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ORIGINAL ARTICLE

Toxicity and biodistribution of a first-generation recombinant adenoviral vector, in the presence of hydroxychloroquine, following retroductal delivery to a single rat submandibular gland

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OBJECTIVE: We examined the toxicity and biodistribution associated with a single administration of a first-generation, serotype 5, adenoviral vector encoding human growth hormone (hGH; AdCMVhGH) to a single rat submandibular gland in the presence of hydroxychloroquine (HCQ). Previously, we showed that hGH is primarily secreted into saliva (approximately ninefold serum level) when expressed as a transgene in salivary glands (e.g. Baum *et al*, 1999), but administration of HCQ substantially increases the hGH levels secreted into the bloodstream (Hoque *et al*, 2001). A potential application of this observation is for patients with adult hGH deficiency.

METHODS: Six groups of male and female adult rats (n = 12 each) were studied, with zero to 1.5×10^{11} particles of AdCMVhGH, ±HCQ, administered retroductally. Multiple clinical and pathological parameters, as well as vector tissue distribution, were assessed.

RESULTS: All animals survived until the scheduled day of sacrifice, and essentially no untoward events were observed clinically or at gross necropsy. We observed no vector-related effects on clinical hematology evaluations and a single, transient significant change on clinical chemistry evaluations (increased serum globulin levels). Three days after AdCMVhGH administration, the vector distributed to all tissues analyzed with the exception of gonads and heart. By day 29, most organs, other than the targeted and contralateral submandibular glands, were negative for the presence of vector. On day 3, none of the animals tested positive for the presence of replication competent adenovirus in either their blood or saliva.

CONCLUSION: Salivary gland delivery of AdCMVhGH ±HCQ appears associated with limited toxicity in rats.
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Keywords: adenovirus; salivary gland; hydroxychloroquine; gene therapy; safety

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Introduction

Previously, we have reported several potential clinical applications of gene transfer to salivary glands (for a review see Baum *et al*, 2002). Among these, correction of systemic, single protein deficiency disorders seems particularly promising (Voutetakis *et al*, 2003; Baum *et al*, 2004). The management of diabetes and many other single protein deficiency diseases currently is achieved by the regular, repeated injection of recombinant proteins (e.g. insulin for diabetes). Gene therapy offers the potential of correcting such deficiencies through the expression of a transgene coding for the required protein. Previous studies have shown that although salivary glands physiologically are best recognized for their exocrine secretion of saliva, they are able to secrete proteins in an endocrine direction (Isenman *et al*, 1999).

Secretory proteins are sorted into either a regulated or constitutive pathway in salivary glands (Loh *et al*, 2002; Voutetakis *et al*, 2003). Secretion of human growth hormone (hGH), as a transgene product from salivary glands, primarily occurs via the regulated pathway, as it does from its physiological site of secretion, somatotrophs in the anterior pituitary gland (Baum *et al*, 1999, 2004). However, in salivary glands regulated pathway secretion goes into saliva, where hGH is not therapeutically useful.

Recently, we showed that it was possible to efficiently direct clinically useful levels of hGH into the

bloodstream following adenoviral vector-mediated hGH gene transfer to rat submandibular glands through use of the approved anti-rheumatic and anti-malarial drug Plaquenil (hydroxychloroquine, HCQ; Hoque *et al*, 2001). Almost all epithelial cell types in rat submandibular glands provide excellent targets for adenoviral vectors (Delporte *et al*, 1997a,b). In anticipation of possible future clinical trials using a gene transfer vector encoding hGH (or another regulated pathway secretory protein), in conjunction with Plaquenil, this study was initiated to determine the tissue distribution and potential systemic toxicity of a single administration of a recombinant first-generation adenoviral vector to the rat submandibular gland in the presence of HCQ.

Materials and methods

This study was designed and conducted to conform with the United States Food and Drug Administration Good Laboratory Practice regulations.

Animals

Fischer 344 rats were obtained from Taconic Farms (Germantown, NY, USA) at 6 weeks of age. Animals were acclimated for 4–6 weeks prior to the study onset. Animals were stratified by body weight (12 rats/treatment group for each gender), and uniquely marked with tail tattoos 1–3 days prior to vector administration (see Table 1). Animals were housed individually in polycarbonate shoebox cages (Lab Products Inc., Seaford, DE, USA) in temperature and humidity controlled rooms. A 12 h light–dark cycle was maintained and animals were given food (irradiated NTP-2000 Open Formula Diet; Zeigler Brothers Inc., Gardners, PA, USA) *ad libitum*, except for overnight fasting prior to scheduled sacrifice. Water was provided each day *ad libitum*.

Recombinant adenovirus construction

The recombinant adenovirus AdCMVhGH is a conventional first generation, adenoviral serotype 5 (Ad5) vector constructed as previously reported (He *et al*, 1998). Briefly, the gene for hGH was sub-cloned into the adenoviral shuttle vector pAC, and then co-transfected into 293 cells along with the adenoviral plasmid pJM17 using calcium phosphate precipitation. After plaque purification, AdCMVhGH was amplified in 293 cells, and a crude viral lysate was obtained. This lysate was subjected to CsCl gradient centrifugation and the purified viral band subsequently isolated, and dialyzed into the following buffer: 10 mM Tris, pH 7.4, 0.5 mM $MgCl_2$, 10% glycerol. Whenever dilutions of the virus were made, a similar buffer, except containing 0.1 mM $MgCl_2$, was used. After determining the virus titer (see below), the vector was stored in 100 μl aliquots at −80°C, and used as needed.

Table 1 Experimental treatment groups[a]

Group	*Treatment (IP)*	*Male (n)*	*Female (n)*	*AdCMVhGH dose (particles)*
1	Saline	12	12	None
2	HCQ	12	12	None
3	Saline	12	12	6×10^9
4	HCQ	12	12	1.5×10^{11}
5	HCQ	12	12	6×10^9
6	HCQ	12	12	2.4×10^8

[a]Each study group consisted of an equal number (*n*) of male and female Fischer 344 rats. Animals were administered either saline (intraperitoneally, IP), or hydroxychloroquine (HCQ; 10 mg kg^{-1}). Groups 3–6 were also administered the indicated dose of AdCMVhGH intraductally in their right submandibular gland, as described in Materials and Methods.

On each experimental day (i.e. when vector administration to rat submandibular glands was to be performed; see below), an appropriate number of frozen aliquots of the vector were removed and diluted in the buffer described above to yield the concentrations required for each experimental group (i.e. either 2.4×10^8, 6×10^9 and 1.5×10^{11} particles per gland). These Ad5 vector doses were chosen because they span a dosage range from a lower level that elicits little to no local salivary gland response (Adesanya *et al*, 1996) to an upper level that is slightly greater than the maximum dose (1×10^{11} particles) reported to be without adverse effects in humans (Crystal *et al*, 2002; Harvey *et al*, 2002). Control animals were treated first, followed by those receiving vector (in ascending dosage order). After vector administration, aliquots of the remaining diluted vector were re-checked to confirm the viral doses delivered, and then used to infect 293 cells to test vector function (i.e. hGH secretion into 293 cell culture medium; see below).

Determination of viral titer

Viral titer was determined as follows. First, physical (virus particles) titer was measured using real time quantitative polymerase chain reaction (QPCR). Briefly, the SYBR Green method was used for QPCR. The SYBR Green PCR master mix was obtained from Perkin Elmer (Applied Biosystems Division, Foster City, CA, USA) and QPCR was performed using an ABI Prism 7700 Sequence Detector. The QPCR conditions used were as follows: 95°C for 2 min; 95°C for 10 min; 95°C for 15 s and 60°C for 1 min for 40 cycles. The two primers used here were from the E2b region of Ad5. The sequences are: E2q1 (5′-GCAGAACCACC-AGCACAGTGT-3′ and E2q2 (5′-TCCACGCATTT-CCTTCTAAGCTA-3′), and these primers amplify a 94 bp fragment. QPCR assays were performed in duplicate. Second, we performed a conventional plaque assay to determine the infectious titer of the virus (in plaque forming units, pfu). For plaque assays we used 293 cells that were infected with serial dilutions of the CsCl purified virus suspended in culture medium. One hour following infection, the vector-containing culture medium was aspirated, replaced with culture medium containing 1% agarose and incubated at 37°C. Subsequently, 2 ml of the agarose/culture medium mixture were added on days 5 and 10, and the number of viral plaques at each dilution was determined on day 14.

AdCMVhGH and HCQ administration

The AdCMVhGH vector administration was staggered so that animals from each group were treated over

several days. A total of six groups per gender were used (Table 1; $n = 12$ per group). On study day −1 (24 h prior to infusion of AdCMVhGH) animals in groups 2, 4, 5, and 6 received HCQ (10 mg kg^{-1} in saline) by intraperitoneal (IP) injection, while animals in groups 1 and 3 received an equal volume of saline by IP injection. On study day 1, each animal in each group was anesthetized as described (Delporte *et al*, 1997a,b) with a solution of ketamine (36–42 mg kg^{-1}) and xylazine (4.8–5.6 mg kg^{-1}). A tapered PE 10 polyethylene cannula was then inserted into the right submandibular duct of each animal and fixed in position with glue. After cannulation each animal received atropine (1 mg kg^{-1}) by intramuscular injection. Approximately 10 min after the atropine injection, either AdCMVhGH (groups 3, 4, 5 and 6) or saline (groups 1 and 2) was infused into the duct via an insulin syringe connected to the cannula, the cannula was left in place for 10 min after administration and thereafter removed. A different syringe and cannula was used for each animal. Immediately after administration of vector or saline, animals in groups 2, 4, 5 and 6 were again given HCQ (10 mg kg^{-1}) by IP injection, while animals in groups 1 and 3 were given an equal volume of saline by the same route. On study day 2, HCQ or saline IP injections were repeated.

Measurement of hGH

The hGH protein was measured using the Nichols Diagnostics (San Clemente, CA, USA) assay kit according to the manufacturers directions, as described (He *et al*, 1998). Each time an assay was performed, a complete set of standards, as obtained from the company, was included. Frequently, two dilutions of each sample were tested, to ensure that all hGH levels measured were within the linear range of the assay.

Clinical assessments

Animals in all treatment groups (Table 1) were observed twice daily for toxicological signs, moribundity and mortality. Additionally, a detailed, hands-on individual clinical evaluation was carried out on days 1, 3 (only those scheduled for sacrifice), 8, 15, 22 and 29. Body weights, shown in Figure 1, were measured on test days −1, 1, 2, 3 (only those scheduled for killing), 8, 15, 22 and 28. Animals were fasted (food only) overnight prior to sacrifice. Food consumption and water intake were measured as indicated in Figures 2 and 3. Blood and saliva collections were staggered to correspond to the vector administration start date, with four rats/group/gender sacrificed on day 3 and the remaining rats sacrificed on day 29. Following anesthesia, as above, animals were given a subcutaneous injection of pilocarpine (5 mg kg^{-1} in double-distilled water) and whole saliva was collected and the volume measured gravimetrically (Delporte *et al*, 1997a,b). Saliva samples were stored at −20°C in two aliquots; one each for QPCR determination of replication competent adenovirus (RCA; see below) and measurement of hGH. Thereafter, blood was collected from the retro-orbital plexus. Whole blood was used for clinical hematology

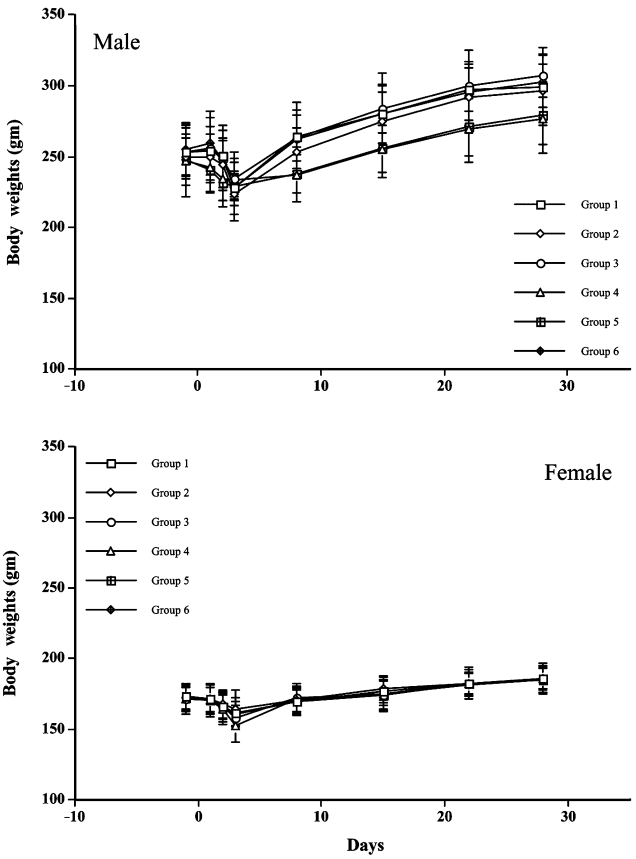

Figure 1 Effect of adenoviral vector ±hydroxychloroquine (10 mg kg^{-1}) on body weight in male and female rats. Group 1, saline; Group 2, hydroxychloroquine (HCQ); Group 3, 6×10^9 particles AdCMVhGH per gland; Group 4, 1.5×10^{11} particles AdCMVhGH per gland +HCQ; Group 5, 6×10^9 particles AdCMVhGH per gland +HCQ; Group 6, 2.4×10^8 particles AdCMVhGH per gland +HCQ. Results with male rats are in the upper panel and results with female rats are in the lower panel. Data shown are mean values ± s.d. See text for additional details

evaluations and for determinations of RCA. The clinical hematology parameters measured included the following: erythrocyte (RBC) count, RBC morphologic assessment, hematocrit, hemoglobin, mean corpuscular volume (MCV), packed cell volume, mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), white blood cell (WBC) count, WBC differential, reticulocyte count, platelet count and morphologic assessment. Serum samples were analyzed for the following components: alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), sorbitol dehydrogenase (SDH), albumin, amylase, creatine kinase (CK), globulin, total protein, urea nitrogen (BUN), creatinine, glucose, total bile acids, chloride and sodium.

Necropsy and tissue handling

To determine the distribution of AdCMVhGH in all animals, as well as the presence of any pathological response to vector delivery, an extensive, systematic necropsy was performed on all animals at the time of scheduled sacrifice. The manner in which this necropsy

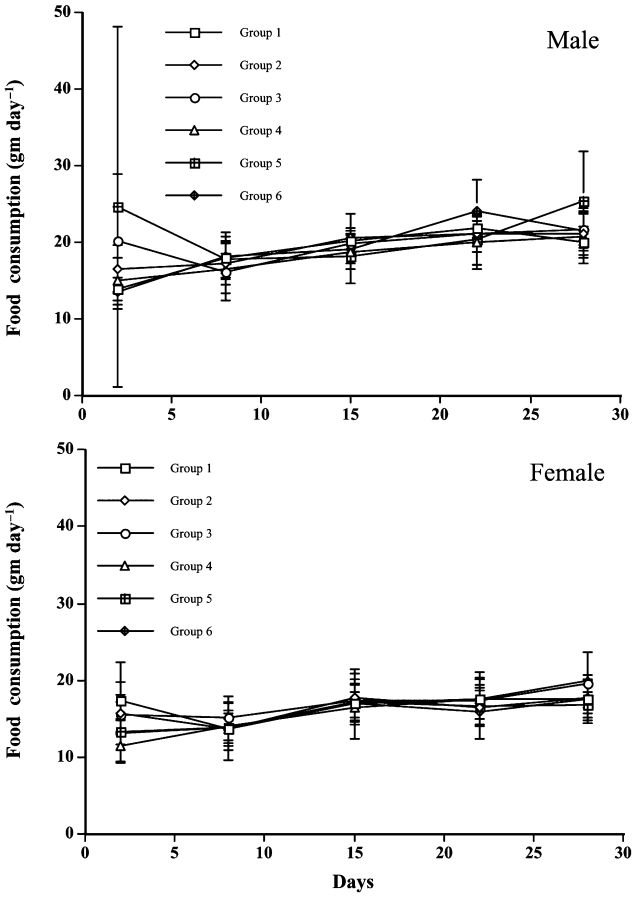

Figure 2 Effect of adenoviral vector ±hydroxychloroquine (10 mg kg^{-1}) on food consumption by male and female rats. Experimental groups are the same as described in the legend to Figure 1. Results with male rats are in the upper panel and results with female rats are in the lower panel. Data shown are mean values ± s.d. See text for additional details

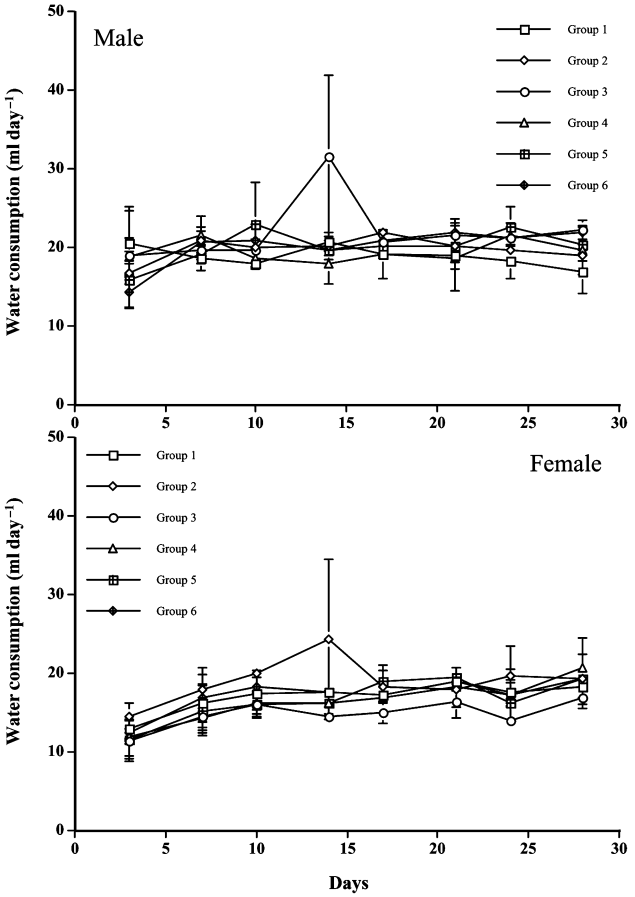

Figure 3 Effect of adenoviral vector ±hydroxychloroquine (10 mg kg^{-1}) on water consumption by male and female rats. Experimental groups are the same as described in the legend to Figure 1. Results with male rats are in the upper panel and results with female rats are in the lower panel. Data shown are mean values ± s.d. See text for additional details

was conducted has been previously described (O'Connell *et al*, 2003). Briefly, gloves were changed between animals or following inadvertent contact with blood or tissue, and individual instrument sets were used for each organ with each animal studied. All instruments were cleaned with Alconox (Alconox Inc., White Plains, NY, USA), sequentially soaked in 0.3 N HCl, 1 N NaOH, 10% bleach, then rinsed with water prior to use. Necropsies were performed in a hood, sprayed with 0.1 N HCl, and animals were placed on fresh absorbent pads prior to the procedure. The order of necropsy was arranged so that animals from treated groups and their appropriate control group were necropsied on the same days, with control animals preceding vector treated animals. Any gross lesions detected were examined in all animals. At necropsy portions of the testis/ovary, spleen, liver, lungs, heart, the draining lymph node, and right and left submandibular glands were flash frozen in liquid nitrogen, stored at −60°C, and subsequently processed for QPCR assays to detect AdCMVhGH (see below). Additionally, we obtained an extensive set of tissues for histopathology (Elmore *et al*, in press). Tissues were trimmed, embedded, sectioned (5–6 μm) and stained with hematoxylin and eosin for evaluation.

Determination of AdCMVhGH tissue distribution

Genomic DNA was extracted from frozen tissue samples using the Qiagen 96 Tissue Kit (Valencia, CA, USA). DNA was quantified by absorbance at A_{260} and stored at −15°C until assayed. Blood samples were stored at 2–8°C until extraction using either the QIAamp Blood kit or QIAamp Spin Blood kit. The positive control was the plasmid pOGH, encoding the hGH gene (Selden *et al*, 1986) and the negative control was rat genomic DNA. In addition, each tissue sample was spiked with 100 copies of pOGH to assess the presence of PCR assay inhibitors. For QPCR we used the following primers to detect the hGH gene: forward primer (5′ GGC TTT TTG ACA ACG CTA TGC 3′) and reverse primer (5′ GTA GGT GTC AAA GGC CAG CTG 3′). These primers were chosen using Primer Express Primer Design software (PE Applied Biosystems), as was the internal fluorogenic probe (5′ CCG CGC CCA TCG TCT GCA 3′; labeled with the 6-FAM reporter dye; PE Applied Biosystems). QPCR was

performed using 1 μg genomic DNA and AmpliTaq Gold DNA polymerase on an ABI Prism 7700/7900 Sequence Detection System (PE Applied Biosystems) with the following optimized conditions: 50°C for 2 min (one cycle), 95°C for 10 min (one cycle), 95°C for 15 s and 60°C for 1 min (40 cycles). This assay was able to detect 10 copies of vector in 1 μg genomic DNA, and was linear over a template range from 10^1 to 10^5 vector copies.

Evaluation of replication competent adenovirus
The QPCR assay for RCA employed a similar approach and methods to those described above for detecting AdCMVhGH. For this assay the positive control was pE1B (BioReliance, Rockville, MD, USA), encoding the adenoviral E1B region, which is absent in AdCMVhGH but present in RCA. As above the negative control was rat genomic DNA, and all tissue samples were spiked with 100 copies of pE1B to assess for assay inhibitors. Saliva (stored at −20°C until assayed) and blood samples were extracted using either the QIAamp Blood kit or QIAamp Spin Blood kit. The primers used were BREL219 and BREL220, and the probe BREL031 (all from BioReliance). This assay used the same conditions as above and also was able to detect 10 copies of the E1B gene in 1 μg genomic DNA, while being linear over a template range from 10^1 to 10^5 vector copies.

Statistical analysis
Body weight data were analyzed with the parametric multiple comparisons procedure of Dunnett (1955). Hematology and clinical chemistry data were analyzed using the nonparametric multiple comparisons procedures of Shirley (1977) and Dunn (1964).

Results and Discussion

Vector characteristics and activity
For the experiments presented herein a single preparation of AdCMVhGH was used (1.1×10^{13} particles ml^{-1}; infectious titer was 1.4×10^{11} pfu ml^{-1}). Prior to *in vivo* experiments, the vector was assayed for expression of hGH by infection of 293 cells *in vitro* (He *et al*, 1998). At a multiplicity of infection (pfu - cell^{-1}) of approximately 16, infection with this preparation led to the production of high levels of hGH in 293 cell culture medium (on average approximately 270 ng ml^{-1}). Additionally, following delivery of the vector to animals, remaining vector in each dosage group (animals groups 3–6) was again tested for function (direction of hGH production). All delivered vector dilutions were functional, and that function was vector dose-dependent (data not shown).

General clinical findings
All male and female rats in this study survived until either the scheduled or terminal sacrifices. There were no toxicologically significant clinical signs noted in rats of either gender over the entire course of this study (Table 2).

Table 2 Effect of adenoviral vector delivery ±HCQ on clinical toxicology[a]

		Clinical signs	
Group	*Mortality*	*Male*	*Female*
1	0/24	0/12	0/12
2	0/24	0/12	Body cuts (2/12)
3	0/24	0/12	0/12
4	0/24	0/12	Body cuts (2/12) Alopecia (2/12)
5	0/24	0/12	Body cuts (2/12) Alopecia (2/12)
6	0/24	0/12	Alopecia (2/12)

[a]Study groups are the same as described in Table 1. Animals were individually examined prior to sacrifice. Groups 2, 4, 5, and 6 received hydroxychloroquine (HCQ, 10 mg kg^{-1}). All rats survived to the scheduled day of sacrifice. None of the male rats showed any clinical toxicological signs, while occasional minor clinical changes (body cuts, alopecia), apparently unrelated to experimental treatments, were seen in a few female mice in each group. See text for additional details.

Rats in all experimental groups gained weight over the course of this study (Figure 1). Initially, all experimental groups showed a transient weight loss immediately following vector delivery. Thereafter, all female rats, and most male rats, began to gain weight. However, for male rats in study groups 4 and 5 (the two highest vector dosage groups; 1.5×10^{11} and 6×10^9 particles per gland, plus HCQ, respectively), this transient weight loss extended from days 2–8. Only after day 8 did these animals begin to gain weight at rates similar to that seen in the other treatment groups (Figure 1), however mean body weights of males in groups 4 and 5 remained significantly lower ($P \leq 0.05$) than either the saline-treated or HCQ-treated groups for the remainder of the study. Mean body weights of males in groups 3 and 6, as well as all groups of females, were not significantly different from those of the saline and HCQ control groups at any point in the study (see legend to Figure 1).

Interestingly, there were no significant differences in food consumption among all studied animal groups in both genders (Figure 2). In general, greatest inter-group variability was seen between days 1 and 2, i.e. immediately following the vector delivery procedures to a single submandibular gland. This variability appeared independent of treatment group and was likely related to anesthesia and other procedures involving duct cannulation in a small animal. Thereafter, food consumption was more consistent, although males generally consumed more food than females. Similarly, we detected no overall group differences in water consumption (Figure 3). While there were sporadic differences in both genders at individual time-points, these differences were few in number, did not occur in either a dose- or treatment-related manner, and thus likely unrelated to the administration of AdCMVhGH.

It is unclear why males in groups 4 and 5 showed a more sustained weight loss initially after vector delivery. Female rats receiving these same doses showed no such effects over this time frame. Furthermore, all animal

groups exhibited similar rates of food and water consumption. If vector per se, or the delivery methods, were locally injurious to salivary glands to a significant extent or more generally intra-orally, we would expect to see similar changes in female rats, which have considerably lower body weights (approximately 30% lower than males). Nonetheless, there is a well-recognized sexual dimporphism in rodent submandibular gland structure and gene expression (e.g. Treister *et al*, 2005) and, conceivably, this may have influenced the observed responses to experimental treatments. In any event, the observed lag in weight gain in these two groups was rectified, and from days 8 through 28 the patterns of body weight gain by male rats in study groups 4 and 5 were parallel to those of all other male rat groups (Figure 1a), although actual weights remained approximately 10% lower. In previous studies, similar doses of adenoviral vector administered to salivary glands in the absence of HCQ were without any effect on body weight in rats of a different strain (O'Connell *et al*, 2003).

Clinical chemistry and hematology parameters

We examined multiple clinical laboratory parameters on study days 3 and 29 (Tables 3 and 4). On day 3, we detected one statistically significant change that occurred in a manner indicating a possible relationship to vector delivery, i.e. elevations in serum globulin levels ($P \leq 0.05$). Serum globulin values were elevated in male rats in all three AdCMVhGH vector (plus HCQ) treatment groups when compared with the HCQ control group. Furthermore, this finding was generally corroborated by findings with female animals in the high-dose vector plus HCQ treatment group, and likely relates to inflammatory reactivity in response to the vector (Elmore *et al*, in press; also see below, histopathological findings). In addition, we found several random statistically significant differences in clinical pathology parameters that did not occur in a manner indicating a possible vector-related effect. These included increases in ALP and amylase levels, which were statistically significant ($P \leq 0.05$) in the mid-dose male rats without HCQ, statistically significant differences for albumin (decrease) and absolute monocytes (increase) in the high-dose female rats plus HCQ, and for hemoglobin and hematocrit (both decreased) in the mid-dose female rats plus HCQ when compared with the HCQ control animals. In general, any differences in clinical pathology observed on day 3 had reversed by day 29. At the latter time-point, a few other random statistically significant differences in clinical pathology parameters again were noted, but as above these did not occur in a manner indicating a possible vector-related effect (not shown).

Table 3 Effect of high dose adenoviral vector delivery ±HCQ on clinical chemistry values[a]

	Saline	*HCQ*	*AdCMVhGH*[b] *+HCQ*
Albumin (g dl^{-1})	4.0 ± 0.1	3.9 ± 0.2	3.7 ± 0.1
ALT (IU l^{-1})	58 ± 13	59.5 ± 15	69 ± 7.3
Amylase (IU l^{-1})	1936 ± 110	1829 ± 363	1693 ± 180
BUN (mg dl^{-1})	18 ± 1.4	19.5 ± 0.6	19.3 ± 1.7
Globulin (g dl^{-1})	2.3 ± 0.1	2.4 ± 0.1	2.85 ± 0.2[c]
Glucose (mg dl^{-1})	131 ± 37	117 ± 12	107 ± 26
LDH (IU l^{-1})	560 ± 179	475 ± 366	448 ± 322
Cl^- (mM)	98.8 ± 1.7	97.8 ± 2.8	91.0 ± 12.7
Na^+ (mM)	146.5 ± 1.7	147.8 ± 0.5	138.8 ± 18
Total protein (g dl^{-1})	6.3 ± 0.2	6.3 ± 0.2	6.6 ± 0.3

[a]Data are the mean ± s.d. of results from male rats at day 3 ($n = 4$ per group) and are representative of all clinical chemistry data. Animals were administered either saline or hydroxychloroquine (HCQ; 10 mg kg^{-1}; intraperitoneally). Results with female rats, and with rats receiving lower doses of AdCMVhGH were similar.
[b]1.5×10^{11} particles administered to the right submandibular gland.
[c]$P \leq 0.05$ compared with the group receiving HCQ alone (all three experimental male groups receiving AdCMVhGH +HCQ showed significant elevations in serum globulin levels). No other biologically significant differences in clinical chemistry values were seen between treatment groups on days 3 and 29. See text for additional details.

Table 4 Effect of high dose adenoviral vector delivery on clinical hematology values[a]

	Saline	*HCQ*	*AdCMVhGH*[b] *+HCQ*
Hemoglobin (g dl^{-1})	15.0 ± 1.0	15.5 ± 0.2	15.0 ± 0.3
Hematocrit (%)	43.1 ± 2.6	44.1 ± 0.5	42.5 ± 0.3
Platelets (10^3 μl^{-1})	530 ± 100	591 ± 76	555 ± 80
RBC (10^6 μl^{-1})	8.0 ± 0.5	8.2 ± 0.9	7.9 ± 0.2
WBC (10^3 μl^{-1})	12.1 ± 1.4	10.0 ± 1.1	11.7 ± 1.2

[a]Data are the mean ± s.d. of results from female rats at day 3 ($n = 4$ per group) and are representative of all clinical hematology data. Animals were administered either saline or hydroxychloroquine (HCQ; 10 mg kg^{-1}; intraperitoneally). Results with male rats, and with rats receiving lower doses of AdCMVhGH were similar.
[b]1.5×10^{11} particles administered to the right submandibular gland. There was no consistent pattern of significant differences in hematology values between treatment groups on days 3 and 29. See text for additional details.

Biodistribution of AdCMVhGH

We next evaluated the biodistribution of AdCMVhGH by QPCR analysis of DNA extracted from multiple tissues using an assay able to detect 10 vector copies μg^{-1} genomic DNA (Tables 5 and 6). With the exception of lung in one animal (Group 2, HCQ alone, day 29; not shown), the tissues from the control animals tested negative for the presence of AdCMVhGH sequences. At day three postadministration (Table 5), a high percentage of vector-treated animals showed a strong positive signal, i.e. over 1×10^4 copies, in the targeted right submandibular salivary gland. Many animals (at the mid- and high-vector doses) also showed evidence for vector presence (10^2–10^3 copies) in the contralateral left submandibular salivary gland. As shown in Table 5, a dose-related increase in AdCMVhGH dissemination was observed in tissues other than the targeted right submandibular gland on day 3. By day 29 post vector administration (Table 6), most of the AdCMVhGH-treated animals showed a positive signal for vector presence in the targeted right submandibular gland at all doses. Overall, the levels of AdCMVhGH in the right submandibular gland had decreased substantially at this later time-point

Table 5 Frequency of detection of AdCMVhGH in rat tissues on day 3

Tissues	*Group 2 (0 particles per gland)*	*Group 4 (1.5×10^{11} particles per gland)*	*Group 5 (6×10^{9} particles per gland)*	*Group 6 (2.4×10^{8} particles per gland)*
Heart	0/7	0/5	0/8	0/6
Lungs	0/6	1/6	1/7	0/8
Liver	0/8	1/4	1/8	0/8
Spleen	0/7	3/4	1/7	0/6
Gonads	0/7	0/7	0/8	0/8
Blood	0/8	1/5	0/7	0/7
Right SMG	0/6	8/8	6/6	8/8
Left SMG	0/8	8/8	4/6	1/4
Total non-SMG tissue	0/43	6/31	3/45	0/43

AdCMVhGH was administered to the right submandibular gland (SMG) of male and female rats. Three days later the indicated tissue samples were obtained at necropsy and later assayed by QPCR for the presence of AdCMVhGH. See Materials and Methods for details. The four groups listed correspond to the AdCMVhGH dose in particles per gland administered that received HCQ. Gonads: testes or ovaries. The data show the number of positive QPCR results (numerator) found for the number of informative assays performed (denominator) with each tissue. Results for groups 1 and 3 were similar to those for groups 2 and 5.

Table 6 Frequency of detection of AdCMVhGH in rat tissues on day 29

Tissues	*Group 2 (0 particles per gland)*	*Group 4 (1.5×10^{11} particles per gland)*	*Group 5 (6×10^{9} particles per gland)*	*Group 6 (2.4×10^{8} particles per gland)*
Heart	0/13	0/14	3/15	0/15
Lungs	1/14	1/9	2/13	0/12
Liver	0/13	2/13	3/15	0/11
Spleen	0/16	3/6	4/12	1/13
Gonads	0/13	1/13	0/12	0/14
Blood	0/14	0/13	2/15	0/13
Right SMG	0/11	6/8	9/12	6/9
Left SMG	0/14	10/11	5/8	0/11
Total non-SMG tissue	1/83	7/68	14/82	1/78

AdCMVhGH was administered to the right submandibular gland (SMG) of male and female rats. On day 29 the indicated tissue samples were obtained at necropsy and later assayed by QPCR for the presence of AdCMVhGH. See Materials and Methods for details. The four groups listed correspond to the AdCMVhGH dose in particles per gland administered that received HCQ. Gonads: testes + ovaries. The data show the number of positive QPCR results (numerator) found for the number of informative assays performed (denominator) with each tissue. Results for groups 1 and 3 were similar to those for groups 2 and 5.

(to approximately 10^2–10^3 copies). We also detected vector in the left submandibular salivary gland at day 29 in a dose-dependent manner and at reduced levels, i.e. <100 copies except for several rats of the high vector dose group (approximately 50%; approximately 10^2–10^3 copies). In rats of both genders, vector was rarely seen in non-salivary gland tissues on day 29 (22 of 228 informative assays). Of these positive results, eight were in spleen and five were in liver).

Overall, these results indicate that 3 days after an intraductal cannulation of the right submandibular gland in rats AdCMVhGH distributes to all the tissues analyzed with the exception of gonads and heart, a finding similar to what we reported previously after administration of a similar first generation adenoviral vector without HCQ (O'Connell *et al*, 2003). In part this is likely because of rat salivary glands being less well encapsulated than their human counterparts. Also similar to our previous report, substantial differences were observed between the various tissues analyzed in terms of level and occurrence of AdCMVhGH. Thus, at the low-dose level (2.4×10^8 particles), AdCMVhGH appeared to localize preferentially in the right submandibular gland where it could persist up to 29 days. At the mid-dose level (6×10^9 particles), the vector was found distributed to all tissues except heart, gonads, right lymph node and blood at day 3. At the high-dose level (1.5×10^{11} particles), the vector was found distributed to all tissues, except heart and gonads at day 3. At day 29, with the exception of right submandibular gland, left submandibular gland, spleen and liver, most organs in the vast majority (approximately 85%) of the animals were negative for the presence of the AdCMVhGH, thus, providing evidence of a trend towards vector clearance over time.

Of note, 3 days after administration of the AdCMVhGH vector to the treatment groups (3, 4, 5 and 6), none of the animals tested positive for the presence of RCA in either the blood or saliva. This is particularly important, as the presence of RCA in saliva, a body fluid with considerable infectious potential, would be a significant concern. This result is similar to what we

reported in our earlier study (O'Connell *et al*, 2003), however, in that study only four animals were tested *vs* all animals sacrificed on day 3 in the present study.

Necropsy and histopathological findings

No abnormal vector-related findings were noted at gross necropsy, for rats examined on both days 3 and 29, suggesting the absence of any effects as a consequence of AdCMVhGH administration ± HCQ. At a microscopic level, with the exception of dose-related active inflammation observed on day 3 in the targeted salivary glands, such as previously reported by us (Adesanya *et al*, 1996; O'Connell *et al*, 2003; Elmore *et al*, in press), there were no other vector-related or toxicological effects that were considered biologically significant. These inflammatory changes in salivary glands were considered reversible, as indicated by the observed resolution or lack of this effect in salivary glands from all day 29 animals that had received vector.

Conclusion

As in our previous study with a different adenoviral vector (O'Connell *et al*, 2003), the AdCMVhGH vector, in the presence or absence of HCQ, appeared to be associated with very limited toxicities, even at the highest dose administered (1.5×10^{11} particles to a single gland). This dose is 50% higher than the dose of similar first generation adenoviral vectors shown to be without adverse effect in humans (Crystal *et al*, 2002; Harvey *et al*, 2002), despite an approximately 200-fold difference in recipient weight. These findings also support the notion that salivary glands may provide a useful gene transfer target site for both local and systemic applications.

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