Local immunomodulatory gene therapy for Sjögren’s syndrome
Lodde, B.M.

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Local Adeno-Associated Virus-Mediated Interleukin-10 Gene Transfer Has Disease Modifying Effects in a Murine Model of Sjögren’s Syndrome

Marc R. Kok¹,², Seichii Yamano¹, Beatrijs M. Lodde¹,², Jianghua Wang¹, Ross I. Couwenhoven³, Shoshana Yakar⁴, Antony Voutetakis⁵, Derek Leroith⁴, Michael Schmidt¹, Sandra Aflone¹, Stanley R. Pillemer¹, Marjorie T. Tsutsui¹, Paul P. Tak⁶, John A. Chiorini¹, Bruce J. Baum¹

¹ Gene Therapy and Therapeutics Branch/NIDCR, National Institutes of Health, DHHS, Bethesda, MD, USA;
² Clinical Immunology and Rheumatology, Academic Medical Center/University of Amsterdam, Amsterdam, the Netherlands;
³ Department of Diagnostic Sciences and Pathology, Dental School, University of Maryland, Baltimore MD, USA;
⁴ Diabetes Branch/NIDDK, National Institutes of Health, DHHS, Bethesda, MD, USA.

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Abstract

OBJECTIVE: Female non-obese diabetic (NOD) mice develop spontaneous autoimmune sialadenitis and loss of salivary flow, and are a widely used model of Sjögren's syndrome (SS). We examined the feasibility of local salivary gland immunomodulatory gene delivery to alter these sequelae in NOD mice. METHODS: We constructed recombinant adeno-associated viral (rAAV) vectors encoding either human interleukin-10 (rAAVhIL-10) or β-galactosidase (rAAVLacZ, control vector). Mice received rAAVhIL-10 or rAAVLacZ by retrograde submandibular ductal instillation either at age 8 weeks (early, before onset of sialadenitis), or at 16 weeks (late, after onset of sialadenitis). As a systemic treatment control, separate mice received intramuscular delivery of rAAVhIL-10 at each time point. RESULTS: Both submandibular and intramuscular delivery of vector led to low circulating levels of hIL-10. After submandibular administration of rAAVhIL-10, salivary flow rates at 20 weeks for both the early and late treatment groups were significantly higher than for both rAAVLacZ-administered and untreated mice. Systemic delivery of rAAVhIL-10 led to improved salivary flow in the late treatment group. Inflammatory infiltrates in submandibular glands, however, were significantly reduced only in mice receiving rAAVhIL-10 locally in the salivary gland compared with mice receiving this vector intramuscularly, rAAVLacZ or no treatment. In addition, after submandibular rAAVhIL-10 delivery, NOD mice exhibited significantly lower blood glucose, and higher serum insulin, levels than all other groups, indicating some systemic benefit of this treatment. CONCLUSIONS: These studies show that expression of hIL-10 by rAAV vectors can have disease-modifying effects in the salivary glands of NOD mice and suggest that local immunomodulatory gene transfer may be useful for managing the salivary gland pathology in SS.
Introduction

Sjögren’s syndrome (SS) is an autoimmune disease, characterized by a focal and diffuse lymphoid cell infiltration into the salivary and lacrimal glands, that affects 0.3-4.8% of the population in the United States. The consequence of chronic immune cell activation in these exocrine glands is diminished secretory function, which leads to symptoms of dry mouth and dry eyes. The non-obese diabetic (NOD) mouse, which is recognized as an animal model for autoimmune type I, insulin-dependent diabetes mellitus, also develops exocrine gland infiltrates and, as in SS, a loss of salivary flow that is age and gender dependent. The female NOD mouse is considered an excellent model for SS. Analyses of lymphocyte populations infiltrating the exocrine tissues have revealed similar profiles in the salivary glands of NOD mice and in the salivary glands of patients with SS, with a predominance of CD4⁺ T cells and significantly fewer CD8⁺ T cells or B cells. In addition, we and others have previously shown that the destructive immune response in salivary glands of NOD mice is biased toward a helper T cell type 1 (Th1) like profile. Upregulation of pro-inflammatory cytokine genes may be responsible or necessary for the development of autoimmune diseases.

Interleukin-10 (IL-10) is a homodimeric cytokine with a wide spectrum of immunosuppressive activities. IL-10 is a potent inhibitor of tumor necrosis factor-α (TNF-α), IL-1, IL-6, IL8, and granulocyte-macrophage colony stimulating factor (GM-CSF) synthesis by monocytes and macrophages. IL-10 also directly suppresses the synthesis of interferon-γ (IFN-γ) by Th1 lymphocytes in vitro. Administration of IL-10 protein has proven therapeutically useful in several preclinical models of autoimmune diseases. Several studies, using both viral and non-viral vectors, have reported that treatment with the IL-10 gene, with or without the IL-4 gene, can inhibit the development of autoimmune diseases, including type I diabetes mellitus in NOD mice. As an alternative to cytokine protein therapeutics, cytokine-based gene therapy would obviate the need for multiple injections as typically required with protein delivery. To our knowledge, there are no published reports examining any effects of either IL-10 protein or gene therapy on the autoimmune sialadenitis that occurs in female NOD mice.

Salivary glands offer an unusual, but potentially useful, target site for both local and systemic gene therapeutics. For example, using recombinant adenoviral vectors we have shown that gene transfer to salivary glands can lead to the augmentation of saliva with an antifungal polypeptide, as well as provide the delivery of biologically active growth hormone into the bloodstream. Furthermore, undesirable vector dissemination is limited beyond the well encapsulated salivary glands. However, in salivary as in other cell types, recombinant ade-
Adeno-associated virus (AAV) is a unique, non-pathogenic member of the Parvoviridae family of small, single-stranded DNA animal viruses. Vectors based on the serotype 2 recombinant AAV (rAAV) have proven to be especially useful for in vivo gene transfer.\textsuperscript{35-37} rAAV vectors infect both dividing and non-dividing cells, elicit relatively modest host immune responses compared with adenoviral vectors,\textsuperscript{38-40} and have demonstrated long-term persistence in a number of in vivo studies. We have shown that rAAV serotype 2 vectors are able to mediate therapeutic gene transfer (human IL-10 [hIL-10], and a plasma membrane water channel, aquaporin 1) to murine salivary glands.\textsuperscript{41,42} Because of the positive features offered by rAAV-mediated gene transfer, we hypothesized that an rAAV encoding hIL-10 could be useful to modulate inflammatory reactions locally in the salivary glands of patients with SS.\textsuperscript{43} In the present study, for the first time, we show the ability of in vivo immunomodulatory gene transfer by rAAVhIL-10 to blunt the immunopathological and functional sequelae characteristic of SS-like autoimmune sialadenitis in NOD mice.

Materials and Methods

Cell lines
The human embryonic kidney (HEK) 293 T cell line expresses the simian virus 40 (SV40) large T antigen in a stable manner.\textsuperscript{44} The COS cell line is derived from monkey kidney epithelial cells.\textsuperscript{45} 293 T and COS cells were grown in Dulbecco’s modified Eagle’s medium (DMEM). All media were supplemented with 10% heat-inactivated (55°C; 30 minutes) fetal bovine serum (Invitrogen Life Technologies, Gaithersburg, MD, USA), 2 mM glutamine, penicillin (100 U/mL), and streptomycin (100 mg/mL; Biofluids, Rockville, MD, USA).

Mice
Animal studies were approved by the National Institute of Dental and Craniofacial Research (NIDCR) Animal Care and Use Committee and the National Institutes of Health (NIH) Biosafety Committee. All procedures were conducted in accordance with IASP (International Association for the Study of Pain) standards. Female NOD/LtJ mice (stock 001976) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA), and male Balb/c mice were obtained from the Division of Cancer Treatment (National Cancer Institute [NCI], Bethesda, MD, USA). Mice were maintained throughout the course of the study in the NIDCR animal facility (Bethesda, MD) in accordance with Federal guidelines.
Blood glucose levels were measured once per week starting at age 12 weeks, using a OneTouch monitor (LifeScan, Milpitas, CA, USA). All mice with blood glucose levels ≥400 mg/dL were given Ultralente insulin (Eli Lilly, Indianapolis, IN, USA) injections (4 U/mouse, every 24 hours) to limit diabetes-related dehydration. We chose not to treat mice until this rather high blood glucose level was reached, because we wished to examine their insulin production. Serum insulin levels were measured with a rat insulin radioimmunoassay kit with a sensitivity of 0.1 ng/mL (LINCO Research, St. Charles, MO, USA).

**Gene transfer, and saliva and serum collection**

The experiments described in this paper represent one of three separate studies, over the indicated 8- to 20-week time course, that we conducted to investigate the effects of rAAVhIL-10 administration on salivary gland function and histopathology in NOD mice. All three studies gave comparable results, regarding the efficacy of salivary gland delivery. However, in two earlier studies that are not presented herein, the only control group used consisted of untreated mice. Mild anesthesia was induced with ketamine (60 mg/mL, 1 mL/kg body weight; Phoenix Scientific, St. Joseph, MO, USA) and xylazine (8 mg/mL; Phoenix Scientific) solution given intramuscularly. Six study groups were employed. After an intramuscular injection of atropine (0.5 mg/kg; Sigma, St. Louis, MO, USA), two groups of female NOD mice received early treatment (8 weeks; before onset of sialadenitis) with either rAAVhIL-10 (n = 4) or rAAVLacZ (n = 3) and two groups received late treatment (16 weeks; after onset of sialadenitis) with either rAAVhIL-10 (n = 6) or rAAVLacZ (n = 4) administered to both submandibular glands by retrograde ductal instillation (2.5x10^{10} genomes per gland). Two additional groups of mice received, at 8 weeks (n = 6) or 16 weeks (n = 5), intramuscular injections of rAAVhIL-10 into the quadriceps muscle (5x10^{10} genomes). Salivary flow rates were measured at 8 weeks (baseline, untreated, not manipulated before saliva collection) and at 12, 16, and 20 weeks of age. Blood glucose and insulin levels were measured at 16 and 20 weeks of age. Whole saliva was collected after stimulation of secretion, using pilocarpine (0.5 mg/kg body weight) administered subcutaneously. Saliva was obtained from the oral cavity by micropipette and placed into pre-weighed 0.5-mL microcentrifuge tubes, and volume was determined gravimetrically as previously described, with comparable results.

**Construction of rAAVhIL-10 and rAAVLacZ**

We previously reported construction of rAAVhIL-10 and rAAVLacZ (encoding β-galactosidase). For the present study, a plasmid containing hIL-10 cDNA (0.6 kb), pH15C, was obtained from the American Type Culture Collection (Manassas, VA, USA), and was amplified to include NotI sites for cloning by PCR employing a GeneAmp PCR reagent kit (Applied Biosystems, Foster City, CA,
USA). The PCR-amplified hIL-10 cDNA was inserted into the NotI site of pAAV to yield the plasmid pAAVhIL-10, with the hIL-10 cDNA driven by the cytomegalovirus promoter-enhancer. To generate the rAAV vectors, the adenoviral helper packaging plasmid pDG (a gift from Prof. J.A. Kleinschmidt) was used. Plates (15 cm) of ~40% confluent 293 T cells were cotransfected with either pAAVhIL-10 or pAAVLacZ, and pDG at a ratio of 1:2, using a calcium phosphate precipitation procedure. Two days after transfection cells were harvested. Clarified cell lysates were adjusted to a refractive index of 1.372 by addition of CsCl and centrifuged at 38,000 rpm for 65 hours at 20ºC. Equilibrium density gradients were fractionated and fractions with a refractive index of 1.369–1.375 were collected and stored at 4ºC. Immediately before use for experiments, the virus was dialyzed against 0.15 M NaCl. The titer of DNA physical particles in rAAV stocks was determined by real-time PCR (see below; Applied Biosystems). Infectious vectors were demonstrated by infecting COS cells with 2 µl of each CsCl fraction in the presence of 2.4x10^8 particles of wild-type adenovirus. As appropriate, either supernatants from infected cells were analyzed in an ELISA for hIL-10 (see below) or cells were stained for β-galactosidase activity with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal).

**Quantification of cytokines**

Secretion of hIL-10 and IFN-γ in cell culture media, mouse sera and saliva was determined with an ELISA, using commercial kits for hIL-10 (R&D Systems, Minneapolis, MN, USA and Biosource International, Camarillo, CA, USA) and mouse IFN-γ (mIFN-γ; Endogen, Woburn, MA, USA). The lower limit of detection was 5 pg/mL for hIL-10 in experiments performed in vitro, 0.2 pg/mL in a high sensitivity assay for hIL-10 in experiments performed in vivo, and 10 pg/mL for mIFN-γ in experiments performed in vivo. Assays were performed according to the manufacturers’ instructions. The levels of several cytokines were also determined after extraction of soluble protein from murine salivary glands. After measuring the wet weight, salivary glands were homogenized in buffer (phosphate buffered saline [PBS] and complete protease inhibitor cocktail; Roche Molecular Biochemicals, Indianapolis, IN, USA). Thereafter, crude extracts were centrifuged at ~325 x g and total protein in the supernatant was determined with a Bio-Rad (Hercules, CA, USA) protein assay according to the manufacturer’s instructions. mIL-4, mIFN-γ, mIL-6, mIL-12, mRANTES, mIL-10, and hIL-10 were measured commercially in SearchLight proteome arrays (Endogen), which are multiplexed assays involving a sandwich ELISA procedure.

**Determination of serum antibodies to hIL-10**

Initially, to determine the presence of antibodies directed against hIL-10 in NOD mouse sera, aliquots of undiluted sera from animals of different rAAV vector treatment groups were obtained at 20 weeks and pooled by group. To 200 mL of
standard concentrations (0.78-50 pg/mL) of recombinant hIL-10 (R&D Systems) 8 ml of pooled serum was added to obtain a final serum dilution of 1:26. The samples were then incubated at 37ºC for 30 minutes. An hIL-10 high sensitivity ELISA, containing polyclonal mouse antihuman antibodies (R&D Systems), was then used to detect hIL-10 protein according to the manufacturer's instructions. A change in the ELISA standard curve provided evidence of the presence of anti-hIL-10 antibodies in NOD mouse sera. Thereafter, serum samples (at dilutions of 1:50-1:2,000) from individual mice were added to 27 pg (in 200 µl) of hIL-10 standard recombinant protein. The samples were incubated at 37ºC for 30 minutes and an hIL-10 ELISA was performed. The maximal serum-mediated reduction in ELISA color development was ~60%. A dilution of serum that reduced this maximum by 50% indicated the titer of anti-hIL-10 antibody present.

Quantitative real-time PCR

Genomic DNA was isolated from submandibular glands, draining lymph nodes, and spleens of all treated mice, using a DNeasy isolation kit (Qiagen, Chatsworth, CA, USA). Quantitative (Q) PCR amplification (final volume 20 µl) of the DNA (100 ng) was performed with an ABI PRISM 7700 sequence detection system (Applied Biosystems) by using the SYBR Green PCR Master Mix (Applied Biosystems) and a specific primer pair (sense: 5'-ATCTACGTATTAGTCATCGCTATTACCATT-3'; and antisense: 3'-TGGAAATCCCCGTGAGTCA-5'; both 0.3 µM) for the cytomegalovirus (CMV) promoter. A PCR thermal profile of holding at 95ºC for 10 min, denaturing at 95ºC for 15 s, and annealing and extending at 60ºC for 1 minute was performed for 40 cycles. A standard curve, employing pAAVCMV and including 100 ng of genomic DNA from untreated animals for each specific tissue, was included for each Q-PCR reaction. Multiple samples of different tissues were included in a single run and the ratio (number of copies) between these samples was used to normalize data obtained from different Q-PCR assays performed during the study. The detection limit of this assay was 10 vector copies.

Histological assessment of submandibular glands

Submandibular glands were removed for histological analyses from NOD mice at the time of sacrifice, at 20 weeks of age, unless otherwise stated, and placed in 10% formalin. After fixation, the tissues were dehydrated in a series of graded ethanol solutions and embedded in paraffin according to standard technique. Sections were cut at a thickness of 5 µm and subsequently stained with hematoxylin and eosin. Histopathologic scoring was performed on the basis of a focus score (in which a focus is an aggregate of 50 or more lymphocytes and histiocytes, per 4 mm²). Foci were counted in at least three different areas of three sections (total of nine fields) from each gland sample, using a graticule at 40x magnification. The results were calculated and expressed as foci per 4 mm².
The focus scores were assessed blindly by three different examiners (MRK, BML, RIC). The mean of the scores determined by each of the three observers was calculated.

Statistical analysis
Descriptive statistics were calculated and reported as means ± SEM. The Student’s t test for unpaired variables was used to compare differences between groups. p-Values less than or equal to 0.05 were considered significant.

Results

Characteristics of rAAVhIL-10 and rAAVLacZ preparations
Aliquots of peak fractions obtained by CsCl centrifugation were pooled after measurement of hIL-10 secretion from, or LacZ expression in, COS cells coinfected with wild-type adenovirus. All experiments reported herein were performed with a single preparation of rAAVhIL-10 or rAAVLacZ having a virus particle titer (i.e., number of genomes) of ~5 x 10^{12} genomes/mL. As reported, we have extensively tested rAAVhIL-10 to show that the encoded transgene product, hIL-10, is functional in vitro and in vivo.42,46

rAAVhIL-10 transduction of salivary glands and quadriceps muscle
Six groups of mice were employed for the present study. NOD mice received either early treatment with rAAVhIL-10 or rAAVLacZ at age 8 weeks or late treatment at 16 weeks, to both submandibular glands by retrograde ductal instillation. The remaining two groups were administered (at 8 or 16 weeks) rAAVhIL-10 via the quadriceps muscle to examine any effect of systemic delivery of the experimental vector. To assess systemic transgene expression, serum hIL-10 levels were determined. At 20 weeks, 3 of 9 early-treated mice (two salivary gland and one intramuscular) and 4 of 11 late-treated mice (two salivary gland and two intramuscular) showed relatively modest, but measurable hIL-10 levels in serum (0.3–9.0 pg/mL; detection limit 0.2 pg/mL). No measurable hIL-10 was detected in serum from animals not treated with rAAVhIL-10. Similar results were found in the two earlier experiments performed after rAAVhIL-10-mediated gene transfer to NOD mouse submandibular glands (data not shown). These results are also comparable to those reported by us after rAAVhIL-10 delivery to salivary glands and quadriceps muscle of Balb/c mice.42,46 In addition, we tested serum samples, obtained at 20 weeks from animals receiving treatment with the rAAVhIL-10 vector at the early time point, for the presence of antibodies to hIL-10. Maximal differences between serum samples were seen at a 1:50 dilution. Most sera tested (8 of 10) from mice receiving rAAVhIL-10 by intramuscular injection and by salivary gland administration exhibited low levels of anti-hIL-10
antibodies (at this dilution leading to ~60% reduction in ELISA color development). Seven of the 10 tested samples showed no detectable serum hIL-10 (all seven sera exhibited the maximal level of anti-hIL-10 antibodies). The remaining three samples had either no detectable antibodies to hIL-10 (two samples, both with clearly measurable serum hIL-10; 3.6 and 9.0 pg/mL) or a relatively low anti-hIL-10 antibody titer and a low level of serum hIL-10 measured (0.3 pg/mL). Importantly, none of the mice (0 of 3) given rAAVLacZ in their salivary glands exhibited antibodies to hIL-10 in their sera (data not shown).

Effect of hIL-10 cDNA delivery on salivary function in NOD mice

The age- and gender-dependent decline in salivary flow rates in NOD mice begins between 8 and 12 weeks of age, and salivary flow rates steadily decrease thereafter. To investigate the effect of rAAVhIL-10 administration on saliva secretion, pilocarpine-stimulated salivary flow rates were measured at 8, 12, 16, and 20 weeks. At 8 weeks, before any treatment, 10 randomly selected mice showed an average salivary flow rate (microliters per 20 minutes; mean ± SEM) of 186 ± 25 (baseline). In the early treatment group, administered rAAVhIL-10 via the submandibular glands, the salivary flow rate was 192 ± 10 at 12 weeks, 168 ± 20 at 16 weeks, and 121 ± 33 at 20 weeks, whereas for intramuscularly treated animals the salivary flow rate was 99 ± 23 at 16 weeks and 100 ± 43 at 20 weeks (Figure 1).

In the late treatment group, the average salivary flow rate (microliters per 20 minutes; mean ± SEM) at 16 weeks (before treatment) was 74 ± 16. After rAAV-hIL-10 administration, salivary flow at 20 weeks was 93 ± 23 with salivary gland delivery and 112 ± 16 with intramuscular treatment. Conversely, for mice receiving rAAVLacZ at week 8 the average salivary flow rate was 125 ± 45 at 12 weeks, 60 ± 16 at 16 weeks (which was similar to the salivary flow rate of untreated mice at this time point), and 28 ± 13 at 20 weeks; and the average salivary flow rate was 31 ± 28 at 20 weeks for mice receiving this vector at week 16. Salivary flow rates of animals receiving rAAVhIL-10 locally in their submandibular glands, both at early and late treatment times, were significantly increased in comparison with those measured in rAAVLacZ-treated mice at both the 16- and 20-week time points (both $p < 0.05$). In two separate earlier experiments conducted by us, salivary flow rates after local administration of rAAVhIL-10 were similarly greater than those measured for untreated controls (data not shown).

Salivary flow rates of mice receiving intramuscular delivery of rAAVhIL-10 were significantly increased in comparison with flow rates measured in rAAVLacZ-treated mice only at the 20-week time point and only for the late treatment (delivery at 16 weeks; $p < 0.05$). The salivary flow rates at 16 weeks among mice receiving rAAVhIL-10 by intramuscular administration at the early time point did
not differ significantly from those of animals receiving rAAVLacZ. We were unable to detect any consistent relationship between salivary flow rates and the presence of blood glucose levels of greater or less than 400 mg/dL in individual NOD mice (data not shown).

![Figure 1](image_url)

Figure 1. Effect of rAAVhIL-10 or rAAVLacZ administration on saliva production in NOD mice
NOD mice received either early treatment (ET) with rAAVhIL-10 (hIL-10) at age 8 weeks, before the onset of sialadenitis, or late treatment (LT) at 16 weeks, after the onset of sialadenitis, via both submandibular glands (SG) by retrograde ductal instillation (2.5x10^10 genomes per gland) or intramuscular injection (IM) to the quadriceps muscle, as a systemic treatment control (5x10^10 genomes). The salivary gland-treated control group received submandibular gland administration of rAAVLacZ (LacZ) at the same time points and doses as the rAAVhIL-10 groups. The untreated control group was not manipulated before saliva collection. Saliva was collected as described in Materials and Methods over a 20-minute period after stimulation with pilocarpine at 8, 12, 16, and 20 weeks of age. The absence of a response column for any treatment group indicates that no saliva collection was performed for that group at that time point. Data represent means ± SEM. Significant differences are indicated and were determined by the Student’s t test.

**Effects of hIL-10 cDNA delivery on inflammatory cell infiltration of salivary glands in NOD mice**

An important feature of this murine model for SS is the focal infiltration of inflammatory cells within the salivary glands. Accordingly, sections from the submandibular glands of rAAVLacZ- and rAAVhIL-10-treated (early and late) NOD mice killed at 20 weeks were examined histologically for inflammatory changes. Early signs of inflammatory cell infiltration can be detected in few mice at 8 weeks, whereas typical signs of autoimmune sialadenitis, similar to that seen in
human SS, are frequently observed in mice of 12 weeks old. At circa 20 weeks of age, moderate to severe inflammatory cell foci in the salivary glands are seen in NOD mice.

As noted earlier, we determined the severity of the sialadenitis according to the number of inflammatory foci present per 4 mm² of salivary tissue (focus score). The number of foci present in salivary glands of mice receiving rAAVhIL-10 locally in their submandibular glands at the early (8-week) time point was clearly reduced compared with submandibular glands from animals administered rAAV-LacZ (Figure 2). Specifically, we observed that at 20 weeks submandibular gland sections from all mice receiving vector in this manner contained fewer inflammatory cell lesions, as well as lesions that demonstrated a more moderate inflammation. In the salivary gland-administered rAAVhIL-10 treatment group no focus score was larger than two, and the range of scores measured was narrow (1.2-2.0; Figure 3). Conversely, in both the rAAVlacZ-treated groups and the mice receiving rAAVhIL-10 via intramuscular injection, large and small foci, which were both perivascular and periductal in location, were seen in the submandibular glands at 20 weeks. Within large and confluent foci in these samples, there was an observable tissue disorganization and replacement of normal salivary gland structure (Figure 2B). Several glands from the rAAVhIL-10 (intramuscularly administered) and rAAVlacZ early treatment groups exhibited high focus scores (≥3) and overall a wider range of scores (2.3-4.4) was detected (Figure 3). The median focus score of mice receiving rAAVhIL-10 in their submandibular glands (1.4) was significantly lower than that of mice receiving rAAVLacZ (3.0) in their submandibular glands (p = 0.013) and that of mice receiving rAAVhIL-10 (2.6) by intramuscular injection (p = 0.004). At the 20-week time point the focus scores of glands from mice treated at 16 weeks with rAAVhIL-10 by salivary administration were also significantly different (p < 0.05) from those of the rAAVlacZ (intramuscularly administered) mice (Figure 3). This same general conclusion was gained from mice examined at 12 and 16 weeks. Focus scores at 12 weeks in mice administered rAAVLacZ via their submandibular glands at 8 weeks averaged 2.9 ± 0.3 (mean ± SEM), whereas animals treated with rAAVhIL-10 had much lower values (1.5 ± 0.3; p = 0.03). Similarly, focus scores of mice administered rAAVhIL-10 by intramuscular injection at 8 weeks averaged 2.2 ± 0.2 at 16 weeks versus 1.1 ± 0.1 (p = 0.02) for mice given this vector via their submandibular glands. These data suggest that rAAVhIL-10 gene transfer can at least in part inhibit SS-like murine salivary gland disease by reducing the severity of the sialadenitis present.
Figure 2. Effect of rAAVhIL-10 administration on the histological appearance of submandibular glands of NOD mice

NOD mice received treatments as described in the legend to Figure 1. Glands were removed for histological analysis from rAAVhIL-10-treated and rAAVLacZ-treated NOD mice at the time of sacrifice (20 weeks). Histopathologic assessment was performed and presented as a focus score (see Materials and Methods and the legend to Figure 3). A. Section from a submandibular gland of a NOD mouse treated early with rAAVhIL-10. A single focus of lymphocytes is detected in the section shown (arrow). B. Foci were more frequently seen in the glands of NOD mice administered rAAVLacZ via their submandibular glands. As shown in this section, the foci were both perivascular and periductal. Within large and confluent foci, there also was observable tissue disorganization and replacement of normal salivary gland structure. Arrows indicate foci in this gland. Hematoxylin and eosin staining; original magnification 40x.
Figure 3. Effect of rAAVhIL-10 administration on inflammatory infiltrates in the submandibular glands of NOD mice

NOD mice received treatments as described in the legend to Figure 1. Submandibular glands were removed for histological analysis at the time of sacrifice (20 weeks). Histopathologic scoring was performed by using the focus score (y axis; focus, an aggregate of 50 or more lymphocytes and histiocytes, per 4 mm²; see description in Materials and Methods; see also Yamano et al.8). Three different examiners assigned focus scores after blindly reviewing at least nine different fields from each gland. Each diamond represents the mean score of a single mouse. Horizontal bars represent the median value for each group. Statistical analyses compared the three different vector treatment groups (rAAVLacZ, rAAVhIL-10 salivary gland [SG] administration, and rAAVhIL-10 intramuscular [IM] administration) within each treatment time (ET – early; and LT – late) separately. Significant differences are indicated (*) and were determined by a Student’s t test.

Effect of hIL-10 cDNA transfer on cytokine expression in submandibular glands

To help understand the mechanism underlying the overall tissue responses seen in salivary glands after rAAVhIL-10 administration, we examined the local expression of several important inflammatory mediators. As shown in Figure 4, we determined the expression of mIL-4, mIFN-γ, mIL-6, mIL-12, mRANTES, mIL-10, and hIL-10 proteins in extracts of salivary glands. Protein was prepared from submandibular glands of NOD mice at 20 weeks of age after treatment with either rAAVhIL-10 (salivary or intramuscular) or rAAVLacZ. For comparison, gland extracts of Balb/c mice (normal control mice) were also tested. None of the inflammatory mediators (shown only for mRANTES in Figure 4) was detectable in the Balb/c mice, whereas all NOD mice exhibited readily measurable values, consistent with the presence of autoimmune disease. Little difference was seen between the treatment groups for mIFN-γ, mIL-10, and mRANTES. However, glands obtained from mice receiving salivary gland administration of rAAVhIL-10 in the early treatment group showed higher levels of mIL-4 (p = 0.06), mIL-6 (p = 0.025), mIL-12 (p = 0.039), as well as hIL-10 (p = 0.031) compared with other treatment groups. Interestingly, animals receiving rAAV-hIL-10 by intramuscular injection at the early time point also exhibited a higher level of hIL-10 (p = 0.009). However, gland extracts from these mice displayed no other significant changes in the profile of inflammatory mediators present. We did not detect hIL-10 in gland extracts from mice receiving salivary gland…
Figure 4. Effect of rAAVhIL-10 administration on cytokine expression in submandibular glands of NOD mice

NOD mice received treatments as described in Materials and Methods and the legend to Figure 1. Submandibular glands were removed at the time of sacrifice from NOD mice, at age 20 weeks. Protein was obtained as described in Materials and Methods and individual cytokines were measured commercially in SearchLight proteome arrays (Endogen), multiplexed assays involving a sandwich ELISA procedure. Data are shown as picograms per milligram wet weight of the submandibular gland and represent means ± SEM. The number of glands assayed per treatment group is the same as indicated in Figure 3. For the salivary gland administered, early treatment group, statistically significant differences are shown (*) in the panels for mouse (m) IL-12, hIL-10 (also for intramuscularly administered, +), and mIL-6, as are the respective probability values, as determined by a Student’s t test. The expression of mIL-4 was of borderline significance (p = 0.06) and is indicated by a solid circle. ET – early treatment; LT – late treatment; LacZ – LacZ vector; hIL-10 – hIL-10 vector; SG – salivary gland; IM – intramuscular.
administration of rAAVhIL-10 in the late treatment group. For this group of mice, vector delivery was four weeks before sacrifice (versus 12 weeks for mice in the early treatment group) and it is likely that hIL-10 protein present in gland extracts was too low to be distinguished from background with our assay.

Detection of vector after administration of rAAV to the salivary glands or to the quadriceps muscle

We next examined salivary glands, draining lymph nodes and spleens of mice by Q-PCR to determine the viral copy number in these different tissues. As shown in Table, after salivary gland administration of either rAAVhIL-10 or rAAVLacZ, little rAAV vector was found in either the draining lymph nodes or spleen. This result strongly suggests that administered vector was minimally disseminated. Interestingly, after intramuscular injection of rAAVhIL-10 at the 16-week time point (late treatment), similarly low levels of viral DNA were found in the spleen, lymph nodes and salivary glands (Table).

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<td>LacZ ET (n = 3)</td>
<td>LacZ LT (n = 4)</td>
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<tr>
<td>Salivary glands</td>
<td>29,700 (29,500)</td>
</tr>
<tr>
<td>Draining lymph nodes</td>
<td>1,060 (1,452)</td>
</tr>
<tr>
<td>Spleen</td>
<td>ND</td>
</tr>
</tbody>
</table>

Table. Presence of rAAV2 vector DNA in salivary glands, draining lymph nodes, and spleens

At 20 weeks after rAAV administration to NOD mice, as described in Materials and Methods and in the legend to Figure 1, the submandibular glands, draining lymph nodes, and spleens were removed at the time of sacrifice. DNA was obtained as described in Materials and Methods. Data represent means (SEM). Multiple Q-PCR runs were performed for each tissue. A separate assay, containing several different samples from all studied tissues, was used to normalize data from the different assays. Tissues from naïve animals (i.e., received no rAAV vector) yielded an average background (in number of vector copies) of 325 ± 185 for salivary gland, 401 ± 203 for draining lymph node, and 678 ± 96 for spleen DNA. This background was subtracted from each experimental value to yield the results presented. Data are shown as DNA copy number per 100 ng total DNA. ND – not detected above background level; ET – early treatment; LT – late treatment; LacZ – LacZ vector; hIL-10 – hIL-10 vector; SG – salivary gland; IM – intramuscular.

Effect of administration of rAAVhIL-10 on blood glucose and insulin levels in NOD mice

Because NOD mice develop severe autoimmune diabetes, throughout this study we monitored blood glucose levels in all mice and administered insulin every 24 hours when they became hyperglycemic (defined for the present study as a
blood glucose level ≥400 mg/dL). Although it was not a focus of this study, we consistently observed that rAAVhIL-10 delivery to salivary glands reduced hyperglycemia in NOD mice, that is, in the present cohort of mice as well as in our earlier two experiments (data not shown). Thus, at 20 weeks the median blood glucose level in mice receiving the rAAVLacZ vector was between 500 and 600 mg/dL, whereas insulin levels were barely detectable (Figure 5). Administration of rAAVhIL-10 by intramuscular injection lowered blood glucose levels (median, ~400 mg/dL) but had no significant effect on insulin levels. Conversely, mice administered rAAVhIL-10 via their salivary glands showed significant reductions in blood glucose by 20 weeks: median, ~190 and ~250 mg/dL in the early (p < 0.05) and late (p = 0.054) treatment groups, respectively. Insulin levels were significantly raised at 20 weeks in mice that had received rAAVhIL-10 in their salivary glands at 8 weeks (early treatment) with a median level of ~0.43 ng/mL (p < 0.05).

Discussion

These studies have directly examined the potential utility of rAAV-mediated hIL-10 gene transfer as a novel approach for the management of the salivary gland pathology and morbidity associated with SS. We hypothesized that rAAV-mediated local hIL-10 gene delivery would be useful to treat the development of murine SS-like disease by modulation of inflammatory reactivity in the salivary glands. As shown herein, as well as in two other similar sets of experiments that are not presented, local delivery of rAAVhIL-10 to salivary glands appears useful to limit SS-like glandular pathology.

Previously, we have demonstrated that an rAAV serotype 2 vector containing the hIL-10 cDNA, rAAVhIL-10, directs functional hIL-10 production in vitro and in vivo. Low levels of hIL-10 were secreted into the bloodstream for more than two months (longest time studied) after local salivary gland delivery of this vector. We further showed that the vector-encoded hIL-10 was biologically active in vivo by challenging rAAVhIL-10-treated IL-10 knockout mice with lipopolysaccharide to induce endotoxic shock eight weeks following systemic vector delivery.

We and others have shown that significant levels of various secretory transgene products can be secreted into the bloodstream directly from salivary glands, using other (adenoviral and non-viral) gene transfer vectors. In the present study, we administered lower (circa 25%) doses of rAAVhIL-10 to salivary glands than we previously reported and only modest hIL-10 levels were detected in serum (and not in all animals); a result that is consistent with our earlier study. We reasoned that employing a lower vector dose should lead to less
Figure 5. Effect of rAAVhIL-10 administration on blood glucose and insulin levels in NOD mice
NOD mice received treatments as described in Materials and Methods and the legend to Figure 1. Blood glucose (A) and insulin (B) levels presented are from mice at 20 weeks. As described in Materials and Methods, mice were treated with insulin when blood glucose measured ≥400 mg/dL. Each diamond represents values from an individual mouse. Circles indicate mice that received insulin treatment (Humalog), 4 U every 24 hours. Horizontal bars represent the median value for all mice in each group. Results with insulin-treated mice were excluded from statistical analyses. Significant differences are as indicated (* or +) and were determined by a Student’s t test.
systemic cytokine delivery, while keeping a more local, intra- and periglandular
distribution of the transgene product. Nonetheless, systemic effects were evident
after salivary gland administration of rAAVhIL-10, i.e., lower blood glucose, and
higher serum insulin, levels (Figure 5). In addition, dissemination of vector
beyond target tissues is a significant concern, which can be minimized after
salivary gland delivery of the transgene, but also not eliminated as shown here-
in. Systemic delivery of a gene transfer vector encoding a cytokine, such as hIL-10, is undesirable because of the considerable potential for adverse effects.
We also observed the formation of low levels of antibodies to the hIL-10 trans-
gene product in most animals (8 of 10 tested) given the rAAVhIL-10 vector at the
early treatment point by intramuscular injection and locally in their submandibu-
lar glands.

Previously, we and others have demonstrated that a decline in salivary flow
rates in NOD mice begins between 8 and 12 weeks of age, and salivary flow
rates steadily decrease thereafter. Thus, an important finding in the present
study, as well as in two similar earlier experiments not reported here, was that
long-term (20 weeks) salivary function is dramatically improved (circa threefold)
in NOD mice following rAAVhIL-10 delivery directly to the submandibular glands
both at the early (8 weeks) and late treatment point (16 weeks). Additionally,
there was clearly some benefit following systemic delivery (intramuscular) of
rAAVhIL-10 observed after late treatment. This latter finding may reflect either
the circulation or accumulation of hIL-10 protein in the salivary glands (Figure 4)
or the dissemination of low levels of rAAVhIL-10 to the submandibular glands
following intramuscular injection (Table). Salivary flow rates in both early-
and late-treated NOD mice (rAAVhIL-10 salivary gland administration) were signifi-
cantly enhanced compared to those seen in the salivary rAAVLacZ-treated NOD
mice. Interestingly, we did not observe a difference in salivary flow between
rAAVLacZ-locally treated mice and untreated mice. This suggests the absence
of any adverse effects of rAAV vectors and β-galactosidase protein on murine
salivary gland function (Figure 1), a finding consistent with our two earlier stu-
dies that employed untreated mice as the only control group (data not shown).

In addition to the effects on salivary function, rAAVhIL-10 administration led to
marked improvements in histologically assessed inflammatory changes (focus
scores) in the submandibular glands. At 20 weeks of age or later, moderate to
severe focal accumulations of inflammatory cells are seen in the salivary glands
of NOD mice (Yamano et al. and herein). However, significantly fewer foci were
found in the glands of mice treated locally in the submandibular glands at the
early time point with rAAVhIL-10 compared to rAAVLacZ-treated animals and
mice receiving intramuscular injections of rAAVhIL-10. Although the focal in-
flammatory changes in the salivary glands of these mice were not completely
eliminated, the occurrence of moderate to severe accumulations of inflammatory cells was dramatically reduced (approximately 50%). These data suggest that rAAV-mediated hIL-10 cDNA delivery to the salivary glands of NOD mice before frank onset of sialadenitis, exerts a local immunomodulatory effect, consistent with our hypothesis. This local effect appears to in part inhibit the progression of SS-like gland dysfunction by reducing the severity of sialadenitis. In the late-treated NOD mice administered rAAVhIL-10 in the submandibular glands, there was also a significant difference in the number of foci present from that observed with the rAAVLacZ-treated group at 20 weeks. This suggests local administration of rAAVhIL-10 can provide potential treatment after disease onset, as is the case with SS patients seen in the clinic. However, since intramuscular injection of rAAVhIL-10 led to some improvement in salivary flow rate, local gene delivery, and subsequent reductions in glandular inflammatory infiltrates, are not entirely responsible for the improvements in salivary gland function observed. Interestingly, we found no consistent relationship between the salivary flow rate and focus score, for individual mice (not shown). Indeed, the relationship between salivary flow rates and extent of sialadenitis, and focus score, in SS patients is still not well understood.51

We originally hypothesized that local salivary gland expression of transgenic hIL-10 would lead to a downregulation of mIFN-γ levels, and a general shift from a Th1-like cytokine profile, compared with results from the untreated or rAAV-LacZ-treated mice. However, the expression of mIFN-γ was minimally affected. Furthermore, after local rAAVhIL-10 administration we saw increased levels of the pro-inflammatory cytokines mIL-6 and mIL-12, as well as an increase in mIL-4 (Figure 4). Thus, despite the overall improvement in salivary function and histological appearance with rAAV-mediated hIL-10 cDNA delivery to submandibular glands of NOD mice, we did not find the hypothesized simple local alteration in cytokine profiles (increase in Th2 versus Th1 cytokines) in treated salivary glands. Clearly, vector copy numbers in glands from the rAAV vector-treated mice were quite high after salivary gland delivery (Table). mRANTES levels in the salivary glands of NOD mice were higher than those in untreated Balb/c animals, consistent with earlier findings that show elevation of RANTES levels in NOD salivary glands as well as in the salivary glands of SS patients.55,56 The lack of a reduction in steady state levels of pro-inflammatory cytokines in the salivary glands of NOD mice may reflect a more important therapeutic role for hIL-10 in the earliest phases of disease development. However, it seems likely that the failure to reduce Th1-like cytokines locally in the glands after rAAV-hIL-10 administration reflects the occurrence of a more complex immune dysregulation than previously considered. Several reports examining the utility of IL-10 gene transfer for the autoimmune diabetes in NOD mice are consistent with these views.18,21,22 Although using higher doses of rAAVhIL-10 could lead to
higher levels of hIL-10 expression in salivary glands, possibly capable of inhibiting steady state pro-inflammatory cytokine expression, unlike our present results, such a maneuver could lead to undesirable immunomodulatory effects systemically. Although, rAAVhIL-10 delivery to salivary glands of NOD mice clearly results in dramatically improved saliva secretion and reduced sialadenitis, more studies are needed to clarify the specific mechanism(s) by which this occurs.

Fleck et al. described a gene transfer approach in another murine model of SS, murine cytomegalovirus-infected B6-*gld/gld* mice. They reported that local adenoviral-mediated FasL gene transfer reduced infiltrating mononuclear cells in salivary glands. Although these results demonstrate proof of concept that local immunomodulatory gene transfer can improve sialadenitis in a murine model of SS, the ultimate clinical utility (restoration of salivary function) of this approach is not clear. In addition, recombinant adenoviral vectors elicit potent host immune responses compared to rAAV vectors; clearly an undesirable feature for managing an autoimmune disease. Although conventional adenoviral vectors direct high levels of transgene expression when compared to rAAV vectors, this expression is close to background levels in salivary glands *in vivo* by two weeks. Thence, rAAV vectors may be especially valuable for the transfer of a variety of immunomodulatory genes in different tissues.

In several studies, it has been reported that systemic (intramuscular, intravenous) treatment with the IL-10 gene can inhibit the development of autoimmune type 1 diabetes mellitus in NOD mice, presumably a result of circulating levels of hIL-10 protein. In the present study, we also found that blood glucose levels at 20 weeks in mice administered rAAVhIL-10 via their salivary glands were significantly lower than those measured in mice receiving rAAVLacZ in this tissue. In addition, median insulin levels were higher in mice administered rAAVhIL-10 than those measured in rAAVLacZ-treated mice at 20 weeks. Two months after local rAAVhIL-10 delivery to salivary glands, high levels of vector were still present in the target gland, but little to no vector could be detected in the spleen, consistent with localization of vector in the encapsulated glands. This suggests that even the low serum hIL-10 levels resulting from salivary gland hIL-10 gene transfer in the present study were partially effective in ameliorating the hyperglycemia of NOD mice. These results also demonstrate that the effects of local salivary gland delivery of a transgene encoding a constitutive pathway secretory protein are not limited to the immediate glandular environment.

**Conclusion**

Our findings represent the first report of successful rAAV-immunomodulatory
gene-based therapeutics in a pre-clinical model of SS. rAAV-mediated delivery of immunomodulatory genes may offer the possibility of persistent local transgene expression to facilitate management of this chronic immunopathological disease.

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References


