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A secretagogue-siRNA conjugate confers resistance to cytotoxicity in a cell model of Sjögren's syndrome

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Abstract

Objective—Sjögren's syndrome (SjS) is characterized by xerophthalmia and xerostomia resulting from loss of secretory function due to immune cell infiltration in lacrimal and salivary glands. Current SjS therapeutic strategies employ secretagogues to induce secretion via muscarinic receptor stimulation. Based on our expertise on muscarinic type-3-receptor (M3R), we are utilizing ligands specific for muscarinic receptor to deliver siRNA into cells via receptor-mediated endocytosis, thereby altering epithelial cell responses to external cues such as pro-inflammatory or death signals while simultaneously stimulating secretion.

Methods—Carbachol was synthesized with an active choline group and conjugated with siRNA targeting caspase-3, and a human salivary gland cell line (HSG) was used to test the efficacy of this conjugate.

Results—Lipofectamine transfection of conjugate into cells resulted in 78%-reduction in caspase-3 gene expression, while external conjugate treatment of HSG cells resulted in similar intracellular calcium release and induction of endocytosis as carbachol stimulation indicating that the siRNA and carbachol portions of conjugate retained function after conjugation. HSG cells treated with conjugate (without Lipofectamine transfection) exhibited a 50% reduction in caspase-3 gene and protein expression indicating our conjugate is effective in delivering functional siRNA into cells via receptor-mediated endocytosis. Furthermore, TNF- α induced apoptosis was significantly reduced in conjugate treated cells.

Conclusions—In conclusion, a secretagogue-siRNA conjugate prevented cytokine-induced apoptosis in salivary epithelial cells, which is critical to maintain fluid secretion and potentially reverse the clinical hallmark of SjS.

Introduction

Sjögren's syndrome (SjS) is common systemic autoimmune disease mainly affecting the salivary and lacrimal glands resulting in secretory hypofunction and dry mouth and dry eye, respectively, which adversely affects quality of life. Despite extensive studies into the

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mechanisms which contribute to the development or pathogenesis of SjS, the events that trigger disease onset in the target exocrine glands remain unknown. Our previous studies examining the salivary glands of the non-obese diabetic (NOD) and more recently, the C57BL/6.NOD-*Aec1/Aec2* mouse models of SjS indicate alterations in the glandular environment even prior to disease onset, including apoptosis of acinar tissues and altered cell proliferation (1-5). Current therapeutic strategies for SjS mainly focus on palliative treatments to stimulate secretion or suppression of immune responses by corticosteroids. However, such treatments do not address the underlying causes of secretory dysfunction, one of which is the loss of acinar cells through apoptotic cell death.

RNA interference (RNAi) is the natural process occurring in most eukaryotic cells in which small double stranded RNA (dsRNA) molecules negatively regulate gene expression by causing the degradation or translation repression of specific mRNA targets (reviewed in (6)). One class of these small dsRNAs are small interfering RNA molecules (siRNA), which are 21 nucleotides long and bind specifically to their target mRNAs via complementary base-pair matching, thus resulting in the cleavage of that mRNA by the RNA-induced silencing complex (RISC, (6)). Since the discovery of the RNAi pathway, there has been a surge in research towards developing siRNA-based therapeutics for otherwise “undruggable” targets. Two critical issues being considered in the development of siRNA therapies are preserving the efficacy and stability of the siRNA molecule in vivo and generating siRNA delivery systems. It has been determined that stability of siRNA in vivo can be achieved through various chemical modifications (7). siRNA delivery can be achieved by a variety of strategies including lipid-based formulations (8), nanoparticles (9), and magnetofection (10). However, these strategies are nonspecific, and cell-type specific delivery is still the most challenging step blocking the progress of RNAi therapy in modern medicine. In order to target siRNA to specific cell or tissue types, specificity must be built into the delivery agents or expressed shRNAs. Some strategies for cell-type specific delivery include antibody targeting (11), cell-penetrating peptides (12), chemical modifications (8), and aptamers (13), but each of these strategies presents certain drawbacks such as cytotoxicity or immunogenicity.

Our strategy is to develop a vehicle that alters molecular signals in the salivary epithelial cells using RNA interference (RNAi)-based strategies. We hypothesized that a ligand for muscarinic type-3 receptor (M3R), carbachol, conjugated with small interfering RNAs (siRNAs), can deliver siRNA into a human salivary gland cell line (HSG) by receptor-mediated endocytosis where it can silence gene expression by RNAi while simultaneously inducing secretion in SjS patients. This carbachol-siRNA conjugate is referred to hereafter as the “conjugate”. For this study, we utilized siRNA targeting caspase-3 in the conjugate to investigate if knockdown of caspase-3 can prevent cytokine-induced apoptosis of HSG cells, mimicking the *in vivo* environment of SjS salivary glands where the fluid-secreting acinar cells undergo apoptosis. Ultimately, our goal is to utilize this siRNA conjugation technique with an FDA-approved MR agonist such as cevimeline (Evoxac). This design is particularly innovative because the siRNA portion of the conjugate can be customized to target any gene of interest, thus RNAi therapy can be delivered to cells in a receptor-specific manner while simultaneously stimulating secretion in SjS patients.

Materials and Methods

Carbachol-siRNA conjugation

Conjugate was ordered and synthesized by Solulink, Inc. (San Diego, CA). siRNA targeting caspase-3 (target sequence: CCGACAAGCUUGAAUUUAU) or a scrambled sequence (GAUAUGUCAACUCAGUACU) were conjugated to carbachol. Conjugate has been

synthesized four times in two years, and experimental variations between batches are minimal based on our functional tests.

Activation of choline—Triethylamine (14 μ L; 100 μ mol) was added to a solution of choline tosylate (13.8mg; 50 μ mol; SigmaAldrich, St. Louis, MO) in anhydrous DMF (250 μ L). A solution of 4-nitrophenyl chloroformate (10.1 mg; 50 μ mol; SigmaAldrich) in anhydrous dichloromethane (100 μ L) was prepared and added directly to the choline/TEA/DMF solution. The reaction was incubated at room temperature for 2 hours. A sample was analyzed by electrospray mass spectrometry and the solution was used directly for modification of the amino-RNA sense strand if the major peak $m/e = 269$.

Choline conjugation to amino-RNA sense strand—Both amino-modified sense and antisense strands were desalted into 100 mmol phosphate, 150 mM NaCl, pH 7.4 using 5K MWCO VivaSpin diafiltration devices (SartoriusStedim, Purchase, NY). The activated choline solution (9.76 μ L; 1.3 μ mol; 20 mol equivalents) was added to the amino-modified sense strand (66.2 nmol; 28.2 μ L; 0.488 OD/ μ L), vortexed, and allowed to stand at room temperature for 1 h and overnight at 4°C. The choline-modified RNA was isolated by desalting into nuclease-free water using a 5K MWCO VivaSpin device. The product was analyzed by MALDI mass spectrometry: expected 6912; found m/e 6925 (starting amino-RNA m/e 6794 + choline-C=O 118).

HSG cell culture

HSG cells were a kind gift from Dr. Joseph Katz at the University of Florida. Cells were maintained in DMEM supplemented with 10% fetal calf serum, penicillin (100U/ml) and streptomycin (100 μ g/ml) (Life Technologies, Burlington, Ontario, Canada). For conjugate treatment, HSG cells were seeded onto 8-chamber slides or 6-well plates in growth media and cultured overnight. The cells were then washed three times with Opti-MEM serum free media (Invitrogen, Carlsbad, CA). The cells were treated with conjugate diluted in Opti-MEM to the specified final concentration. Appropriate controls were included in each experiment. Negative control conjugate (“Neg Ctl Conjugate”) was synthesized by Solulink as described previously using a scrambled siRNA sequence and carbachol. “Free siCaspase-3” indicates siRNA targeting caspase-3 was added to culture media in the absence of transfection reagent to serve as an additional control. “Carbachol only” indicates 100 μ M carbachol was added to the culture media. “siCaspase-3” or “transfected siCaspase-3” indicates that the cells were transfected with 40nM siRNA targeting caspase-3 to serve as a positive control for caspase-3 knockdown.

Transfection and qRT-PCR

HSG cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. siRNAs targeting human caspase-3 (target sequence 5'CCGACAAGCUUGAAUUUAU3') and human GAPDH (target sequence 5'GGUCAUCCAUGACAACUUUTT3') were purchased from Dharmacon (Lafayette, CO) and Applied Biosystems (Carlsbad, CA), respectively, and transfected into cells at a final concentration of 40nM. Caspase-3, OAS-1, and MX-1 gene expression in HSG cells was analyzed after transfection or conjugate treatment using quantitative real time RT-PCR (qRT-PCR). Total RNA was extracted using the miRVana miRNA Isolation kit (Ambion, Austin, TX) in accordance with the manufacturer's protocol. RNA concentrations were determined, and 50ng of each RNA sample was used for qRT-PCR which was performed using the TaqMan High-Capacity cDNA Reverse Transcription Kit, TaqMan Fast PCR Master Mix, and Taqman mRNA assay primers (Applied Biosystems). All reactions were analyzed using StepOne Real-Time PCR System (Applied Biosystems). The levels of mRNA were normalized to 18S controls. The cycle threshold values, corresponding to the

PCR cycle number at which fluorescence emission reaches a threshold above baseline emission, were determined and the relative mRNA expression was calculated using the $\Delta\Delta C_t$ method (14).

Calcium Release Assay

Untreated, conjugate treated and carbachol-treated HSG cells were monitored using Invitrogen's Fluo-4 No Wash Calcium assay kit in accordance with the manufacturer's protocol. Briefly, 30,000 cells per well were cultured in a 96-well plate overnight in growth media. Before the assay, the growth media was removed and 100 μ l of dye-loading solution was added to each well. Carbachol or conjugate was added to the cells at the indicated concentrations, and emitted fluorescence (516nm) was measured using a fluorescent plate reader.

Calcium Imaging

ParC5 cells were loaded with Fura 2-AM (15). Briefly, a 1 mM solution of Fura 2-AM was made in DMSO containing 20% pluronic acid. This was then diluted 100-fold in physiological solution. The dye was filled in a pipette and pressure injected for 30–45 min. Cells were incubated in 20 μ M Fura 2-AM in 0.2% pluronic acid for an hour at RT and allowed to recover a further hour. Imaging was performed using a Zeiss Axioskop-2 upright microscope using a water-immersion 40 $\times$ lens (numerical aperture, 0.8). Images were acquired using a cooled CCD camera (Cooke Sensicam; PCO, Kelheim, Germany). Excitation was achieved using a Sutter Instruments (Novato, CA) DG-4 fast wavelength switcher and standard Fura 2 excitation and emission filters were used (Chroma Technology, Brattleboro, VT). Images were acquired and processed using the SlideBook software (Intelligent Imaging Innovations, Denver, CO). Ratiometric data were acquired at 0.1–1 Hz. Analyzed data were graphed using the Microcal Origin software. Calcium signals were expressed as changes in the 340/380 ratio. Agonist was applied using pressure application via a patch pipette placed $\sim$ 20 μ m above the slice for 30 s.

Endocytosis assay

ParC5 cells were transfected with the Yellow Fluorescent Protein (YFP)-tagged NBCe1 construct (NBCe1-EYFP). 100 μ M CCh or 1:100 diluted conjugate (8.71 μ M) were added to serum-free culture media. Cells were then washed with PBS, fixed, permeabilized, and labeled with anti-Early Endosomal Antigen 1 (EEA1) primary antibodies followed by Alexa Fluor-Texas red secondary antibodies, washed and mounted with Vectashield on slides for qualitative analysis through a confocal microscopy. Fluorescent images were taken using an Olympus Spinning Disk confocal microscope controlled by Slidebook 4.0.10.2. Confocal images were captured in z-stack intervals of 1 μ m using a 60 $\times$ oil immersion objective (1.45NA).

In situ hybridization

HSG cells were washed once with PBS, fixed in 4% paraformaldehyde for 15 minutes at room temperature, permeabilized in 0.5% Triton X-100 for five minutes, and dehydrated with 70, 90, and 100% ethanol for one minute each. The FAM-labeled DNA oligo probe specific for the antisense strand of caspase-3 siRNA (Exiqon Woburn, MA) was diluted to 80nM in PBS and heated to 80°C for two minutes before addition to the cells. Hybridization was performed at 47°C for 45 minutes, and the cells were then washed and mounted in Vectashield Mounting Medium containing DAPI (Vector Labs, Burlingame, CA). Images were taken with Zeiss Axiovert 200 M microscope and a Zeiss AxioCam MRm camera.

Immunocytochemistry

Caspase-3 protein was detected with rabbit anti-caspase-3 antibodies (Abcam, Cambridge, MA) used at 1:200 dilution. Secondary antibodies used were Alexa Fluor 568 goat anti-rabbit IgG (1:400) from Molecular Probes (Carlsbad, CA). Slides were mounted using Vectashield Mounting Medium containing DAPI (Vector Labs) and images were taken with Zeiss Axiovert 200 M microscope and a Zeiss AxioCam MRm camera. Image J analysis software was used to measure relative caspase-3 protein expression.

Apoptosis assay

HSG cells were treated with TNF- α (50ng/ml, BD Biosciences, San Jose, CA) and cycloheximide (10 μ g/ml, Sigma, St. Louis, MO) for eight hours. Cycloheximide is required to sensitize the cells to TNF- α induced apoptosis (16, 17). The cells were then washed once with PBS, briefly trypsinized, and pelleted. The cells were then stained with FITC-conjugated Annexin V (BD Biosciences) and propidium iodide (BD Biosciences) for 15 minutes at room temperature. The stained cells were analyzed by flow cytometry (FACS Calibur, BD Biosciences) to determine the percentage of early (annexin-V positive) and late (double positive) apoptotic cells.

Statistical Analysis

Statistics were calculated using GraphPad Prism 4 software (GraphPad Software, La Jolla, CA). Student t tests or one-way ANOVA were used as indicated, with $p < 0.05$ considered statistically significant.

Results

Conjugation of carbachol with siRNA

Our conjugate design aims to utilize the specificity of MR agonist carbachol to deliver siRNA into MR-expressing cells of the salivary glands via receptor-mediated endocytosis. Ultimately, our goal is to apply this technique to an FDA-approved MR agonist such as cevimeline or pilocarpine. Carbachol was synthesized with an active choline group which was linked to an siRNA targeting caspase-3 via a 5' amino group as described in Methods (Figure 1A).

Carbachol and siRNA portions of conjugate retained function after conjugation

After conjugation, we set out to determine if the carbachol and siRNA portions of the conjugate retained function after the conjugation process. First, we transfected the conjugate into HSG cells in varying concentrations from 100nM to 10 μ M using Lipofectamine 2000, incubated the cells for 48 hours, and analyzed caspase-3 gene expression by qRT-PCR. Cells were also transfected with a negative control conjugate containing a scrambled siRNA sequence. Figure 1B shows that transfected conjugate resulted in significant knockdown of caspase-3 gene expression compared to cells transfected with negative control conjugate ($*p < 0.01$) as determined by one-way ANOVA with Dunnett's multiple comparison test). Interestingly, 100nM and 1 μ M transfected conjugate resulted in 78% and 71% reduction in caspase-3 gene expression, respectively, while 10 μ M transfected conjugate resulted in only a 38% reduction (Figure 1B). This is most likely due to saturation of the endogenous RISC machinery in the cells. Unconjugated caspase-3 siRNA was transfected into cells in parallel and resulted in a 53% reduction in caspase-3 gene expression, while free siRNA and carbachol added to the cells alone had no effect on caspase-3 gene expression. These data indicate that the siRNA portion of the conjugate retained function after the conjugation process, and the conjugated carbachol did not influence siRNA efficacy on gene knockdown.

Next, we tested the carbachol portion of the conjugate in a calcium release assay that quantitatively measures intracellular calcium release from HSG cells. MR agonists such as carbachol, bind MR on the cell surface, initializing signal transduction which results in intracellular calcium release from the endoplasmic reticulum, ultimately leading to fluid secretion (18). Therefore, intracellular calcium release is monitored as a measure of carbachol function. HSG cells were treated with 1-100 μ M carbachol and 100nM-87 μ M conjugate in parallel, and as shown in Figure 1C, conjugate treatment resulted in similar levels of intracellular calcium release as carbachol treatment. To independently verify these results, calcium ratiometric analysis was performed using the rat parotid cell line, ParC5. In this sensitive assay, cells were treated with 100 μ M carbachol or 8.71 μ M (1:100 dilution) of conjugate or a free siRNA control. Single cells stimulated with conjugate produced similar levels of calcium release as those stimulated with carbachol, as presented in peaks (Figure 1D). These data indicate that the conjugation process did not alter the efficacy of the carbachol.

Another measure of carbachol function of importance to our study is its known ability to induce muscarinic receptor-mediated endocytosis. To monitor carbachol-induced endocytosis with our conjugate, ParC5 cells were transfected with the Yellow Fluorescent Protein (YFP)-tagged NBCe1 construct (NBCe1-EYFP), which is an electrogenic Na⁺-HCO₃⁻ cotransporter (NBCe1) known to be endocytosed upon cholinergic stimulation (19), and then treated with 100 μ M carbachol or 8.71 μ M conjugate. Colocalization (yellow) between NBCe1-EYFP (green) and EEA1-endosomal marker (red) indicates the induction of endocytosis. As shown in Figure 2, the merged images suggest that carbachol and the conjugate similarly stimulate endocytosis of basolateral NBCe1 in ParC5 cells, indicating that the carbachol portion of the conjugate is capable of inducing receptor-mediated endocytosis.

Conjugate entry detected in HSG cells

Once we verified that both the carbachol and siRNA portions of the conjugate retained function after conjugation, we set out to determine if the conjugate could enter cells through receptor-mediated endocytosis. A FAM-labeled DNA oligo probe was designed to specifically bind the antisense strand of the caspase-3 siRNA, and in situ hybridization was utilized to visualize the entry of conjugate into HSG cells (Figure 3). Cells were treated with 5 μ M conjugate for 30 minutes to 24 hours or left untreated and fixed after each time point. As shown in Figure 3, conjugate is detected in the cytoplasm (arrows) as soon as 30 minutes after treatment and up to 24 hours after treatment, although notably, conjugate was detected in only approximately 30% of cells. This experiment was repeated six times over a several month period, with reproducible results; however, the percentage of cells which contained conjugate varied with cell batches. This may be due to non-uniform M3R expression on HSG cells in response to subtle changes in culture condition overtime. This also reflects the importance of receptor density in the receptor-mediated endocytosis. Nonetheless, our data indicate that conjugate entry into cells was successful.

Conjugate treatment results in caspase-3 gene and protein reduction

We next sought to determine if the conjugate was capable of knocking down caspase-3 gene/protein expression after entry into the cells. Cells were treated with 8.71 μ M conjugate for 4-6 hours in serum free media which was then replaced with growth media. After 48 hours of incubation, caspase-3 gene expression was analyzed by qRT-PCR. As shown in Figure 4A, conjugate-treated cells showed a 50% reduction in caspase-3 gene expression compared to cells treated with negative control conjugate (*p<0.01 as determined by one-way ANOVA with Dunnett's multiple comparison test). Caspase-3 gene expression in MR-negative HeLa cells treated with 5 μ M conjugate was not affected (Figure 4B), indicating

that the conjugate specifically targets MR-expressing cells. HSG cells were also treated with 50nM to 1 μ M concentrations of free siCaspase-3 to demonstrate that siRNA alone is unable to enter the cells. As expected, free siCaspase-3 treated cells showed no reduction in caspase-3 gene expression (Supplemental Figure 1). To ensure that the reduced caspase-3 gene/protein expression was not due to a non-specific interferon response, mRNA expression of interferon response genes OAS1 and MX1 was monitored and shown to remain unchanged after conjugate treatment (Figure 4C). After 72 hours of incubation, caspase-3 protein expression was analyzed by immunofluorescence. As shown in Figure 5A, caspase-3 staining was drastically reduced in conjugate-treated and caspase-3 siRNA-transfected cells (red) compared to untreated cells. Transfected GAPDH siRNA, carbachol only, and free siCaspase-3 had no effect on caspase-3 protein levels. Caspase-3 siRNA was detected in transfected cells (green), but conjugate was minimally detected, indicating that the conjugate may be degraded after 72 hours. Quantitative analysis of caspase-3 protein levels carried out using Image J software (Figure 5B) shows that caspase-3 protein levels were reduced by 50% in conjugate-treated cells, similarly to caspase-3 siRNA transfected cells. Western blot was also performed to further demonstrate the reduction in caspase-3 protein levels after conjugate treatment. As shown in Figure 5C, cells treated with 5 μ M conjugate exhibited greater than 50% reduction in caspase-3 compared to mock transfected or cell treated with negative control conjugate. Taken together, these data demonstrate that our conjugate is effective in knocking down caspase-3 gene and protein expression and reveal a novel strategy for RNAi therapies in conjugating siRNAs directly with specific receptor ligands.

Conjugate treatment prevents TNF- α induced apoptosis of HSG cells

In SjS, the presence of inflammatory cytokines in target tissues contributes to apoptosis of surrounding cells. Hence, our strategy is to prevent cytokine-induced apoptosis of acinar cells using the conjugate to knock down caspase-3 expression. HSG cells were treated with 10 μ M conjugate or negative control conjugate or transfected with caspase-3 siRNA and incubated for 96 hours to allow for complete caspase-3 knockdown. The cells were then treated with TNF- α (50 ng/ml) and cycloheximide (10 μ g/ml) for eight hours, and then stained with Annexin-V and propidium iodide (PI) as described in Methods. The percent of early apoptotic cells (Annexin-V positive) was reduced in caspase-3 transfected cells by 25% and in conjugate treated cells by 33% after TNF- α /cycloheximide treatment (Figure 6A, $p < 0.02$ compared to negative control conjugate-treated cells as determined by t test). The percent of late apoptotic cells (Annexin-V/PI positive) was reduced by 58% in caspase-3 transfected cells and by 25% in conjugate treated cells (Figure 6B, $p < 0.01$ compared to negative control conjugate-treated cells as determined by t test). Apoptosis in cells treated with negative control conjugate with a scrambled siRNA sequence was not significantly different from mock transfected cells. These data indicate the therapeutic potential of conjugate in preventing cytokine-induced apoptosis in salivary acinar cells thus maintaining secretory function in SjS.

Discussion

The goal of this study was to determine if siRNA targeting caspase-3 conjugated to the MR agonist carbachol can be delivered to cells via receptor-mediated endocytosis hence providing a novel approach for cell type-specific RNAi therapy to preserve epithelial cells and maintain secretory function in SjS. Our data indicated that both the siRNA and carbachol portions of the conjugate retained function after the conjugation process (Figures 1 and 2), and conjugate entry into HSG cells was detectable using a FAM-labeled probe (Figure 3). Conjugate treatment of cells resulted in a 50% reduction in caspase-3 gene and protein expression (Figures 4 and 5), indicating that this siRNA-carbachol conjugate is

successfully delivered into cells via MR receptor-mediated endocytosis. Conjugate treatment also resulted in reduced apoptosis after TNF- α /cycloheximide treatment (Figure 6) demonstrating its therapeutic potential.

Our current study supports the further development of this novel type of RNAi therapy which could potentially have broad applications. To our knowledge, our strategy of directly conjugating siRNA molecules to a receptor ligand has not been attempted to date. This design has multiple advantages over current strategies, as well as some limitations. For example, the small size of most receptor ligands (carbachol's molecular weight is 182.6g/mol) coupled with the small size of siRNAs results in the increased likelihood that the siRNA's function will not be affected by the conjugated ligand. Also, by avoiding relatively bulky protein complexes such as a biotin-streptavidin bridge, the overall size of our conjugate is small and it did not induce an immune response (Figure 4C). Other strategies that employ biotin-streptavidin linking of antibodies to siRNA make use of the specificity of antibody targeting, but once inside the cell the siRNA most likely would have to be cleaved free from the antibody-biotin-streptavidin complex in order to be functional, and the large size of the complex increases the possibility of triggering a cellular response. However, conjugating siRNA to receptor ligands is limited to the chemical structure and flexibility of those ligands. Ligands with more complex structures may be more difficult to modify in order to link an siRNA without disrupting the ligand's capacity to bind its target receptor.

In our studies, we are aiming to utilize our carbachol-siRNA conjugate for the treatment of SjS. Our previous studies have shown that acinar cells show signs of apoptotic cell death even prior to disease onset in our SjS-prone mouse model system (C57BL/6.NOD-*Aec1Aec2*). Therefore, we set out to reverse apoptotic cell death in the acinar cell population by knocking down caspase-3 while simultaneously stimulating fluid secretion with carbachol. However, the beauty of our strategy is that any gene of interest can be targeted by simply changing the target sequence of the siRNA. Other potential applications include but are not limited to: 1) Alteration of molecular events in the epithelial cells to down-regulate cytokine secretion, co-stimulatory molecules or HLA class II expression for antigen presentation; 2) Inhibition of epithelial cell apoptosis to determine its role in the onset and progression of SjS and to enhance saliva secretion from acinar cells; 3) Improvement of mild cognitive impairment or fatigue in a subset of patients by regulating secretion of pro-inflammatory cytokines from target tissues of SjS; 4) Targeting macrophage-specific receptor to regulate caspase-1, which is important for pro-inflammatory cytokine secretion that leads to epithelial cell death in the glands prior to disease onset; 5) Targeting viral antigens detected in the salivary glands to reduce immunogenicity; 6) Differentiation of the hyperproliferative ductal cells in SjS into secretory acinar cells *in situ* in the diseased glands by identifying master transcription factors, activators or repressors for differentiation.

Early intervention is more effective and beneficial than late intervention with regards to maintaining saliva flow in SjS patients. Modulating host cell or innate immune response around the time of disease onset or while acinar cells are still viable in the diseased salivary glands would be as critical as regulating autoreactive immune cells to prevent severe secretory dysfunction. Unfortunately, pre-diagnostic markers for SjS are not available, let alone SjS-disease specific diagnostic markers. Our research has focused on identifying such markers by investigating the disease pathogenesis in the salivary glands of SjS mouse models from pre-disease stage to full-blown disease stage (2, 3, 20-29). Regulating gene expression by siRNA will become an invaluable tool to reshape the responses of epithelial cells from the early age on. Furthermore, in cases of advanced stages of disease, targeting hyperproliferative ductal cells in SjS glands to differentiate ductal cells into secretory acinar cells with RNAi may also be a possibility.

Further studies into the efficacy of this conjugate in our SjS-prone mouse model are needed, and modifications to increase the stability of the siRNA for in vivo applications may be necessary. Also, the development and optimization of in vivo delivery techniques for our conjugate will be required prior to clinical applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Carbachol and siRNA portions of conjugate retained function after conjugation. (A) Scheme presenting the two-step activation and conjugation of choline to 5'-amino-modified RNA. Activation of choline was carried out by reaction with 4-nitrophenyl chloroformate followed by reaction with amino-modified RNA. (B) Caspase-3 gene expression after transfection of siRNA, conjugate, or negative control conjugate. Free siCaspase-3 treatment indicates naked siRNA targeting caspase-3 was added to the cells in the absence of transfecting reagent. Negative control conjugate contains a scrambled siRNA sequence. Asterisks indicate $p < 0.05$ compared to negative control conjugate as determined by one-way ANOVA with Dunnett's multiple comparison test. (C) Conjugate was added to HSG cells as indicated and intracellular calcium release was measured as fluorescence intensity. Bars represent mean with standard error ($n=3$ independent experiments), and dashed line indicates level of background fluorescence. (D) Calcium imaging of Parc5 cells treated with 100 μM carbachol, a free siRNA control, or 8.7 μM conjugate. Each colored line represents calcium response in a single cell.

Figure 2.

Conjugate induces receptor-mediated endocytosis. ParC5 cells transfected with YFP-tagged NBCe1 (green) were treated with carbachol or conjugate and costained with anti-EEA-1 endosomal marker (red). Colocalization (yellow) of NBCe1 and EEA-1 indicated by yellow. Nuclei counterstained with DAPI (blue, merged images). Images shown at 630X magnification, insets are enlarged to view colocalization.

Figure 3. Conjugate detected in HSG cell cytoplasm within 30 minutes of incubation. HSG cells were treated with 5 μ M conjugate for the indicated times before being fixed and used for in situ hybridization with a FAM-labeled DNA oligo probe specific for the antisense strand of caspase-3 siRNA. Arrows indicate conjugate detected in cytoplasm of cells (green). Nuclei counterstained with DAPI (blue). Images shown at 200X magnification.

Figure 4.

Conjugate treatment reduced caspase-3 mRNA expression in HSG cells. A) Conjugate had comparable caspase-3 knockdown efficiency compared to transfected siRNA. HSG cells were treated with 8.71μM conjugate or negative control conjugate and incubated for 48 hours. Caspase-3 gene expression was then analyzed by qRT-PCR. Asterisks (*) indicate $p < 0.01$ compared to negative control conjugate treated cells as determined by one-way ANOVA with Dunnett's multiple comparison test. Bars represent mean with standard error (n=5 independent experiments). B) Conjugate had no effect on M3R-negative cells. HeLa cells were treated with conjugate or transfected with caspase-3 siRNA, incubated for 72 hours, and caspase-3 gene expression was analyzed by qRT-PCR. Bars represent mean with standard error (n=2). C) Conjugate incubation did not induce interferon response. HSG cells were treated with conjugate or transfected with caspase-3 siRNA as previously described and incubated for 72 hours. qRT-PCR was used to analyze mRNA expression of interferon response genes OAS1 (white bars) and MX1 (black bars) which remained unchanged. Bars represent mean with standard error (n=3).

Figure 5.

Conjugate treatment reduced caspase-3 protein expression in HSG cells. HSG cells were treated with 8.71μM conjugate and incubated for 72 hours. Caspase-3 protein expression was analyzed by immunofluorescence (A) using rabbit anti-caspase-3 antibodies and alexa fluor 568 goat anti-rabbit IgG secondary antibodies (red). Cell nuclei were counterstained with DAPI (blue), and caspase-3 siRNA or conjugate was detected by in situ hybridization (green). Images shown at 100X magnification. (B) Quantitation of caspase-3 protein levels were measured using Image J image analysis software and normalized to untreated cells. Asterisks (*) indicate $p < 0.05$ as determined by student t test. Bars represent mean with standard error ($n = 3$ independent experiments). (C) Lysates of cells treated as described above were analyzed for caspase-3 and GAPDH protein expression by Western blot.

Figure 6.

Conjugate treatment prevented TNF- α induced apoptosis in HSG cells. HSG cells were treated with 10 μ M conjugate, 10 μ M negative control conjugate, or transfected with caspase-3 siRNA and incubated for 96 hours. The cells were then treated with TNF- α (50ng/ml) and cycloheximide (10 μ g/ml) for eight hours and stained with Annexin-V and propidium iodide. (A) Flow cytometry was used to assess early apoptotic cells (Annexin-V positive) and (B) late apoptotic cells (Annexin-V/PI positive) in treated (black bars) and untreated (white bars) cells. The percent of early and late apoptotic cells after TNF- α /cycloheximide treatment was significantly reduced in caspase-3-transfected and conjugate-treated cells compared to cells treated with negative control conjugate (asterisks p<0.01 as determined by t test). Bars represent mean with standard error (n=4 independent experiments).