Local immunomodulatory gene therapy for Sjögren’s syndrome

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CHAPTER 5

Effect of Human Vasoactive Intestinal Peptide Gene Transfer in a Murine Model of Sjögren’s Syndrome

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Abstract

OBJECTIVE: Sjögren’s syndrome (SS), an autoimmune exocrinopathy mainly affecting lacrimal and salivary glands, results in ocular and oral dryness (keratoconjunctivitis sicca and xerostomia). The etiology and pathogenesis are largely unknown; currently, only palliative treatment is available. Gene transfer of vasoactive intestinal peptide (VIP), based on its immunomodulatory properties, potentially may be useful in management of SS. METHODS: A serotype 2 recombinant adeno-associated virus encoding the human VIP transgene (rAAV2hVIP) was constructed and its efficacy tested in the female non-obese diabetic (NOD) mouse model for SS following retrograde instillation in salivary glands. \(10^{10}\) particles/gland of rAAV2hVIP or rAAV2LacZ (encoding β-galactosidase; control vector) were administered at 8 weeks of age (before sialadenitis onset). Salivary flow rates were determined before vector delivery and at time of sacrifice (16 weeks). After sacrifice, saliva, serum, and salivary glands were harvested. Analysis of salivary output, inflammatory infiltrates (focus scores), VIP protein expression, cytokine profile, and serum anti-VIP antibodies was performed. RESULTS: rAAV2hVIP led to significantly improved salivary flow, increased salivary gland and serum expression of VIP, and reduction of salivary gland cytokines IL-2, IL-10, IL-12(p70) and TNF-α, and serum RANTES, compared with control vector. There was no difference in focus scores or apoptotic rates; neutralizing antibodies were not detected. CONCLUSIONS: This study shows local delivery of rAAV2hVIP can have disease-modifying and immunosuppressive effects in salivary glands of the NOD mouse model of SS. The novel strategy of employing VIP prophylactically may be useful for both understanding and managing the salivary component of SS.
Introduction

Sjögren’s syndrome (SS) is an autoimmune exocrinopathy of unknown etiology, predominantly affecting peri- and postmenopausal women. Although the main symptoms consist of ocular and oral dryness (xerophthalmia and xerostomia), there are also systemic effects. Affected glands are featured by infiltrating, apoptosis-resistant CD4+ T cells, and to a lesser extent CD8+ T cells, B cells and macrophages, and by pro-inflammatory cytokines secreted from both lymphocytes and epithelial cells, together leading to inflammatory infiltrates, acinar atrophy and destruction. At present, patients are only offered symptomatic treatment, which is often unsatisfactory.

Initially discovered as a gastrointestinal hormone, vasoactive intestinal peptide (VIP) exhibits abundant functions, ranging from neurotransmitter, vasodilator, and bronchodilator effects to acting as a trophic agent, secretagogue, and immunomodulator. VIP belongs to the glucagon/secretin superfamily. Its precursor protein, prepro-VIP/PHM-27, encoded on human chromosome 6, is a 8,837 bp gene, containing seven exons and six introns and yielding the 28-amino acid VIP. The amino acid sequence has been completely preserved in humans and mice and transgenic human VIP (hVIP) has been shown to act through mouse VIP receptors in transgenic mice.

Based on its immunomodulatory properties, VIP possibly may be useful in the management of several autoimmune disorders, but a short half-life could limit the applications of protein-based therapy. In contrast, gene transfer potentially offers a means of sustained expression of a transgene like VIP. Since high serum VIP levels are associated with secretory diarrhea in patients with a VIPoma, local therapy of VIP is preferable. Salivary glands provide an excellent target site for localized gene transfer following retrograde ductal infusion of vectors.

Recently, we have constructed a recombinant serotype 5 adenovirus encoding the human VIP cDNA (rAd5CMVhVIP) and shown expression and function of the transgene. Recombinant adenoviral vectors offer strong, but short-term, protein expression due to a potent immune response by the host. Adeno-associated virus (AAV), a small, single-stranded DNA, non-pathogenic virus, has shown considerable promise as a viral vector for gene therapy. For recombinant serotype 2 AAV vectors (rAAV2) this is attributable to its capacity to infect numerous mammalian cells, dividing as well as non-dividing, and a minimal immune response. In previous in vivo studies we have shown therapeutic effects of different transgenes encoded by rAAV2 vectors, currently the most widely used serotype, when delivered to murine submandibular glands, including local
delivery of an rAAV2 encoding human interleukin-10 (hIL-10) to the non-obese diabetic (NOD) mouse. The NOD mouse develops, besides type I, insulin-dependent diabetes mellitus, exocrine gland infiltrates and decreased glandular secretion, which are age and gender dependent, making it the most useful, commonly available animal model to study the disease properties of SS.

In the present study, we have constructed the vector rAAV2hVIP and examined its ability to alter the progressive, SS-like dysfunction in NOD mice after local salivary gland delivery before disease-onset.

Materials and Methods

Construction of viral vector encoding functional hVIP
We previously reported construction of the hVIP cDNA and the generation of a recombinant serotype 5 adenoviral vector rAd5CMVhVIP. The cytomegalovirus (CMV) promotor/enhancer, hVIP cDNA and simian virus 40 (SV40) poly adenylation signal from pAC-CMV-hVIP were subcloned into the rAAV2 plasmid pDT1.1 (containing the ampicillin resistance gene; described as pAAV-MCS2.7 in Braddon et al.), resulting in pAAV2CMVhVIP (6391 bp, 1937 bp between inverted terminal repeats, ITRs). The correct construct was verified by restriction digests and sequencing, and the presence of the ITRs was confirmed by a SmaI digest (not shown). rAAV2hVIP was generated by subsequently cotransfecting this plasmid with the adenoviral helper packaging plasmid pDG, a generous gift from Prof. J.A. Kleinschmidt, at a ratio of 1:3 in 15 cm plates of ~40% confluent 293 T cells using a calcium phosphate precipitation procedure. Additional details can be found in the supplementary information.

Infectious vectors were demonstrated by transducing $1.4 \times 10^4$ human embryonic kidney (HEK) 293 cells on 96-well plates with serial dilutions of each aliquoted CsCl fraction in the presence of $1.5 \times 10^7$ particles of wild-type adenovirus. After 24 hours of incubation, supernatants from infected cells were analyzed with an ELISA for VIP (see below).

Construction of rAAV2LacZ
Previously, we reported construction, expression, and function of rAAV2LacZ (encoding β-galactosidase; described as rAAVRnLacZ in Chiorini et al.).

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
Gene transfer, and saliva, serum and salivary gland collection
Initially, to investigate the effects of rAAV2hVIP administration on salivary gland function in NOD mice, we tested two rAAV2hVIP vector doses (10^9 and 10^10 particles, 7 animals/group) per salivary gland (both glands targeted), with the latter dose yielding the best results. For the second study, we instilled only one dose, 10^10 particles in each gland (8 animals/group). The experiments described herein report the 10^10 particles/gland data from the two separate studies, over the indicated 8- to 16-week time course. Both studies gave comparable results.

Vector delivery and saliva collection (at 8 weeks of age), and saliva, salivary gland and blood collections (16 weeks) were performed, as described in previous reports from our laboratory. Additional details can be found in the supplementary information.

Transgene expression
VIP expression and secretion were measured by an ELISA (Peninsula Laboratories, San Carlos, CA, USA) of cell supernatants, and murine saliva, serum, and salivary gland extracts. The assay sensitivity was ~0.016 ng/mL; the polyclonal rabbit antibody was known to cross-react with mouse and rat VIP. Murine samples were spiked with standard concentrations of recombinant VIP (Peninsula Laboratories), and different dilutions in assay buffer were used to investigate assay interference in biological samples.

Determination of serum antibodies against VIP
Serum antibodies to VIP were determined as follows. Aliquots of undiluted mouse sera were obtained at 16 weeks and pooled by vector group. To 50 µl of standard concentrations (0.016-10 ng/mL) of recombinant VIP (Peninsula Laboratories), 2 µl of pooled serum was added to obtain a final serum dilution of 1:26. Next, the samples were incubated at 37°C for 30 minutes. The VIP ELISA, containing polyclonal rabbit anti-VIP antibodies (Peninsula Laboratories), was
then used to detect unbound VIP protein, according to the manufacturer’s instructions. A change in the ELISA standard curve, where the optical density (OD) is inversely related to the protein concentration, was used to detect the presence of anti-VIP antibodies in NOD mouse sera.

**Quantification of cytokines**

IL-2, IL-4, IL-6, IL-10, IL-12(p70), interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and RANTES were measured commercially in SearchLight proteome arrays (Pierce Biotechnology, Woburn, MA, USA), which are multiplexed assays involving a sandwich ELISA procedure. Additional details can be found in supplementary information.

**Histological assessment of salivary glands**

Part of each salivary gland was fixed in 10% formalin overnight. Thereafter, the tissues were dehydrated in a series of graded ethanol solutions and embedded in paraffin. Three sections (5 µm thick), each one 50 µm apart from the previous, were mounted on poly-L-lysine coated slides and stained with hematoxylin-eosin. Histopathologic scoring of all three sections was performed by counting the number of foci present (one focus is an aggregate of 50 or more lymphocytes or histiocytes per 4 mm²). The scoring was done blindly by three examiners (BML, FM, APC) and the mean of all focus scores per animal was then calculated.

**Apoptosis of salivary gland epithelial cells**

VIP reportedly can protect cells from apoptosis. Apoptotic epithelial cells were identified in salivary gland paraffin-embedded sections by a Terminal Deoxynucleotidyl Transferase (TdT)-Mediated dUTP-Biotin Nick-End Labeling (TUNEL) Assay using a cell death detection kit (Chemicon, Temecula, CA, USA), according to the manufacturer’s instructions and as previously described. During subsequent visualization by light microscopy, the number of apoptotic epithelial cells was counted blindly by three examiners (BML, FM, APC) and the apoptotic index determined as the percentage of apoptotic cells divided by total amount of epithelial cells (counted with Scion Image Software [Scion Corporation, Frederick, MD, USA]). For each animal, three different fields in one section were examined. The mean of all apoptotic indices per animal was then calculated.

**Statistical analysis**

Data analysis consisted of descriptive statistics, reported as means ± SEM or medians, unpaired Student’s t tests and Mann-Whitney Rank Sum Tests, as appropriate.
Results

**VIP expression by rAAV2hVIP in vitro**

The highest particle titers of rAAV2hVIP were typically found in fractions with a refractive index of ~1.372, equivalent to a buoyant density of ~1.4 g/mL, and corresponded to $1.4 \times 10^{13}$ particles/mL. VIP expression after transduction of HEK 293 cells with these fractions showed that $4.9 \times 10^3$ particles rAAV2hVIP per cell resulted in 1.5 ng VIP/mL culture medium (sevenfold increase over background; Figure 1). Functional activity of the hVIP transgene expressed from this transgene cassette has been previously described.\(^{17}\)

**VIP expression in vivo**

VIP expression was measured in saliva, serum, and protein extracts of salivary glands. Reliable results were obtained for serum and salivary gland extracts (see supplementary Table 1). Animals administered the rAAV2hVIP vector exhibited significantly higher levels of VIP (ng/mL, mean ± SEM) compared to control in salivary gland extracts (0.49 ± 0.03 vs. 0.36 ± 0.05, \(p = 0.039\)), and serum (0.16 ± 0.01 vs. 0.13 ± 0.01, \(p = 0.030\)). It was not possible to discern salivary VIP expression accurately, because of a complex cross-reactivity in salivary samples (data not shown).

![Figure 1. In vitro VIP expression directed by rAAV2hVIP](image)

Transduction of HEK 293 cells with rAAV2hVIP (multiplicity of infection [MOI] $4.9 \times 10^3$), in the presence of wild-type adenovirus (MOI $1.0 \times 10^3$); control represents identical amount of HEK 293 cells infected with wild-type adenovirus alone. This experiment was analyzed in duplicate and is representative of four separate experiments.

**Serum anti-VIP antibodies**

To determine the presence of serum anti-VIP antibodies pooled sera of 16-week-old mice treated with rAAV2LacZ or rAAV2hVIP were incubated with standard concentrations of recombinant VIP and assessed by an ELISA. No differences were seen between the two vector groups indicating local delivery of rAAV2hVIP did not result in the development of serum anti-VIP antibodies eight
weeks after vector delivery (see supplementary Figure 2).

**Effect of rAAV2hVIP and rAAV2LacZ on salivary function in NOD mice**
Female NOD mice show a progressive decline in salivary flow rates starting between 8 and 12 weeks of age. We examined salivary flow before virus administration at 8 weeks of age (baseline) and at the time of sacrifice (16 weeks). At baseline, the average salivary flow rate (microliters/body weight [grams] in 20 minutes; mean ± SEM) was 3.81 ± 0.47. At 16 weeks of age, eight weeks after vector delivery, the salivary flow rates for the rAAV2LacZ and rAAV2hVIP group were, respectively, 2.05 ± 0.36 and 4.32 ± 0.47 (p < 0.001; Figure 2). The decrease in flow rate of mice treated with rAAV2LacZ at 16 weeks compared with 8 weeks was also statistically significant (p = 0.01).

![Figure 2. Effect of rAAV2hVIP and rAAV2LacZ on salivary flow rate in NOD mice](image)

**Effect of rAAV2hVIP and rAAV2LacZ on inflammatory infiltrates in salivary glands of NOD mice**
Female NOD mice start developing characteristic focal inflammatory infiltrates in salivary glands after 8 weeks of age; such local changes also form an important clinical feature in SS patients. Salivary gland sections of 16-week-old rAAV2hVIP-treated mice showed no difference in focus scores (mean ± SEM) from the group treated with rAAV2LacZ (1.93 ± 0.11 versus 1.85 ± 0.26, p = 0.75).
**Effect of rAAV2hVIP and rAAV2LacZ on apoptotic rate in salivary glands of NOD mice**

The median apoptotic index, as an indication of the apoptotic rate, of epithelial cells in salivary glands was not different between the rAAV2LacZ and rAAV2hVIP group (medians 0.15 vs. 0.16, respectively, \( p = 0.45 \)).

**Effect of rAAV2hVIP and rAAV2LacZ on cytokine expression in salivary gland extracts and sera of NOD mice**

VIP can act as an immunomodulator, inhibiting levels of pro-inflammatory cytokines and increasing levels of anti-inflammatory cytokines. We examined protein expression of several pro- and anti-inflammatory cytokines and one chemokine, locally in extracts of salivary glands, as well as systemically in serum, in treated mice at 16 weeks of age. Aqueous salivary gland extracts from NOD mice treated with rAAV2hVIP showed lower levels of IL-2 (\( p = 0.001 \)), IL-10 (\( p = 0.054 \)), IL-12(p70) (\( p = 0.008 \)), and TNF-\( \alpha \) (\( p = 0.021 \)) than those from mice treated with rAAV2LacZ (Table). IL-4, IL-6, IFN-\( \gamma \) and RANTES levels in salivary gland extracts were not significantly different between these groups. Serum levels of all measured cytokines were not different between the two vector groups (supplementary Table 2). However, levels of RANTES in serum were significantly lower in rAAV2hVIP-treated mice (6.8 vs. 12.2 pg/mL, \( p = 0.040 \)).

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>LacZ (SEM)</th>
<th>hVIP (SEM)</th>
<th>( p )-Value</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.01 (0.01)</td>
<td>0.001</td>
</tr>
<tr>
<td>IL-4*</td>
<td>0.00</td>
<td>0.00</td>
<td>1.000</td>
</tr>
<tr>
<td>IL-6*</td>
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<td>0.00</td>
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<tr>
<td>IL-10*</td>
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<td>0.00</td>
<td>0.054</td>
</tr>
<tr>
<td>IL-12(p70)</td>
<td>0.10 (0.00)</td>
<td>0.05 (0.01)</td>
<td>0.008</td>
</tr>
<tr>
<td>TNF-( \alpha )*</td>
<td>0.05</td>
<td>0.00</td>
<td>0.021</td>
</tr>
<tr>
<td>IFN-( \gamma )</td>
<td>6.46 (1.19)</td>
<td>5.97 (0.59)</td>
<td>0.708</td>
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<tr>
<td>RANTES</td>
<td>3.75 (0.39)</td>
<td>2.68 (0.54)</td>
<td>0.142</td>
</tr>
</tbody>
</table>

**Table. Levels of inflammatory molecules in salivary gland extracts**

Protein expression of immunomodulatory molecules (pg/mg wet weight) in salivary gland extracts after administration of rAAV2LacZ (LacZ, \( n = 7 \)) or rAAV2hVIP (hVIP, \( n = 8 \)). Means (SEM) are shown unless otherwise noted. *Mann-Whitney Rank Sum Test with median values.

**Discussion**

This is the first report examining the efficacy of transgenic VIP in a model of SS.
Additionally, we are not aware of any publications using protein-based VIP therapy to alter the disease course of SS, although administration of the protein VIP has been successfully employed in other models of autoimmune diseases, such as collagen-induced arthritis\textsuperscript{35,36} and experimental autoimmune uveoretinitis.\textsuperscript{37} An rAAV2hVIP was constructed and its immunomodulatory and clinical efficacy tested in the NOD mouse model for SS. For the present study, we chose to treat the animals early before disease onset, i.e., a prevention, not treatment, model. Based on the results, in the future, we will test rAAV2hVIP’s effects upon administration after disease of onset, more closely resembling a clinical situation. Installation of rAAV2hVIP in the salivary glands of NOD mice led to higher salivary flow rates, increased salivary gland and serum expression of VIP, a reduction of cytokines IL-2, IL-10, IL-12(p70) and TNF-\(\alpha\) in salivary gland extracts and of serum RANTES, but no difference in focus scores or apoptotic rates, compared with results with the control vector.

Salivary gland cells are polarized and can secrete transgene-encoded proteins either via the constitutive pathway across the basolateral membrane, or upon stimulation via the regulated pathway into saliva.\textsuperscript{38-40} Pre/pro-VIP protein contains a recognition signal directing it into secretory granules during post-translational processes.\textsuperscript{41} Since the hVIP cDNA used here should contain that signal,\textsuperscript{17} salivary cells are to secrete hVIP mostly via the regulated pathway into saliva, with only a small proportion exiting to the interstitium and serum.\textsuperscript{38,39,42} VIP is a small neuropeptide, quickly degraded and inactivated, rendering a limited bioavailability.\textsuperscript{43} However, by adding protease inhibitors/EDTA to samples herein, as well as by performing multiple dilution and spiking experiments, we were able to determine accurate VIP levels in both serum and salivary gland extracts. rAAV2hVIP-treated mice showed significantly higher VIP expression in salivary gland extracts and serum than control rAAV2LacZ-treated mice. Unfortunately, due to a complex cross-reactivity pattern, we were unable to discern reliable salivary VIP expression, despite multiple controls and precautions. We have not studied VIP levels in human saliva, but this was previously done in studies employing the same precautions as we did.\textsuperscript{44,45} The protein levels expressed from the rAAV2hVIP vector \textit{in vivo}, while low, are reasonable and comparable to those of other transgenic secretory proteins in rAAV2 experiments.\textsuperscript{21,22}

We observed a decrease in the levels of several pro-inflammatory cytokines, IL-2, IL-12(p70) and TNF-\(\alpha\), but also in the anti-inflammatory cytokine IL-10, in salivary gland extracts after hVIP gene transfer. Importantly, serum cytokines were not different between the two rAAV vector groups, with only levels of the chemokine RANTES being lower in serum of rAAV2hVIP-treated animals. Several previous studies with untreated NOD/LiJ mice, of approximately the same age as the NOD mice studied by us, showed comparable serum cytokine levels.
to our data (e.g., see Yang et al.\textsuperscript{46,47}). To our knowledge, submandibular gland cytokine protein levels of 16-week-old NOD/LtJ mice have not been previously reported.

In the immune system VIP acts as an autocrine regulator.\textsuperscript{32} The VIP/PACAP receptor family consists of three G-protein coupled receptors, two of which, VPAC\textsubscript{1} and VPAC\textsubscript{2}, are present on macrophages, and CD4\textsuperscript{+} and CD8\textsuperscript{+} T lymphocytes.\textsuperscript{48,49} Effects of VIP are broad, including the inhibition of several macrophage functions, T cell proliferation, lymphocyte migration, and expression of chemokines and pro-inflammatory cytokines.\textsuperscript{7,32,33} Conversely, the production of IL-10 is reportedly stimulated by VIP.\textsuperscript{34} Pozo et al. postulated VIP to be a Th2 cytokine with a key role in neuroimmunology,\textsuperscript{34,50} i.e., VIP production by Th2 cells, as well as VIP stimulation of Th2, and inhibition of Th1, functions. While VIP may exert these effects in some autoimmune diseases with a balance shifted towards Th1 response, such as rheumatoid arthritis,\textsuperscript{51} our results here suggest it may be a different case in the NOD mouse model and SS. Upon gene transfer of hVIP, we did not observe a significant shift from Th1 to Th2 cytokine production. In fact, hVIP gene transfer led to an unexpected downregulation of salivary gland IL-10 levels and could indicate that VIP acts as a more overall immunosuppressant than strict Th2 cytokine in this SS model. In SS patients, salivary gland and serum IL-10 levels are actually increased, depending on disease stage and activity, and might be a sign of B cell activation and lymphoma development.\textsuperscript{52-55} As recently stated by Delaleu et al., “further approaches modulating cytokines are warranted in SS.”\textsuperscript{56}

Although the exact immunopathogenesis still waits to be elucidated, SS is thought to resemble an imbalance in cytokine production, locally as well as systemically, depending on disease stage and severity.\textsuperscript{3,56} However, the cytokine profile in submandibular gland biopsies with simultaneous expression of IFN-\gamma, IL-2, IL-4 and IL-13 provides an argument against a simple Th1 or Th2 predominance in SS.\textsuperscript{3} Clearly, hVIP gene transfer to submandibular glands in the present study resulted in local immunomodulatory activity.

Salivary flow rates were increased in the rAAV2hVIP group, but lymphocytic infiltrates (focus scores) were unaffected. Although historically xerostomia in SS patients was solely attributed to a destruction of glandular tissue, a well-recognized incongruity exists between the reduction in salivary flow and the extent of focal lymphoid infiltration.\textsuperscript{57,58} Hypofunction of salivary epithelial cells might be, in addition, due to the effects of cytokines or autoantibodies (e.g., anti-muscarinic receptor antibodies) in SS patients.\textsuperscript{59,60} Thus, it is not entirely unexpected that a dissociation was seen in rAAV2hVIP effects on salivary flow rate and focal salivary gland inflammatory infiltrates.
Salivary glands consist of acinar cells, forming the primary saliva, and ductal cells, which primarily reabsorb NaCl, but are relatively impermeable to water.\textsuperscript{16} rAAV2 vectors appear to transduce only ductal cells, not acinar cells.\textsuperscript{21,22} Therefore, we suggest that transgenic hVIP secreted by the murine transduced ductal cells will bind to VIP receptors on surrounding lymphocytes, as well as conceivably membranes of adjacent acinar cells, thereby influencing the immune milieu and/or directly facilitating enhanced salivary secretion.

In human submandibular glands, VIP receptors are found on both luminal and basal membranes of mucous acinar cells, as well as intercalated ducts.\textsuperscript{61} In human labial salivary glands, VIP binding sites are detected on the basal membranes of mucous acini\textsuperscript{62} and the presence of a functional VIP receptor system \textit{in vitro} has been demonstrated on isolated acinar cells.\textsuperscript{63} There are no data on VIP receptors in epithelial cells of mouse submandibular glands. In addition, several studies of SS patients have shown the existence of immunoreactive VIP nerve fibers in human submandibular glands\textsuperscript{61} and human labial salivary glands,\textsuperscript{63,64} where VIP containing nerves are in close contact with the acinar cells, inside the acinar basement membrane.\textsuperscript{64} Taken together, it is thought that VIP plays a role in several components of reflex salivary secretion.\textsuperscript{62,65}

**Conclusion**
This is the first study describing the effect of VIP gene transfer in a model of SS. We have employed local administration of an rAAV2 vector encoding hVIP to murine salivary glands and shown local disease modifying and immunosuppressive effects. The data suggest that VIP may be useful in the management of the salivary component of SS.

**Acknowledgements**
We would like to thank Dr. Christine Delporte for her expert knowledge on VIP and helpful suggestions.

**SUPPLEMENTARY INFORMATION**

**Materials and Methods**

*Cell lines*
The cell lines used were HEK 293 cells and 293 T cells, which express the simian virus 40 (SV40) large T antigen in a stable manner\textsuperscript{66} (ATCC, Manassas, VA, USA). HEK 293 cells were grown in Improved Minimum Essential Medium,
Eagle’s (IMEM) and 293 T cells in Dulbecco’s Modified Eagle’s Medium (DMEM). All media were supplemented with 10% 55°C heat-inactivated fetal bovine serum (Life Technologies, Rockville, MD, USA), 2 mM glutamine, penicillin (100 U/mL), and streptomycin (100 mg/mL; Biofluids, Rockville, MD, USA).

**Construction of viral vector encoding functional hVIP**

Two days after transfection, cells were harvested. Clarified cell lysates were treated with benzonase (100 U/mL lysate, incubated for 45 minute at 37°C) and 0.5% sodium deoxycholate, adjusted to a refractive index of 1.372 by addition of CsCl and centrifuged at 38,000 rpm for 65 hr at 20°C. Equilibrium density gradients were fractionated and fractions with a refractive index of 1.366–1.3765 were collected and stored at 4°C. The particle titer was determined by real-time quantitative (Q) PCR using an ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA), the SYBR Green PCR Master Mix (Applied Biosystems) and a specific 5’ (0.3 µM; GATGCCTTTTGGCAGTACATC) and 3’ (0.3 µM; TGGGGTGGAGACTTGGAAATC) primer pair for the CMV promoter. The Q-PCR was performed with the following conditions: holding at 95°C for 10 min, denaturing at 95°C for 15 s, and extension and annealing at 60°C for 1 minute for 40 cycles. A standard curve employing pAAV2CMVhVIP was included and duplicate samples were assayed; the detection limit of the assay was 10 vector copies.

**Mice**

Over the past two years, we have observed variability in many different cohorts of NOD mice purchased from The Jackson Laboratory with regard to prevalence and severity of sialadenitis and type I diabetes mellitus. More specifically, the salivary flow rate reductions and focus scores in these cohorts were not to the same extent as we have seen previously. Therefore, we compared untreated NOD mice from two different sources, i.e., Taconic (Germantown, NY, USA) and The Jackson Laboratory, as SS models. In our study, Taconic NOD mice showed no decrease in salivary flow rate, lower focus scores and decreased prevalence of type I diabetes mellitus at 20 weeks of age, compared to mice from The Jackson Laboratory. We concluded the Taconic NOD mice did not comprise a reliable SS model in which to study the efficacy of gene transfer procedures, and all studies reported here were conducted with NOD mice purchased from The Jackson Laboratory.

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

Mild anesthesia was induced with a ketamine (100 mg/mL, 1 mL/kg body weight; Fort Dodge Animal Health, Fort Dodge, IA, USA) and xylazine (20 mg/mL, 0.7 mL/kg body weight; Phoenix Scientific, St. Joseph, MO, USA) solution given intramuscularly. After an intramuscular injection of atropine (0.5 mg/kg body
weight; Sigma, St. Louis, MO, USA) rAAV2hVIP or rAAV2LacZ (n = 15 each) were administered to both submandibular glands of NOD mice by retrograde ductal instillation (10^{10} genomes per gland) at 8 weeks of age. Salivary flow rates were measured at 8 weeks (baseline, untreated, not manipulated before saliva collection) and 16 weeks of age (time of sacrifice). After induction of anesthesia and stimulation of secretion, using pilocarpine (0.5 mg/kg body weight; Sigma) administered subcutaneously,^{21,28} whole saliva was collected from the oral cavity with a microhematocrit capillary tube (Fisher Scientific, Hampton, NH, USA). This microcapillary tube was placed in an ice-cold pre-weighed 0.5-mL microcentrifuge tube, containing complete protease inhibitor cocktail (Roche Molecular Biochemical, Indianapolis, IN, USA) and 10 mM Ethylenediaminetetraacetic Acid (EDTA), and saliva volume was determined gravimetrically as previously described, with comparable results.^{21,23,28} After sacrifice, blood was collected in ice-cold 1.1-mL Z-gel tubes (Sarstedt, Newton, NC, USA), containing complete protease inhibitor cocktail (Roche) and 3.4 mM EDTA, and kept on ice. Blood samples were centrifuged at 10,000 rpm for 5 minutes and serum was collected. All samples were stored at -80°C until analysis.

Supplementary Figure 1. Study design

Two days before viral vector delivery pilocarpine-stimulated salivary flow rates were measured in 8-week-old female NOD mice. On day 0, 10^{10} particles of rAAV2hVIP or rAAV2LacZ (n = 7 first study, n = 8 second study, each) were instilled in murine salivary glands (SG). From age week 10-16 blood glucose levels and body weights were measured. At 16 weeks of age salivary flow rates were determined, all mice were sacrificed, and saliva, serum, and salivary glands were collected and stored at -80°C. Thereafter, analysis of salivary output, inflammatory infiltrates (focus scores), VIP protein expression, cytokine profile, and serum anti-VIP antibodies was performed. For a detailed description refer to text.
Quantification of cytokines
Cytokine levels were determined in salivary glands (see text), after extraction of soluble protein, and serum. Immediately after sacrifice, salivary glands were snap-frozen in 2-methylbutane on dry ice and stored at -80ºC until further analysis. Wet weight was measured and the glands were homogenized in ice-cold buffer (phosphate-buffered saline [PBS] and complete protease inhibitor cocktail; Roche) on ice. Thereafter, homogenates were centrifuged at 1,500 x g for 15 minutes at 4ºC and amount of total protein in the supernatants was determined with a Bio-Rad (Hercules, CA, USA) protein assay according to the manufacturer’s instructions.

Results

Experimental study design
A time line of the studies is shown in supplementary Figure 1. Two diabetic mice treated with rAAV2LacZ died during the study and were not included. Another mouse in the LacZ group and one in the hVIP group developed diabetes during the study; both were treated with insulin daily. Blood glucose levels and body weights at 16 weeks of age were not different between the two groups (data not shown).

<table>
<thead>
<tr>
<th></th>
<th>LacZ (SEM)</th>
<th>hVIP (SEM)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum‡</td>
<td>0.13 (0.01)</td>
<td>0.16 (0.01)</td>
<td>0.030</td>
</tr>
<tr>
<td>SG*</td>
<td>0.36 (0.05)</td>
<td>0.49 (0.03)</td>
<td>0.039</td>
</tr>
</tbody>
</table>

Supplementary Table 1. VIP levels in serum and salivary gland extracts
VIP expression (ng/mL) in vivo after administration of rAAV2LacZ (LacZ) or rAAV2hVIP (hVIP) in serum and salivary glands (SG). Means (SEM) are shown. ‡LacZ, n = 13; hVIP, n = 15. *LacZ, n = 7; hVIP, n = 8

Discussion
Local pro-inflammatory cytokines, such as TNF-α, were decreased after rAAV2hVIP delivery, although randomized, blind clinical trials using systemically administered TNF-α protein antagonists have not proven beneficial for SS. This could be due to the fact these studies featured recombinant proteins, with a limited half-life, administered intramuscularly and, therefore, distally. Local immunomodulatory gene transfer could perhaps change the outcomes.
Salivary gland delivery is relatively non-invasive with easy access through the excretory duct orifices. Salivary glands are also non-critical for life organs, and they are well-encapsulated, therefore limiting undesirable viral spread. Furthermore, high systemic VIP concentrations, e.g., as seen with a VIPoma, are disadvantageous. The results of the present study indeed suggest predominantly local vector effects. Although not shown herein, blood glucose levels showed no differences between the two vector treatment groups, suggesting no influence by transgenic hVIP after salivary gland delivery on developing diabetes in NOD mice.

**Supplementary Figure 2. Evaluation of serum anti-VIP antibodies in NOD mice**

Pooled sera of 16-week-old mice treated with rAAV2LacZ or rAAV2hVIP were incubated with standard concentrations of recombinant VIP and assessed by an ELISA, as described in Materials and Methods. The standard VIP protein was also tested in the absence of sera. The optical density (OD) of these reaction mixtures is shown. Note that there are no differences between VIP standard protein incubated with sera from both vector treatment groups. The figure represents the results from samples obtained with the second study and is representative of both studies.

**Supplementary Table 2. Levels of inflammatory molecules in serum**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>LacZ (SEM)</th>
<th>hVIP (SEM)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>23.04 (0.70)</td>
<td>19.80 (1.87)</td>
<td>0.150</td>
</tr>
<tr>
<td>IL-4*</td>
<td>0.00</td>
<td>0.00</td>
<td>0.189</td>
</tr>
<tr>
<td>IL-6</td>
<td>80.40 (33.49)</td>
<td>34.85 (5.74)</td>
<td>0.175</td>
</tr>
<tr>
<td>IL-10</td>
<td>7.14 (3.10)</td>
<td>6.11 (0.74)</td>
<td>0.736</td>
</tr>
<tr>
<td>IL-12(p70)</td>
<td>11.84 (0.73)</td>
<td>9.93 (0.84)</td>
<td>0.113</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.00</td>
<td>0.00</td>
<td>1.000</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>620.56 (84.23)</td>
<td>682.90 (71.23)</td>
<td>0.579</td>
</tr>
<tr>
<td>RANTES*</td>
<td>12.20</td>
<td>6.80</td>
<td>0.040</td>
</tr>
</tbody>
</table>

Protein expression of immunomodulatory molecules (pg/mL) in serum after administration of rAAV2LacZ (LacZ, n = 7) or rAAV2hVIP (hVIP, n = 8). Means (SEM) are shown unless otherwise noted. *Mann-Whitney Rank Sum Test with median values.
mice. Importantly, none of the serum cytokines studied by us were different between both treatment groups, with the only significant change observed in serum levels of the chemokine RANTES. The significance of this latter effect is unclear at present. Additionally, we saw no differences in anti-VIP neutralizing antibody levels when comparing the sera from rAAV2LacZ- and rAAV2hVIP-treated mice.

References

Chapter 5


