EXTENDED REPORT

Effect of human vasoactive intestinal peptide gene transfer in a murine model of Sjögren's syndrome

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Background: Sjögren's syndrome (SS), an autoimmune exocrinopathy mainly affecting lachrymal and salivary glands, results in ocular and oral dryness (keratoconjunctivitis sicca and xerostomia). The aetiology and pathogenesis are largely unknown; currently, only palliative treatment is available.

Objective: To determine whether gene transfer of vasoactive intestinal peptide (VIP), based on its immunomodulatory properties, might be useful in the management of SS.

Methods: A recombinant serotype 2 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 after retrograde instillation in submandibular glands (SMGs). 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 death (16 weeks). After death, saliva, serum, and SMGs were harvested. Salivary output, inflammatory infiltrates (focus scores), VIP protein expression, cytokine profile, and serum anti-VIP antibodies were analysed.

Results: rAAV2hVIP significantly improved the salivary flow, increased SMG and serum expression of VIP, and reduced SMG cytokines interleukin (IL) 2, IL10, IL12 (p70), and tumour necrosis factor α, and serum RANTES, compared with the control vector. No difference in focus scores or apoptotic rates was found; neutralising antibodies were not detected.

Conclusions: Local delivery of rAAV2hVIP can have disease modifying and immunosuppressive effects in SMGs of the NOD mouse model of SS. The new strategy of employing VIP prophylactically may be useful for both understanding and managing the salivary component of SS.

S jögren's syndrome (SS) is an autoimmune exocrinopathy of unknown aetiology, predominantly affecting peri- and post-menopausal women.[1] Although the main symptoms consist of ocular and oral dryness (xerophthalmia and xerostomia), there are also systemic effects. Features of affected glands are infiltrating, apoptosis resistant CD4+ T cells, and to a lesser extent, CD8+ T cells, B cells, and macrophages, and proinflammatory cytokines secreted from both lymphocytes and epithelial cells, together leading to inflammatory infiltrates, acinar atrophy, and destruction.[2 3] At present, patients are only offered symptomatic treatment, which is often unsatisfactory.

Vasoactive intestinal peptide (VIP), initially discovered as a gastrointestinal hormone, exhibits abundant functions, ranging from neurotransmitter, vasodilator, and bronchodilator effects to acting as a trophic agent, secretagogue, and immunomodulator.[4–7] VIP belongs to the glucagon/secretin superfamily.[8] Its precursor protein, prepro-VIP/PHM-27, encoded on human chromosome 6,[9] is an 8837 bp gene, containing seven exons and six introns and yielding the 28-amino acid VIP.[10] The amino acid sequence has been completely preserved in humans and mice[11 12] and transgenic human VIP (hVIP) has been shown to act through mouse VIP receptors in transgenic mice.[13]

Based on its immunomodulatory properties, VIP possibly may be useful in the management of several autoimmune disorders,[14] but its short half life may limit the applications of protein based treatment. In contrast, gene transfer potentially offers a means of sustained expression of a transgene like VIP.[7] Because high serum VIP levels are associated with secretory diarrhoea in patients with a VIPoma,[15] local treatment with VIP is preferable. Salivary glands provide an excellent target site for localised gene transfer after retrograde ductal infusion of vectors.[16]

Recently, we have constructed a recombinant serotype 5 adenovirus encoding the human VIP cDNA (rAd5CMVhVIP) and shown expression and function of the transgene.[17] Recombinant adenoviral vectors offer strong, but short term, protein expression due to a potent immune response by the host.[18] 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 the ability to infect numerous mammalian cells, dividing as well as non-dividing, and a minimal immune response.[18 19] In previous in vivo studies we have demonstrated the therapeutic effects of different transgenes encoded by rAAV2 vectors, currently the most widely used serotype, when delivered to murine submandibular glands (SMGs),[20–22] including local delivery of an rAAV2 encoding human interleukin 10 (hIL10) to the non-obese diabetic (NOD) mouse.[23] The NOD mouse develops, besides type I insulin dependent diabetes mellitus, exocrine gland infiltrates and decreased glandular secretion, which are dependent on age and sex,[2 24 25] making it the most useful, commonly available animal model to study the disease properties of SS.

In this study we have constructed the vector, recombinant serotype 2 adeno-associated virus encoding the human VIP

transgene (rAAV2hVIP), and examined its ability to alter the progressive SS-like dysfunction in NOD mice after local SMG delivery before disease onset.

MATERIALS AND METHODS
Construction of a viral vector encoding functional hVIP

We previously reported construction of the hVIP cDNA and the generation of a recombinant serotype 5 adenoviral vector rAd5CMVhVIP.[17] The cytomegalovirus (CMV) promoter/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[20]), resulting in pAAV2CMVhVIP (6391 bp, 1937 bp between inverted terminal repeats). The correct construct was verified by restriction digests and sequencing, and the presence of the inverted terminal repeats was confirmed by an SmaI digest (not shown). rAAV2hVIP was generated by subsequently cotransfecting this plasmid with the adenoviral helper packaging plasmid, pDG (a generous gift of Professor JA Kleinschmidt), at a ratio of 1:3 in 15 cm plates of ~40% confluent 293 T cells using a calcium phosphate precipitation procedure.[26] Additional details can be found in the supplementary information (available at http://www.annrheumdis.com/supplemental).

Infectious vectors were demonstrated by transducing 1.4×10^4 293 HEK cells on 96 well plates with serial dilutions of each aliquoted CsCl fraction in the presence of 1.5×10^7 particles of wild-type adenovirus. After 24 hours of incubation, supernatants from infected cells were analysed with an enzyme linked immunosorbent assay (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).[23][27]

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 the International Association for the Study of Pain (IASP) standards. Female NOD/LtJ mice (stock 001976) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Additional details can be found in the supplementary information (http://www.annrheumdis.com/supplemental).

Mice were maintained throughout the course of the study in the NIDCR animal facility (Bethesda, MD, USA) in accordance with Federal guidelines. Starting at age 10 weeks, body weights were measured weekly, as well as blood glucose levels (obtained by tail cut), using a OneTouch monitor (LifeScan, Milpitas, CA, USA). All mice with blood glucose levels ⩾22.2 mmol/l were given Ultra-Lente insulin (Eli Lilly, Indianapolis, IN, USA) injections subcutaneously (5 U/mouse, every 24 hours) to limit diabetes related dehydration, as described.[23]

Gene transfer, and salivary, SMG, and serum 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, seven animals/group) for each SMG (both glands targeted); the latter dose yielded the best results. For the second study, we instilled only one dose, 10^{10} particles in each gland (eight animals/group). The experiments described herein report the 10^{10} particles/SMG data from the two separate studies, over the indicated 8–16 week time course. Both studies gave comparable results. Vector delivery and saliva collection (at 8 weeks of age), and saliva, SMG, and blood collections (16 weeks) were performed as described in previous reports from our laboratory.[20–23] Additional details can be found in the supplementary information (http://www.annrheumdis.com/supplemental).

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 SMG 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 serum 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℃ 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 is inversely related to the protein concentration, was used to detect the presence of anti-VIP antibodies in NOD mouse sera.

Quantification of cytokines
IL2, IL4, IL6, IL10, IL12 (p70), interferon γ (IFNγ), tumour necrosis factor α (TNFα), and RANTES were measured commercially in SearchLight proteome arrays (Pierce Biotechnology, Woburn, MA, USA), which are multiplexed assays involving a sandwich ELISA procedure.[23] Additional details can be found in the supplementary information (http://www.annrheumdis.com/supplemental).
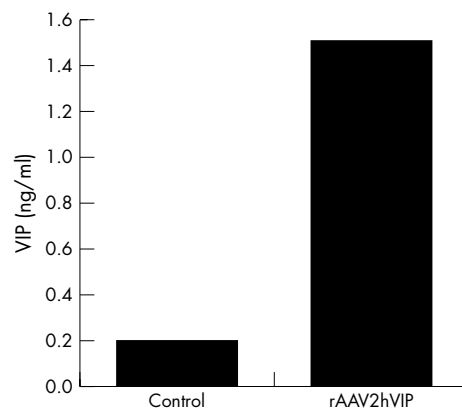
Figure 1 In vitro VIP expression directed by rAAV2hVIP. Transduction of 293 HEK cells with rAAV2hVIP (multiplicity of infection (MOI) = 4.9×10^3), in the presence of wild-type adenovirus (MOI = 1.0×10^3); control represents identical amount of 293 HEK cells infected with wild-type adenovirus alone. This experiment was analysed in duplicate and is representative of four separate experiments.

Histological assessment of SMGs

Part of each SMG 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 50 μm apart from the previous one, were mounted on poly-L-lysine coated slides and stained with haematoxylin-eosin. Histopathological 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 mm[2]).[23 28 29] The scoring was done blindly by three examiners (BML, FM, APC), and the mean of all focus scores for each animal was then calculated.

Apoptosis of SMG epithelial cells

VIP reportedly can protect cells from apoptosis.[30] Apoptotic epithelial cells were identified in SMG paraffin embedded sections by a terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick-end labelling (TUNEL) assay using a cell death detection kit (Chemicon, Temecula, CA, USA), according to the manufacturer's instructions and as previously described.[31] During subsequent visualisation 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 the 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 for each animal was then calculated.
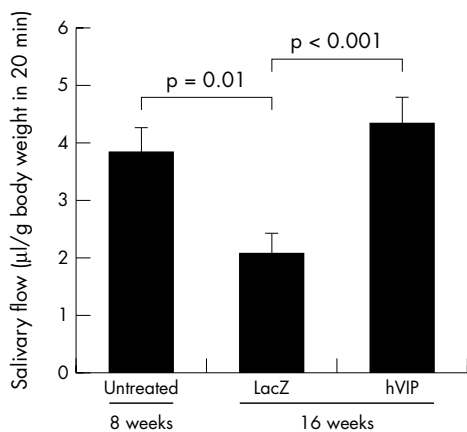
Statistical analysis

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 titres 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 1.4×10^{13} particles/ml. VIP expression after transduction of 293 HEK cells with these fractions showed that 4.9×10^3 particles rAAV2hVIP per cell resulted in 1.5 ng VIP/ml culture medium

Table 1 Levels of inflammatory molecules in SMG extracts

Cytokine	LacZ	hVIP	p Value
IL2	0.13 (0.03)	0.01 (0.01)	0.001
IL4*	0.00	0.00	1.000
IL6*	0.00	0.00	0.397
IL10*	0.05	0.00	0.054
IL12 (p70)	0.10 (0.00)	0.05 (0.01)	0.008
TNFα*	0.05	0.00	0.021
IFNγ	6.46 (1.19)	5.97 (0.59)	0.708
RANTES	3.75 (0.39)	2.68 (0.54)	0.142

Protein expression of immunomodulatory molecules (pg/mg wet weight) in SMG 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.

(sevenfold increase over background) (fig 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 SMGs. Reliable results were obtained for serum and SMG extracts (see supplementary table 1 (http://www.annrheumdis.com/supplemental)). Animals given the rAAV2hVIP vector exhibited significantly higher levels of VIP (ng/ml, mean (SEM)) than controls in SMG extracts (0.49 (0.03) v 0.36 (0.05), p = 0.039), and in serum (0.16 (0.01) v 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).

Serum anti-VIP antibodies

To determine the presence of serum anti-VIP antibodies, pooled serum samples 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 that local delivery of rAAV2hVIP did not result in the development of serum anti-VIP antibodies 8 weeks after vector delivery (see supplementary fig 2 (http://www.annrheumdis.com/supplemental)).

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.[28] We examined salivary flow before virus administration at 8 weeks of age (baseline) and at the time of death (16 weeks). At baseline, the mean (SEM) salivary flow rate was 3.81 (0.47) μl/body weight (grams) in 20 minutes. At 16 weeks of age, 8 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; fig 2). The decrease in flow rate of mice treated with rAAV2LacZ at 16 weeks compared with 8 weeks was also significant (p = 0.01).

Effect of rAAV2hVIP and rAAV2LacZ on inflammatory infiltrates in SMGs of NOD mice

Female NOD mice start developing characteristic focal inflammatory infiltrates in SMGs after 8 weeks of age[28]; such local changes also form an important clinical feature in patients with SS. SMG 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) v 1.85 (0.26), p = 0.75).

Figure 2 Effect of rAAV2hVIP and rAAV2LacZ on salivary flow rate in NOD mice. Mice were anaesthetised and pilocarpine stimulated whole saliva was collected, as described in ''Materials and methods''. Salivary flow rate (μl/body weight (grams) in 20 minutes) at 8 weeks (n = 19; randomly selected from both groups, untreated) and mice at 16 weeks of age either treated with rAAV2LacZ (LacZ, n = 13) or rAAV2hVIP (hVIP, n = 15) is shown. Bars represent means (SEM). Student's t tests were performed and the p values for the differences are indicated.

Effect of rAAV2hVIP and rAAV2LacZ on apoptotic rate in SMGs of NOD mice

The median apoptotic index, as an indication of the apoptotic rate, of epithelial cells in SMGs did not differ between the rAAV2LacZ and rAAV2hVIP groups (medians 0.15 v 0.16, respectively, p = 0.45).

Effect of rAAV2hVIP and rAAV2LacZ on cytokine expression in SMG extracts and sera of NOD mice

VIP can act as an immunomodulator, inhibiting levels of proinflammatory cytokines[32 33] and increasing levels of anti-inflammatory cytokines.[34] We examined protein expression of several pro- and anti-inflammatory cytokines and one chemokine, locally in extracts of SMGs, as well as systemically in serum, in treated mice at 16 weeks of age. Aqueous SMG extracts from NOD mice treated with rAAV2hVIP showed lower levels of IL2 (p = 0.001), IL10 (p = 0.054), IL12 (p70) (p = 0.008), and TNFα (p = 0.021) than those from mice treated with rAAV2LacZ (table 1). IL4, IL6, IFNγ, and RANTES levels in SMG extracts did not differ significantly between these groups. Serum levels of all measured cytokines did not differ between the two vector groups (supplementary table 2 (http://www.annrheumdis.com/supplemental)). However, levels of RANTES in serum were significantly lower in rAAV2hVIP treated mice (6.8 v 12.2 pg/ml, p = 0.040).

DISCUSSION

As far as we know, 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 treatment 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[35 36] and experimental autoimmune uveoretinitis.[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—that is, a prevention, not treatment, model. Based on the results, in the future, we will test the effects of rAAV2hVIP upon administration after disease onset, more closely resembling a clinical situation. Instillation of rAAV2hVIP in the SMGs of NOD mice led to higher salivary flow rates, no difference in focus scores or apoptotic rates, increased SMG and serum expression of VIP, a reduction of cytokines IL2, IL10, IL12 (p70), and TNFα in SMG extracts and of serum RANTES, compared with the results with the control vector.

Salivary gland cells are polarised and can secrete transgene encoded proteins either via the constitutive pathway across the basolateral membrane, or on stimulation via the regulated pathway into saliva.[38–40] Pre/pro-VIP protein contains a recognition signal directing it into secretory granules during post-translational processes.[41] Because the hVIP cDNA used here should contain that signal,[17] salivary cells should secrete hVIP mostly via the regulated pathway into saliva, with only a small proportion exiting to the interstitium and serum.[38 39 42] VIP is a small neuropeptide, quickly degraded and inactivated, giving it a limited bioavailability.[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 SMG extracts. rAAV2hVIP treated mice showed significantly higher VIP expression in SMG extracts and serum than control rAAV2LacZ treated mice. Unfortunately, owing 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.[44 45] The protein levels expressed from the rAAV2hVIP vector in vivo, while low, are reasonable and comparable to those of other transgenic secretory proteins in rAAV2 experiments.[21 22]

We observed a decrease in the levels of several proinflammatory cytokines, IL2, IL12 (p70), and TNFα, but also in the anti-inflammatory cytokine IL10, in SMG 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 the serum of rAAV2hVIP treated animals. Several previous studies with untreated NOD/LtJ mice, of about the same age as the NOD mice studied by us, showed comparable serum cytokine levels to our data (for example, see Yang et al[46 47]). To our knowledge, SMG 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.[32] The VIP/PACAP receptor family consists of three G-protein coupled receptors, two of which, VPAC$_1$ and VPAC$_2$, are present on macrophages, and CD4$^+$ and CD8$^+$ T lymphocytes.[48 49] The effects of VIP are wide ranging, including the inhibition of several macrophage functions, T cell proliferation, lymphocyte migration, and the expression of chemokines and proinflammatory cytokines.[7 32 33] Conversely, the production of IL10 is reportedly stimulated by VIP.[34] Pozo et al postulated that VIP is a Th2 cytokine with a key role in neuroimmunology[34 50]—that is, VIP production by Th2 cells, as well as VIP stimulation of Th2, and inhibition of Th1, functions. Although VIP may exert these effects in some autoimmune diseases with a balance shifted towards Th1 response, such as rheumatoid arthritis,[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 down regulation of SMG IL10 levels and may indicate that VIP acts as a more overall immunosuppressant than strict Th2 cytokine in this SS model. In patients with SS, salivary gland and serum IL10 levels are actually increased, depending on the disease stage and activity, and might be a sign of B cell activation and lymphoma development.[52–55] As recently stated by Delaleu et al, ''further approaches modulating cytokines are warranted in SS''.[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.[3 56] However, the cytokine profile in SMG biopsies with simultaneous expression of IFNγ, IL2, IL4, and IL13 provides an argument against a simple Th1 or Th2 predominance in SS.[3] Clearly, hVIP gene transfer to SMGs 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 patients with SS was solely attributed to a destruction of glandular tissue, a well recognised incongruity exists between the reduction in salivary flow and the extent of focal lymphoid infiltration.[57 58] Hypofunction of salivary epithelial cells might be, in addition, due to the effects of cytokines or autoantibodies (for example, anti-muscarinic receptor antibodies) in patients with SS.[59 60] Thus, it is not entirely unexpected that a dissociation was seen in rAAV2hVIP effects on salivary flow rate and focal SMG 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.[16] rAAV2 vectors appear to transduce only ductal cells, not acinar cells.[21 22] Therefore, we suggest that the 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 SMGs, VIP receptors are found on both luminal and basal membranes of mucous acinar cells, as well as intercalated ducts.[61] In human labial salivary glands, VIP binding sites are detected on the basal membranes of mucous acini,[62] and the presence of a functional VIP receptor system in vitro has been demonstrated on isolated acinar cells.[63] There are no data on VIP receptors in epithelial cells of mouse SMGs. In addition, several studies of patients with SS have shown the existence of immunoreactive VIP nerve fibres in human SMGs[61] and human labial salivary glands,[63 64] where VIP containing nerves are in close contact with the acinar cells, inside the acinar basement membrane.[44] Taken together, it is thought that VIP has a role in several components of reflex salivary secretion.[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 SMGs 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.

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