rat and minipig (below) studies, was made for experimental convenience. The results, however, were inconsistent, and only 2 of the 4 AdhAQP1-treated monkeys displayed any increase of salivary flow rates compared to the single animal administered an irrelevant virus [26]. Possible reasons for the disparity from the rat studies include an inadequate perfusion of the virus into the primate glands, differences between these animal models in the distribution of viral receptor on the luminal surfaces of gland cells, as well as physiological or pathophysiological differences in the target cells. Subsequently, we repeated studies in irradiated rats and generally confirmed the efficacy of the AdhAQP1 vector in restoring salivary fluid output [22,27]. Thus, following the non-human primate study, it was not clear whether the strategy of hAQP1 cDNA transfer would be useful for correction of existing IR damage in salivary glands of animals larger than rats.

In order to test further whether AdhAQP1 would be effective at improving salivary secretion in irradiated salivary glands of a large animal, we chose to evaluate a different model, the miniature pig (minipig; ~35 kg), which is more readily, and affordably, studied [28,29]. Sixteen weeks following IR (20 Gy) salivation from the targeted parotid gland was

Table 1
Effect of AdhAQP1 on salivary secretion in irradiated animals[a]

Species	Vector	Salivary flow (% control[b])
Rat	AdhAQP1	83.6
	Addl312[c]	36.1
Minipig	AdhAQP1	81
	AdCMVLuc[d]	30

[a] This table is a summary of data previously reported in Delporte et al. [22] and Shan et al. [30]. For the rat experiments, animals received 21 Gy, while the minipigs received 20 Gy, each in a single dose. The data shown are the average percent of control salivary flow results seen three days following vector delivery. 100% would be equivalent to control (i.e., normal) salivary flow.
[b] Control data in the rat experiments were derived from animals that were not irradiated but infected with a control vector. Control data in the minipig experiments were derived from the pre-irradiation salivary flow rates in the same animals.
[c] Addl312 is a rAd5 vector without any encoded transgene.
[d] AdCMVLuc is a rAd5 vector encoding firefly luciferase.

decreased by >80% [30]. Administration of AdhAQP1 resulted in a dose-dependent increase in parotid salivary flow to ~80% pre-IR levels on day 3 (Table 1; [30]). A control rAd5 vector encoding firefly luciferase was without significant effect on salivary flow. The effective dose of AdhAQP1 used was 10^9 pfu /gland, equivalent to 2.5×10^5 pfu/μL infusate, a dose leading to a comparable (scaled) transgene expression in murine and minipig salivary glands [28]. It is noteworthy that this effective dose was only 20% of the total dose shown to be effective in our earlier irradiated rat studies [22].

The only minipig parotid cell types that were found to express the transgenic hAQP1 in immunohistochemical studies were intralobular and interlobular ductal cells. No evidence was found for AdhAQP1-mediated transgenic hAQP1 expression in surviving parotid acinar cells. Our previously reported studies in non-irradiated minipigs [28] showed that the volume in which we infused administered vector (4000 μL) was sufficient to permit reasonable vector contact with acinar cells and indeed those studies clearly demonstrated that rAd5 vectors could transduce minipig parotid acinar cells [28]. In irradiated rat submandibular glands [22], AdhAQP1 administration can effectively result in transduced acinar cells. Since the hAQP1 transgene was expressed only in parotid ductal cells, the implication of our minipig study is that the increased salivary secretion observed was likely due to enhanced water permeability in the normally water-impermeable duct cells [30].

The specific mechanism (osmotic gradient) by which fluid secretion occurred from the hAQP1-expressing duct cells has not been established, however. We hypothesized, based on the irradiated rat study [22], that AdhAQP1 likely elicited fluid secretion from ductal cells via a $KHCO_3$-generated gradient (lumen>interstitium). Our minipig study suggests this mechanism is still possible, but we have yet to account for all of the required osmolytes [30]. Although the osmotic mechanism is not clear, our aggregate findings in minipigs demonstrated that localized delivery of AdhAQP1 to IR-damaged parotid glands is effective in increasing salivary secretion in a large animal model, without any significant general adverse events (see below), and lend support for the notion that hAQP1 cDNA transfer may be clinically useful for patients with IR-induced salivary hypofunction.

4. Pre-clinical toxicology studies

To advance this potential treatment into clinical trials, in addition to efficacy studies, it is necessary to conduct detailed toxicological and bio-distribution studies to ensure patient safety. GLP is an acronym for Good Laboratory Practice, a defined level of conduct for non-clinical studies established by the US Food and Drug Administration (FDA; [31]). Accordingly, soon after we initially demonstrated that AdhAQP1 led to increased salivary flow in irradiated rats [22], we began to conduct both non-GLP and GLP-level studies with AdhAQP1 and related first generation rAd5 vectors [32–34]. Herein, we use the term non-GLP to identify high quality laboratory research studies. Good Manufacturing Practice (GMP) is the level of conduct required for materials intended for use in human (clinical) studies [35].

4.1. Non-GLP studies with AdhAQP1

All of the non-GLP level safety studies that we have conducted so far with any rAd5 vector have indicated the same conclusion regardless of species tested; evidence of local, dose-dependent vector related inflammatory changes in the target gland, but no evidence for significant systemic manifestations of vector delivery to salivary glands [26,28,32]. A detailed description of rAd5 vector-induced inflammatory changes in salivary glands was previously reported [36]. Initially, we conducted a study examining the safety of AdhAQP1 administration to rat submandibular glands [32]. In that study, we administered either buffer, a control rAd5 vector not encoding a transgene (Addl12) or AdhAQP1, at a high vector dose, 5×10^9 pfu/gland (i.e., the dose used in rat efficacy studies [22]), to male Wistar rats (250–300 g). Animals were followed for up to 9 days, and detailed serum chemistries and a complete gross and limited (liver, spleen, pancreas, lung, kidney, stomach) microscopic necropsy were performed after sacrifice. On days 1 and 9, most clinical chemistry (blood urea nitrogen, creatinine, total protein, albumin, total bilirubin, cholesterol, calcium, sodium, globulin) and hematology (hemoglobin, hematocrit, red blood cells, mean corpuscular volume, mean corpuscular hemoglobin concentration) values were not different between buffer- and AdhAQP1-treated rats. However, on days 1 and 9 when compared to the buffer-treated rats, systemic evidence of inflammation (i.e., increased levels of lactate dehydrogenase and white blood cells) was seen, after AdhAQP1 delivery to salivary glands at this high vector dose.

The non-GLP level study performed in non-human primates, while equivocal with respect to AdhAQP1 efficacy, as described above, indicated that administration to parotid glands of either 2×10^9 or 1×10^8 pfu/gland AdhAQP1 was without untoward local (salivary) or systemic (serum chemistry, hematology) effects [26]. Similarly, our non-GLP level studies with rAd5 vectors in minipigs (described above; [28,30]) showed that all animals remained healthy and exhibited no clinically significant

adverse reaction, locally or generally, after administration to their parotid glands. As in rat studies, minipig parotid gland tissue samples revealed the presence of a dose-dependent inflammatory response [28]. While there were several transient alterations in clinical laboratory parameters after rAd5 vector delivery to minipig parotid glands, we found no significant differences between the values measured before rAd5 vector delivery and those after 14 days; essentially all clinical chemistry and hematology parameters were within normal limits [28]. For these studies, the analyses performed were extensive and included serum calcium, potassium, sodium, chloride, glucose, total protein, albumin, globulin, aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, blood urea nitrogen, alkaline phosphatase, and creatinine. Hematology included the number of white blood cells, red blood cells and concentration of hemoglobin present. All minipigs could tolerate a rAd5 dose of 2×10^9 pfu (i.e., vector administered to both glands) without clinically untoward effects.

4.2. GLP studies with rAd5 vectors

Two GLP-grade toxicology and biodistribution studies have thus far been completed using rAd5 vectors and previously reported (Table 2) [33,34]. In the first study, groups of fifteen male and fifteen female Wistar rats were administered, via the right submandibular duct, a single dose, either 10^6, 3×10^7 or 10^9 pfu (~100:1 particle: pfu), of a rAd5 vector, AdCMVH3, identical to AdhAQP1, except for the encoded transgene (histatin 3; H3). An additional group of fifteen males and fifteen females were administered virus dilution buffer. Animals were observed for up to 15 days for clinical signs of toxicity, and body weights and food consumption were measured. An extensive and specialized PCR necropsy was performed for scheduled sacrifices [33] and collected tissues were either snap frozen in liquid nitrogen for PCR analysis or fixed in 10% formalin for histopathological evaluation.

All animals survived to their scheduled sacrifice and showed no unusual clinical signs or effects on body weight or food consumption (Table 2). Potential rAd5 vector-related changes were observed during gross and microscopic evaluation of the submandibular/sublingual salivary glands from male and female rats from the high dose group at all time points and were similar to those reported by us earlier [36]. The "no observed effect level" was determined to be 10^6 pfu for both male and female animals. All other lesions observed microscopically were considered to be incidental findings. Additionally, we found little change in the extensive set of serum chemistry and hematology parameters that we measured between the various animal groups studied. As in our non-GLP level studies, we found in this GLP level study that rAd5 vector was without significant general adverse effect even at absolute doses ≥ those planned for future clinical use.

Using a PCR assay (able to detect 100 viral particles/0.5 μg extracted DNA), various organs, as well as oral tissues, were tested for the presence of AdCMVH3. At the 15-day time point, no virus was detectable in the tissues of any of the control animals. Virus was also undetectable in the gonads of animals in all groups at day 15. Occasional positive results were seen in organs other than salivary glands or oral tissues, most commonly at the highest vector dose (see [33]). The saliva of four animals (two male, two female; treated with 10^9 pfu AdCMVH3 and exhibiting strongly positive PCR results with DNA from their targeted submandibular gland indicating significant vector levels) was tested for the presence of replication competent adenovirus (RCA). RCA generation following salivary gland administration of recombinant vector would be a significant concern because of the possibility for shedding of virus in a body fluid with considerable infectious potential. All four saliva samples were negative for RCA [33].

Recently, we reported toxicological and biodistribution findings in a GLP study of a different rAd5 vector encoding growth hormone (AdCMVhGH), administered to a single rat submandibular gland in the presence of the anti-malarial/anti-rheumatic drug hydroxychloroquine [34]. The AdCMVhGH vector was associated with very limited toxicities, similar to what was found in the AdCMVH3 study described above (see Table 2). Thus far, both our non-GLP and GLP level studies show that first generation rAd5 vectors result in no significant general adverse effects after administration to mammalian salivary glands. At present, we have almost finished the in-life

Table 2
Summarized results of completed GLP toxicological studies of rAd5 vector delivery to rat submandibular glands[a]

Vector	AdCMVH3	AdCMVhGH
Reference	[33]	[34]
Doses[b]	$\leq10^9$ pfu	$\leq10^{11}$ particles
Length of study	15 days	28 days
Survival[c]	100%	100%
Clinical signs[d]	No significant	No significant
Food consumption	No change	No change
Weight gain	No change	No change
Histopathology[e]	Targeted SMG	Targeted SMG
Clinical chemistry[f]	No change	Globulin increased
Hematology[g]	NR[h]	No change
RCA[i]	None detected	None detected

[a] Results presented are from comparisons with controls within the same study. Similar results were found for both male and female rats.
[b] For AdCMVH3 the following doses were administered: 0, 10^6, 3×10^7 or 10^9 pfu (~100 particles/pfu). For AdCMVhGH the following doses were administered: 0, 2.4×10^6, 6×10^9 or 1.5×10^{11} viral particles. Zero indicates diluent buffer was administered.
[c] Percentage of animals surviving until day of scheduled sacrifice.
[d] No toxicologically significant clinical signs were noted in rats of either gender.
[e] The only consistent histopathological change observed that was considered vector-related was a vector dose-related inflammation in the targeted submandibular gland (SMG).
[f] Serum samples were analyzed for the following components: alanine aminotransferase, alkaline phosphatase, lactate dehydrogenase, sorbitol dehydrogenase, albumin, amylase, creatine kinase, globulin, total protein, blood urea nitrogen, creatinine, glucose, total bile acids, chloride and sodium.
[g] The hematology parameters measured included red blood cell (RBC) count, RBC morphologic assessment, hematocrit, hemoglobin, mean corpuscular volume, packed cell volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, white blood cell (WBC) count, WBC differential, reticulocyte count, platelet count and morphologic assessment.
[h] NR, not reported due to excessive hemolysis.
[i] Replication competent adenovirus.

phase of a GLP level study with the AdhAQP1 vector delivered to a single rat submandibular gland.

5. Rationale for gene therapy with Ad vectors for IR-induced parotid hypofunction

The use of a rAd5 vector for initial hAQP1 gene transfer studies in adults with IR-induced parotid gland hypofunction is based upon several considerations. First, rAd5 vectors are extremely efficient at transferring genes to many non- or slowly dividing epithelial cells, like salivary glands [17]. Second, having a vector that leads to robust expression is advantageous from a safety perspective because less of the vector is needed to generate therapeutic transgenic protein levels. Third, the rAd5 vector will be administered locally into the well-encapsulated parotid gland thus reducing the risk of systemic exposure. Finally, in the event the AdhAQP1 strategy is not useful in human parotid glands, vector activity will be short-lived. However, if the strategy is useful, choice of a rAd5 vector initially does not preclude subsequent use of a vector with persistent expression for stable hAQP1 gene transfer, e.g., a serotype 2 adeno-associated viral vector [17,19,37].

5.1. Specific aim of clinical trial

The primary purpose of the proposed clinical protocol is to test the safety of AdhAQP1 with some measures of efficacy (i.e., it is a Phase I/II, dose escalation study) in patients with established IR-induced parotid gland hypofunction. The principal outcome measure for biological efficacy will be parotid gland salivary output. The proposed AdhAQP1 doses to be studied will increase from 1.4×10^8 particles/gland (estimated particle: pfu ratio of ~15:1) to a maximum of 1×10^{11} particles/gland (Table 3). This maximal vector dose has been shown to be without significant untoward effects clinically in other target organs [38,39].

For each patient, only one gland will be infused with vector in ~1.0 mL infusion buffer, based on optimal gland infusion volumes obtained from pre-entry sialograms. The study population will include 15 individuals with IR-induced parotid salivary hypofunction, defined as having an absence of unstimulated (resting) parotid salivary flow and a stimulated parotid salivary flow in the vector targeted gland >0 and <0.2 mL/min/gland. Enrolled patients will be individuals aged 18–65 years old, who are able to provide informed consent and who have received IR for head and neck cancer at least 5 years previously, and have no evidence of recurrent tumor. The choice of at least a 5-year interval between IR and AdhAQP1 delivery for the clinical study was made because patients who are tumor free for 5 years after initial treatment have an excellent prognosis for long-term survival [40]. Patients will receive AdhAQP1 at the start of the study and be evaluated thereafter on days 1 (at multiple times), 2, 3, 7, 14, 28, 42, 90, 120, 150, 180 and 360, and annually thereafter for 15 years, to assess salivary flow as well as numerous routine general and gene therapy-related safety-related parameters.

Table 3
Proposed AdhAQP1 clinical dose escalation scheme

Patient number	Dose in particle units	"MOI" in pfu/μL infusate
1–3	1.4×10^8	9.3×10^3
4–6	8.4×10^8	5.6×10^4
7–9	3.75×10^9	2.5×10^5
10–12	1.67×10^{10}	1.1×10^6
13–15	1×10^{11}	6.7×10^6

For dosage comparison, in 1999 a rAd5 study at the University of Pennsylvania resulted in the death of a young man [42]. The fatal (highest) dose delivered was 3.8×10^{13} rAd5 particles, and the starting dose for that study was 1.86×10^{11} particles [42], with all doses administered intravascularly. The maximum dose in the proposed study will be <0.3% that of the fatal dose in the University of Pennsylvania study. The starting dose, 1.4×10^8 particles/gland, corresponds to a MOI (defined here as the viral plaque forming [infectious] units/μL infusate) of ~9.3×10^3 (assuming a 15:1 particle: pfu ratio and an infusion volume of 1 mL, while the maximum dose proposed (1×10^{11} particles/gland) corresponds to a MOI of ~6.7×10^6 pfu/μL infusate. We previously reported that there is comparable (scaled) transgene expression in murine submandibular glands and minipig parotid glands with a MOI of ~2.5×10^5 pfu/μL [28]. This is also the same MOI that led to a restoration of parotid salivary flow in irradiated minipigs [30] (see Table 1).

A separate but important issue related to safety is consideration of the possible risks, versus the potential benefits of increased salivary secretion, to a patient as a result of hAQP1 cDNA transfer. Based on the pre-clinical animal model studies described above, there appears to be little to no risk of hAQP1 cDNA gene transfer *per se*. For example, we found that rAd5 vectors identical to AdhAQP1, except for the encoded transgene (i.e., encoding instead of hAQP1 either H3, hGH or luciferase), all showed similar safety profiles, i.e., the transgene encoded provided no additional effect [e.g., 30,33,34]. Furthermore, although epithelial cells of salivary duct do not normally express hAQP1, it is widely distributed in a spectrum of different tissues. In several tissues, it is extremely abundant, e.g., there are ~150,000 copies of monomeric AQP1 in every human red blood cell and AQP1 represents ~2.4% of total membrane protein, while in the kidney cortex this figure is ~1% [41]. Additionally, we have shown that polarized salivary epithelial cells, after in vitro infection with AdhAQP1 at a MOI=1 pfu/cell, respond well physiologically to imposed osmotic gradients even as steep as 300 mosm (i.e., 640 mosm apical; 340 mosm basal) [24]. This MOI, which leads to high levels of AQP1 expression (~30% that seen in 5 μg crude rat kidney membranes), as well as this osmotic gradient, are far in excess of that which cells can reasonably be expected to be exposed in vivo in patients in the proposed clinical study. Thus, taken together these observations support the notion that the risk: benefit ratio for hAQP1 cDNA transfer to patients in the proposed clinical trial is quite low.

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