Late responses to adenoviral-mediated transfer of the aquaporin-1 gene for radiation-induced salivary hypofunction

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We evaluated late effects of AdhAQP1 administration in five subjects in a clinical trial for radiation-induced salivary hypofunction (http://www.clinicaltrials.gov/ct/show/NCT00372320?order=). All were identified as initially responding to human aquaporin-1 (hAQP1) gene transfer. They were followed for 3–4 years after AdhAQP1 delivery to one parotid gland. At intervals we examined salivary flow, xerostomic symptoms, saliva composition, vector presence and efficacy in the targeted gland, clinical laboratory data and adverse events. All displayed marked increases (71–500% above baseline) in parotid flow 3–4.7 years after treatment, with improved symptoms for ~2–3 years. There were some changes in [Na+] and [Cl−] consistent with elevated salivary flow, but no uniform changes in secretion of key parotid proteins. There were no clinically significant adverse events, nor consistent negative changes in laboratory parameters. One subject underwent a core needle biopsy of the targeted parotid gland 3.1 years post treatment and displayed evidence of hAQP1 protein in acinar, but not duct, cell membranes. All subjects responding to hAQP1 gene transfer initially had benefits for much longer times. First-generation adenoviral vectors typically yield transit effects, but these data show beneficial effects can continue years after parotid gland delivery.

INTRODUCTION

Head and neck cancers are among the most common malignancies worldwide, with the majority of patients being treated at least in part with radiation. It has long been recognized that during radiation therapy damage can occur to healthy salivary glands.2,1 This is surprising given the generally low rate of turnover of mammalian salivary epithelial cells.3,4 Although methods of radiation have greatly improved,2,5 and can significantly limit the damage to normal tissue adjacent to the tumor, radiation-induced salivary hypofunction is still a significant clinical problem because of (i) the large number of patients with already existing radiation-induced gland damage and (ii) the fact that the most technologically advanced instruments to focus radiation and minimize gland damage are primarily found in academic medical centers in relatively wealthy countries. Accordingly, the approved protocol was modified based on our observations with the first responder-subject (#19, see below), who exhibited a positive response to gene transfer on day 7.12 Although his initial peak increase in saliva flow rate on days 180 and 360, both of which were well above his baseline value (see below). Accordingly, the approved protocol was modified to permit all responders to AdhAQP1 administration to be evaluated for two additional time points, at least 1 and 2 years following their completion of the original 360-day protocol. All five responder-subjects consented to this extended evaluation. It is the purpose of the present study to describe results from all five responder-subject evaluations following the initially reported day 42-time period.12

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RESULTS

Supplementary Table 1 provides several general clinical characteristics of the five responder-subjects studied herein; most of these were reported earlier.\textsuperscript{12}

Evaluation of adverse events and clinical laboratory parameters

All adverse events (AEs) occurring after day 42 that were reported by these five subjects were analyzed. Our previous report\textsuperscript{12} described AEs occurring through day 42. The subjects reported a total of 21 AEs during the post-treatment period from day 42 through the final time point (~3–4.7 years). Of these 21 AEs, 18 were regarded as unrelated to either the treatment with AdhAQP1 or to the study procedures used. Of the remaining three AEs, one (soreness in a parotid duct) was considered unrelated to treatment, but definitely related to a study procedure. The second AE (an oral candidal infection) was considered unrelated to treatment and unlikely related to the study procedures. The third (an upper respiratory tract infection) was considered unlikely related to treatment and unrelated to study procedures. Of the 21 AEs, 18 were considered mild (grade 1), but three, all unrelated to treatment or study procedures, were serious and required hospitalization (hip fracture from a motorcycle accident; elective surgery for degenerative hip disease and an episode of severe abdominal pain).

All clinical laboratory parameters measured (clinical chemistries and hematology), for all five subjects from day 90 through the completion of all follow-up visits, were evaluated. There were no

Figure 1. Stimulated parotid salivary flow rates of the targeted gland at key time points from each subject responding positively to AdhAQP1 treatment. The color coding for subjects in this figure are the same as in Figure 3, and as those published in Baum et al.\textsuperscript{12} The Y axis shows salivary flow in ml min\textsuperscript{-1} from the targeted parotid gland. The X axis shows five key time points in this study, with the specific days for each indicated above data points in the figure. Baseline represents each subject’s initial visit to the NIH Clinical Center prior to any procedures being performed. The initial peak increase in salivary flow represents the time point (varied from day 7 to 42) following AdhAQP1 administration when the subject’s parotid salivary flow was maximal. Both the baseline and initial peak data were presented previously in Baum et al.\textsuperscript{12} The end point of the originally approved study was on the day 360 visit after vector delivery for each subject. Thereafter, the original clinical protocol was amended and we received permission to evaluate the effects of AdhAQP1 administration to all five responder-subjects for an additional 2 years of follow-up. The exact days following vector administration for the two follow-up visits are different for each subject (shown also in Table 1). They are shown in the figure and are as follows: #19 (days 1124 and 1708, respectively), #50 (1132 and 1531), #99 (721 and 1141), #103 (797 and 1119) and #118 (721 and 1086).

Table 1. Parotid salivary flow rates at key time points\textsuperscript{a}

| Subject | Baseline | Peak 1 flow rate (day) | Day 360/end-point flow rate | Follow-up 1 flow rate (day) | Follow-up 2 flow rate (day) |
|---------|----------|------------------------|----------------------------|-----------------------------|-----------------------------|
| 19 Treated | 0.073 | 0.403 (7) | 0.191 | 0.405 (1124) | 0.447 (1708) |
| Untreated | 0.13 | NR | NR | 0.068 | 0.268 |
| 50 Treated | 0.145 | 0.291 (42) | 0.239 | 0.209 (1132) | 0.248 (1531) |
| Untreated | 0.0 | 0.0 | 0.0 | 0.13 |
| 99 Treated | 0.092 | 0.497 (14) | 0.366 | 0.128 (721) | 0.225 (1141) |
| Untreated | Tubing | Tubing | Tubing | Tubing | 0.03 |
| 103 Treated | 0.136 | 0.221 (28) | 0.172 | 0.144 (797) | 0.456 (1119) |
| Untreated | 0.458 | 0.256 | 0.107 | 0.103 | 0.3 |
| 118 Treated | 0.044 | 0.107 (28) | 0.089 | 0.085 (721) | 0.107 (1086) |
| Untreated | 0.173 | NR | 0.0 | 0.145 | 0.238 |

Abbreviation: NR, no data recorded for that time point. \textsuperscript{a}Parotid saliva flow rates are given in ml per min per gland and were obtained as described previously.\textsuperscript{12} All five subjects positively responding to AdhAQP1 treatment were seen for two additional follow-up visits after the original final visit at day 360. The number of days after AdhAQP1 administration for each of the initial and follow-up visits is shown in parentheses. Baseline represents salivary flow rate in the targeted and contralateral gland prior to vector delivery. Data are shown for both the treated and the untreated, that is, contralateral glands. Tubing means saliva was in the tubing of the collection device, but was unable to be quantified.
consistent, significant changes related to the study (AdhAQP1 administration or related procedures) for any parameter measured.

Parotid salivary secretion
None of the non-responder subjects showed any increase in parotid flow rate after the original day 42 data reported. Figure 1 shows the individual salivary flow rates, at several key time points, obtained from each of the five subjects considered to be positive responders to hAQP1 gene transfer,12 over their entire course of observation. All subjects were seen for minimally 3 years after gene transfer, whereas the first two responders sequentially were seen over a longer interval (#19 for ~4.7 years; #50 for ~4.2 years). Each person’s parotid salivary flow results were then compared with their baseline parotid flow rate at the first pre-vector delivery visit (baseline). It is clear visually in Figure 1 and in tabular form for these key time points (Table 1) that following their initial peak in flow rate at the first pre-vector delivery visit (baseline). Model 2a, shown in Table 2 (16 visits including baseline visit), reports the generalized estimating equation (GEE) modeling with uniform correlation structure with the Huber White estimator of variance.14 A graphic representation of some of these data, the dynamics of salivary flow from the targeted parotid gland, is shown in Figure 2a. Consistent with this visual impression, the GEE model indicated that the stimulated parotid salivary flow from the targeted parotid gland continued to be statistically significantly higher at follow-up visit 1 with a 0.17 ml min⁻¹ (95% confidence interval (CI): 0.06–0.28, s.e.: 0.06, P-value = 0.003) increase from baseline flow rate, and at follow-up visit 2 with a 0.27 ml min⁻¹ (95% CI: 0.14–0.40, s.e.: 0.07, P-value < 0.001) increase from baseline flow rate. Furthermore, the stimulated parotid saliva flow

| Time         | SPFR | 95% CI     | Robust s.e. | P-value |
|--------------|------|------------|-------------|---------|
| **Visit 2**  | 0.142| 0.102, 0.182| 0.020       | < 0.001*|
| **Visit 3**  | −0.008| −0.043, 0.026| 0.018       | 0.632   |
| **Visit 4**  | 0.087| 0.000, 0.175| 0.045       | 0.051   |
| **Visit 5**  | 0.145| −0.008, 0.298| 0.078       | 0.063   |
| **Visit 6**  | 0.177| 0.062, 0.291| 0.058       | 0.002*  |
| **Visit 7**  | 0.152| −0.007, 0.311| 0.081       | 0.061   |
| **Visit 8**  | 0.131| 0.093, 0.169| 0.019       | < 0.001*|
| **Visit 9**  | 0.114| 0.039, 0.189| 0.038       | 0.003*  |
| **Visit 10** | 0.099| 0.048, 0.151| 0.026       | < 0.001*|
| **Visit 11** | 0.176| 0.113, 0.239| 0.032       | < 0.001*|
| **Visit 12** | 0.146| 0.038, 0.254| 0.055       | 0.008*  |
| **Visit 13** | 0.207| 0.031, 0.384| 0.090       | 0.021*  |
| **Visit 14** | 0.184| 0.098, 0.270| 0.044       | < 0.001*|
| **Visit 15** | 0.166| 0.055, 0.277| 0.057       | 0.003*  |
| **Visit 16** | 0.269| 0.140, 0.397| 0.066       | < 0.001*|

**Abbreviations:** CI, confidence interval; GEE, generalized estimating equation; SPFR, stimulated parotid gland salivary flow rate. *Statistically significant. Model 1a—all 16 visits in the model with visits treated as a categorical time variable. Visit 1 is the reference (baseline). Model 1b—all 16 visits in the model with visits treated as continuous time variable. Visit 1 is the reference (baseline). Model 2a—4 visits (stimulated parotid salivary flow rate at peak 1, follow-up 1 and follow-up 2 visits were compared with baseline flow rates) in the model with visits treated as categorical time variable. Visit 1 is the reference (baseline). Model 2b—4 visits in the model with visits treated as continuous time variable. Estimates are rounded to three decimal places.

Late effects of AdhAQP1 for IR-induced xerostomia
I Alevizos et al
Gene Therapy (2017) 176–186
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with every sequential visit, when only the peak 1, follow-up visit 1 and follow-up visit 2 were compared with the baseline visit (Model 2b).

As noted above, we also examined stimulated parotid flow rates in the contralateral parotid glands in more detail using GEE modeling. However, again, we could not detect any consistent effects of AdhAQP1 treatment on stimulated saliva in the contralateral parotid glands (Table 3). A graphic representation of the dynamics of salivary flow from the contralateral parotid gland is shown in Figure 2b.

Evaluating xerostomic symptoms
For the present study we evaluated the same two, key xerostomic symptoms previously reported using a validated visual analogue scale (VAS, Supplementary Figure 1).\(^1,\(^5\) The subjects were asked to rate (i) the dryness in their mouth (dry mouth; Figure 3a) and (ii) how much saliva was in their mouth (amount of saliva; Figure 3b) at the beginning of each study visit prior to any saliva collections. The time points shown in Figure 3 are the same key time points shown in Figure 1 and Table 1. As reported previously,\(^12\) when compared with the baseline visit each of these five subjects showed improvement (that is, a lower VAS score) in these two subjective assessments at the time of their initial elevation in parotid salivary flow (during days 7–42). Thereafter, the results were more variable, but all five individuals reported some improvement in these two symptoms at either (or both) the 2- or 3-year time point. However, by the second follow-up visit, three subjects (19, 50 and 99) felt that their mouths were almost as dry as at the start of the trial, and both subjects 19 and 99 believed the saliva in their mouth was at or near the baseline levels (Figures 3a and b).

Serum neutralizing antibodies
Previously, we have reported serum neutralizing antibody levels at baseline\(^12\) and over the initial 42 days post-AdhAQP1 treatment.\(^16\) As shown in Figure 4, compared with baseline serum neutralizing antibody levels measured in the five responder-subjects, little change occurred throughout the study, extending to 3+ years post treatment, with serum neutralizing antibody titers at or below those measured at baseline.

Analysis of saliva composition
Table 4 shows the concentration of Na\(^+\), K\(^+\) and Cl\(^-\) in parotid saliva at baseline and at or near the initially observed increase in salivary flow. For both subjects with saliva samples available for analysis at the first salivary peak (#19 and 50), there was a dramatic increase in the concentrations of all three electrolytes. Subject #99 showed a similar increase, albeit not as marked, with a saliva sample obtained at day 7. His initial peak of salivary flow was at day 14, but the day 7 flow rate was still >2-fold that at baseline. Subjects 103 and 118 had their initial peak salivary flow rates on day 28, however, samples were only available for analysis on day 7. Flow rates for both the subjects were ~30% above their baseline levels, and electrolyte levels were only modestly elevated, if at all (Table 4).

There were sufficient quantities of parotid saliva available to evaluate key parotid acinar cell secretory proteins in subjects 19, 50 and 99 (Figure 5). When comparing levels of amylase, histatins 1, 3 and 5, and acidic proline-rich proteins 1 and 2 at baseline (B) with the initial peak salivary flow increase for #19 (day 7) and #50 (day 42) no consistent pattern of change was observed. For subject #99 there was only a minimal amount of baseline saliva available, and none of the peak saliva, so useful comparisons were made on day 28 with day 3 saliva samples, which were of a similar level to the peak (day 14) and baseline flow rates, respectively. Again, the general impression is one of no consistent change in rates from the treated gland increased by 0.01 ml min\(^{-1}\) (95% CI: 0.005–0.013, s.e.: 0.002; P-value < 0.001) with every sequential visit (Model 1b). Models 2a and 2b (four visits including baseline visit), shown in Table 2A, also reported stimulated parotid salivary flow rate at three key time points, namely the study visit with the initial peak salivary flow rate (peak 1), follow-up visit 1 and follow-up visit 2. The GEE model (Model 2a) with uniform correlation structure with the Huber White estimator of variance indicated that the stimulated parotid saliva flow rate from the treated gland continued to be statistically significantly higher on these three visits, with a 0.26 ml min\(^{-1}\) (95% CI: 0.13–0.40, s.e.: 0.07, P-value < 0.001) increase in flow rate at peak 1, a 0.15 ml min\(^{-1}\) (95% CI: 0.04–0.26, s.e.: 0.06, P-value = 0.007) increase in flow rate at follow-up visit 1, and a 0.26 ml min\(^{-1}\) (95% CI: 0.13–0.38, s.e.: 0.06, P-value < 0.001) increase in flow rate at follow-up visit 2, compared with baseline flow rate. Furthermore, the stimulated parotid saliva flow from the treated gland increased by 0.05 ml min\(^{-1}\) (95% CI: 0.006–0.091, s.e.: 0.02, P-value = 0.025)

Figure 2. Depiction of the changes from baseline for stimulated parotid salivary flow rates at each time point in this clinical trial from the AdhAQP1-treated gland (a) and the contralateral untreated gland (b). Statistical significance is indicated using the dark (P < 0.05) and light blue (P > 0.05) shading of each bar, and the 95% confidence intervals are depicted. Bars above the line represent an increase in salivary flow rate, whereas those below indicate a decrease in salivary flow rate. Statistical analysis used GEE modeling as presented in Tables 2A and 3. Visit numbers represent the following time points: 1 (baseline), 2 (6 h), 3 (day 1), 4 (day 2), 5 (day 3), 6 (day 7), 7 (day 14), 8 (day 28), 9 (day 42), 10 (day 90), 11 (day 120), 12 (day 150), 13 (day 180), 14 (day 360), 15 (follow-up visit 1), 16 (follow-up visit 2). The exact times for the two follow-up visits are presented in Figure 1 and its legend.
the output of the examined key parotid secretory proteins (Figure 5).

Analysis of parotid gland biopsy samples
Two subjects underwent a modified sialoendoscopic biopsy of the targeted parotid gland at the time of their first long-term follow-up visit. DNA isolated from both samples was examined for the presence of AdhAQP1 using a conventional PCR assay. The appropriate PCR amplicon was detected in the sample from only one subject, #99, (Figure 6 left panel; #103 was negative). As can also be seen in Figure 6 (right panel), when this PCR reaction was performed on a sample of normal human parotid gland no amplicon was detected. An ultrasound-guided core needle biopsy was performed on subject #19, and yielded considerably more tissue. That tissue was examined by light and immunofluorescence microscopy (Figure 7). Figure 7a depicts the general histological appearance of the biopsied tissue, showing the presence of acinar and duct cells with hematoxylin and eosin (H&E) staining. Figure 7b shows results of immunostaining with a control antibody, and only the appearance of DAPI-stained nuclei is visible. Figure 7c shows the tissue sample stained with an antibody to aquaporin-5, which is a normal component of the luminal membranes of human parotid acinar cells (a) but not present in duct cells (d) except those of the intercalated duct region.

Figure 3. Visual analogue scale (VAS) measurements of two key xerostomic symptoms at key time points in responder-subjects. (a) Rate the dryness in your mouth; (b) Rate how much saliva is in your mouth. A lower score indicates an improvement in the symptom. The color coding for subjects in this figure are the same as in Figure 1, and as those published in Baum et al. Baseline and initial peak data were presented previously in Baum et al. As noted in the legend of Figure 1, the end point of the originally approved study was on the day 360 visit after vector delivery for each subject. The two follow-up visits are different for each subject (presented in Figure 1). They are as follows: #19 (days 1124 and 1708, respectively), #50 (1132 and 1531), #99 (721 and 1141), #103 (797 and 1119) and #118 (721 and 1086). The Y axis represents the visual analogue scale numerical value (10 cm scale, with 10 being the driest mouth (a) or the lowest amount of saliva (b) imaginable). The form used for the visual analogue scale measurements can be found in Supplementary Figure 1 and was derived from Pai et al.
Figure 4. Anti-Ad5 serum neutralizing antibody titerd in responder-subjects. Anti-Ad5 neutralizing serum antibodies employed a previously described assay. The assay tests the ability of serum dilutions to block the transduction of 293 cells by an Ad5 vector, AdCMVLuc encoding luciferase. The titers (Y axis) represent serum dilutions resulting in a 50% inhibition of transduction. The X axis represents time points in the study. All time points shown are days following AdhAQP1 administration. Individual subject symbols are indicated. No sample was available to perform this assay at the second follow-up visit of subject 19. The exact days of each follow-up visit are listed in the legends of Figures 1 and 3, and Table 1. Note that serum neutralizing antibody titers found in all time points through day 42 have recently been reported and were generally similar to those shown in the figure. B, baseline.

Figure 7d shows the tissue sample stained with an antibody to hAQP1, which normally is only present in myoepithelial and vascular endothelial cells in the human parotid gland. Figure 7e is an enlarged region of a hAQP1-negative acinus, with only immunostained myoepithelial and endothelial cells observed, whereas Figure 7f shows an enlarged region of an acinus that was transduced by AdhAQP1. This acinus and many adjacent acini are still expressing hAQP1 in their luminal and basolateral membranes 3.1 years after AdhAQP1 treatment. Figure 7g shows the results obtained when a biopsy specimen from a normal human parotid gland was stained with anti-human AQP1 antibody. The immunofluorescence staining is found only in two cell types: myoepithelial and vascular endothelial cells (Figure 7h shows an enlarged view), and is comparable to the results shown in Figure 7e.

DISCUSSION

First-generation Ad5 vectors, such as AdhAQP1, have been used frequently in pre-clinical and clinical gene therapy studies. Although these vectors lead to very efficient gene transfer and high levels of transgenic protein production, they are also considered problematic because they elicit potent innate, cellular and humoral immune responses. In great part, because of this immunoreactivity, first-generation Ad5 vectors are considered only to yield transient expression of the delivered transgene, typically for no more than a week or two, with a peak at - days 2 or 3. Compared with Ad5 vector use systemically (intravascular delivery) or to a variety of organs, relatively few studies have involved delivery of a first-generation Ad5 vector to salivary glands. All but one of these studies were performed in pre-clinical, animal models (mouse, rat, miniature pig and macaque), and all studies in these animal models displayed the typical pattern of transgene expression described above.

There is only a single human study reported involving gene transfer to a salivary gland, the same as the one described herein. This study employed the first-generation Ad5 vector, AdhAQP1. Our previous report described initial results from this study, through day 42 following delivery of AdhAQP1 to a single, irradiation-damaged salivary gland. The present study reports results from the long-term follow-up of the five subjects described previously to have responded positively to AdhAQP1 delivery. The initial report of this clinical trial was remarkable in showing that the peak of transgene expression, inferred to be the peak of increased parotid saliva flow rate, occurred on days 7-42, that is, over a much later time frame than anticipated. Studies with AdhAQP1 and other Ad5 vectors in salivary glands in all animal models were quite similar to each other and, as indicated above, showed peak transgene expression on days 2 or 3, which then returned to background levels by 2 weeks. The present report is even more remarkable in that all five subjects who responded positively to hAQP1 gene transfer initially, still displayed substantially elevated levels of parotid saliva flow 3-4 years following AdhAQP1 administration. In addition, we showed that most subjects also experienced relief from two key xerostomic symptoms for at least 2 years after treatment. Because of the unique nature of our results it is important to try to understand how these findings might have occurred.

Globally, there are likely two key reasons. First, it is generally considered that the significant immune response to a first-generation Ad5 vector delivery results in the complete removal of the vector from the targeted tissue. However, as we have shown previously in rat salivary glands, that is not the case. Following administration of a dose of 10^9 vector particles/ rat submandibular

| Table 4. Parotid saliva electrolyte composition |
|------------------|----------|----------|----------|
| Subject (electrolyte) | Baseline | Day 7    | Day 42   |
| Na^+              | 18.8     | 110.6    | 55       |
| K^+               | 15.8     | 27.3     | 16.3     |
| Cl^-              | 22.6     | 109.9    | 57       |
| Na^+              | 12.8     | 35.2     | 83       |
| K^+               | 12.9     | 10.6     | 16.7     |
| Cl^-              | 17.1     | 36.2     | 81.3     |
| Na^+              | 4.1      | 37.9     | 0.0      |
| K^+               | 18.5     | 22       | 25.6     |
| Cl^-              | ND       | 40.1     | 22.2     |
| Na^+              | 0.0      | 7.7      | 0.0      |
| K^+               | 22       | 24.5     | 20.6     |
| Cl^-              | 10.2     | 19       | 12.1     |
| Na^+              | 0.9      | 4.3      | 2.7      |
| K^+               | 21.9     | 25.8     | 29.9     |
| Cl^-              | 14.6     | ND       | 19.5     |

Abbreviation: ND, not determined. *The bold values represent electrolyte concentrations (mM) in samples from the subject’s initial peak flow, that is, day 7 for #19 and day 42 for #50. †The initial peak flow rate for this subject was on day 14. The day 7 flow was 0.24 ml min^-1 per gland, and was substantially above baseline (0.092). ‡The initial peak flow rate for this subject was on day 28. The day 7 flow was 0.184 ml min^-1 per gland, 36% above baseline (0.135). §The initial peak flow rate for this subject was on day 28. The day 7 flow was 0.058 ml min^-1 per gland, 31% above baseline (0.044).
Late effects of AdhAQP1 for IR-induced xerostomia

I Alevizos et al

Late effects of AdhAQP1 for IR-induced xerostomia

I Alevizos et al

Late effects of AdhAQP1 for IR-induced xerostomia

I Alevizos et al

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I Alevizos et al

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I Alevizos et al

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A final major finding in the present study supports a key conclusion of the initial 42-day report, that is, that AdhAQP1 delivery to a single human parotid gland is safe. Herein we found no significant vector or procedure-related AEs following day 42 of the study until the final follow-up subject visits, 3–4 years later. In addition, there were no consistent, significant changes related to the study in any clinical chemistry or hematology parameter measured.

The original purpose of the AdhAQP1 clinical trial (http://www.clinicaltrials.gov/ct/show/NCT00372320?order=) was considered essentially to be a proof of concept, that is, that hAQP1 gene transfer to an irradiation-damaged salivary gland would lead to increased fluid secretion from the targeted gland. It was expected that a positive result would unlikely lead to a long-term benefit and that the AdhAQP1 vector would be rapidly cleared from the targeted gland (http://osp.huhn.de/phase1/RAC_minutes_12-05.pdf). First-generation Ad5 vectors may not be ideal gene therapy vectors. However, when used at modest doses and delivered locally to a parotid gland, conditions that do not elicit a marked immune response, Ad5 vectors can be useful for salivary gland gene transfer in humans. As shown herein, all five responder-subjects in the AdhAQP1 clinical trial experienced significant objective and subjective benefits over a considerable time period following vector administration.

MATERIALS AND METHODS

General methods

As previously described, the Phase I/II clinical trial (NIH protocol 06-D-0206) to test the safety and efficacy of AdhAQP1–mediated gene transfer to a single, previously irradiated parotid gland was approved by the NIDCR Institutional Review Board, the NIH Biosafety Committee, the Recombinant DNA Advisory Committee, the FDA (IND #83-13,102), as well as an Independent Data and Safety Monitoring Board. Five enrolled subjects (all males; #19, 50, 99, 103 and 118) were considered to be positive responders based on increased parotid salivary flow rates, following 2% citric acid stimulation (dorsal surface swabbing of the tongue), as well as a measured improvement in two key xerostomic symptoms using a visual analogue scale developed by Pai et al. Parotid salivary flow rates measured by the methods used herein are widely employed, but can show from 15 to 45% variability according to several studies. The two xerostomic symptoms reported earlier and herein exhibit moderate (rate the dryness of your mouth) and marginal (rate how much saliva is in your mouth) reproducibility. Although these two questions could reflect independent symptoms, they were used to indicate self-perceived oral dryness and oral moisture, respectively. Both are reported herein because of their use in our earlier publication in defining possible responder-subjects. The responder-subjects were in the first three AdhAQP1 vector dosage groups: 4.8x10^11 (#19), 2.9x10^10 (#50, 99) and 1.3x10^10 (#103, 118) vector particles per gland. As noted earlier, general clinical characteristics of these subjects were previously reported in Baum et al. and a summary also can be found in Supplementary Table 1. All methods were as previously reported, except for the following used only for the present study.

Additional methods

Anti-Ad5 neutralizing serum antibodies employed a previously described assay. The assay tests the ability of serum dilutions to block the transduction of 293 cells by an Ad5 vector, AdCMVLuc encoding luciferase. All assays reported herein were performed at one time and the titers indicated represent serum dilutions resulting in a 50% inhibition of transduction. Some samples of all five subjects’ parotid saliva, at or near the initially observed increase in salivary flow, were available for ionic composition analysis (Na+, K+, Cl−). This was performed using previously reported methods. In addition, parotid saliva samples from three subjects (#19, 50 and 99) were available for secretory protein analysis using well-described polycrylamide gel electrophoresis methods. In addition, two duct cells expressing hAQP1 in acinar cells in situ results from the hCMV promoter likely not being methylated in transduced acinar cells, the promoter may have been methylated in duct cells targeted by the vector. There was no way for us to examine this possibility under the protocol approved for the present study, but it should be testable in the future.
subject #99 and 103 were analyzed for the presence of vector DNA using PCR (see below), whereas the tissue obtained from the ultrasound-guided core needle biopsy was examined by immunofluorescence microscopy (see below).

PCR assay
The tissue samples from subject #99 and 103 were placed in 100 μl saline solution and genomic DNA directly extracted using the QIAampDNA blood mini kit (QIAGEN, Gaithersburg, MD, USA). One-half of the obtained DNA

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**Figure 7.** Images from core needle biopsy specimen obtained from subject #19 at follow-up visit 1 (day 1124 after AdhAQP1 administration). (a) H&E staining of the parotid gland tissue sample showing the presence of acini and ducts. (b) Control for immunofluorescence staining using normal rabbit IgG as the primary antibody. The nuclei are stained using DAPI and have a blue color. (c) Tissue stained with an antibody to human AQPS. The immunofluorescence staining observed is localized only to the luminal membrane of acinar cells (a) and the closely adjacent intercalated duct region. Larger ducts (d) do not express AQPS and are unstained. (d) Tissue stained with an antibody to human AQP1. The immunofluorescence staining is found in three cell types: myoepithelial, vascular endothelial and acinar. Normally, AQP1 is only present in myoepithelial and vascular endothelial cells (Gresz et al.17). Acinar cells that can be seen expressing AQP1 (right central and bottom portion of panel) were transduced with AdhAQP1 administered to subject #19 1124 days previously. (e) An enlarged region of (d) showing the presence of AQP1 in myoepithelial (yellow arrows) and vascular endothelial cells (red, smaller arrows), and the negative staining of a non-transduced acinus. (f) An enlarged region of (d) showing the abundant presence of AQP1 in the basolateral and luminal membranes of a transduced acinus. (g) AQP1 localization in a biopsy specimen from a normal, male human volunteer's parotid gland, that is, without AdhAQP1 transduction. There is no immunofluorescence staining in acinar cells. (h) An enlarged region of (g) clearly showing the absence of AQP1 staining in acinar (a) and duct (d) cells, but its presence in myoepithelial cells (yellow arrows) and vascular endothelial cells (smaller red arrows). See Materials and Methods section for details on the staining procedures and antibodies used.
(from 20 to 146 ng) was used in a conventional PCR assay. Primer 1 (5′-CGTAGCCGAGCGATCTATATAA-3′) is a forward primer from the hCMV promoter, which was used to drive hAQP1 expression in AdhAQP1. Primer 2 (5′-TACAGAGGGCCGATGCC-3′) is a reverse primer from sequences in hAQP1. PCR was performed at 50 °C for 1 min, 72 °C for 1 min and 94 °C for 1 min, for 40 cycles. About 10 μl of the PCR reaction mixture was electrophoresed on a 1% agarose gel. If the expected band was not present (it was not for both subjects’ samples), then 5 μl from that first PCR reaction was used to perform a second round of PCR under the same conditions. Again, the expected band was not present on the resulting agarose gel, so 5 μl from second reaction mixture was used to perform a third and final round of PCR. The positive control was DNA extracted from the pure AdhAQP1 viral vector, and that PCR was performed separately from both the subjects’ samples. The resulting 565 bp amplion from this PCR assay could only be derived from tissue samples in which the AdhAQP1 vector genome was present.

Immunofluorescence staining

The ultrasound-guided core needle biopsy sample obtained from #19’s targeted parotid gland was fixed in 10% formalin and embedded in paraffin. Sections (5 μm) were treated exactly as previously described.29 The primary antibodies used herein were rabbit monoclonal anti-aquaporin-1, rabbit monoclonal anti-aquaporin-5 and normal rabbit IgG as an antibody control (all from Abcam Inc., Cambridge, MA, USA). The secondary antibody was Alexa Fluor 488 donkey anti rabbit IgG (H+L) (Invitrogen, Carlsbad, CA, USA) and used as described.29 In addition, sections of this sample were stained conventionally with H&E.

Statistical analyses

To determine if the change in salivary flow rates in the AdhAQP1-targeted parotid gland of all previously designated responder-subjects was statistically significantly different over time, longitudinal analyses were undertaken. First, exploratory data analysis was performed. Next, correlation structure between multiple salivary flow measurements was analyzed. Models with independent, exchangeable/uniform, autoregressive, and unstructured correlation structures were compared. Autocorrelation function and quasi-Akaike Information Criterion were computed for model selection. GEE models with uniform correlation structure with Huber White estimator of variance were then constructed.1 These models were constructed to: (1) compare stimulated parotid salivary flow rate at all post-treatment visits to baseline values; (2) compare baseline flow rates with those at the initial peak (during the first 42-study days; reported25) and both long-term follow-up visits (days 721–1708 following AdhAQP1-mediated gene transfer); and (3) determine the change in flow rate with time, that is, over a period of 16 visits and over a period of 4 keys visits (baseline, initial peak, follow-up 1 and 2). Similarly, GEE models were constructed to assess changes in stimulated parotid salivary flow rates in the contralateral, non-targeted parotid gland.

CONFLICT OF INTEREST

BJ Baum currently serves as a consultant to GSK, but was not so engaged during this study. The remaining authors declare no conflict of interest.

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Late effects of AdhAQP1 for IR-induced xerostomia
I Alevizos et al

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