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SHORT COMMUNICATION

CFTR antisense phosphorothioate oligodeoxynucleotides (S-ODNs) induce tracheo-bronchial mucin (*TBM*) mRNA expression in human airway mucosa

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Mucus hypersecretion is a critical component of cystic fibrosis (CF) pathogenesis. The effects of dysfunction of the cystic fibrosis transmembrane regulator (CFTR) on mucin expression were examined using the tracheo-bronchial mucin (*TBM*) gene as an indicator. *TBM* mRNA expression was assessed in a human bronchial epithelial cell line (HBE1) and human nasal mucosal explants *in vitro.* Antisense phosphorothioate oligodeoxynucleotides (S-ODN) to *TBM* suppressed baseline expression of *TBM* mRNA in both systems, but had no effect on glyceraldehyde phosphate dehydrogenase mRNA (*GAPDH*) expression. Sense and missense (multiple scrambled control oligonucleotides) S-ODNs had no effect. 8Br-cAMP and PGE1 significantly elevated *TBM* mRNA expression. These increases were also specifically inhibited by the antisense S-ODNs. In order to induce a CF-like state, S-ODN to *CFTR* were added to explants. Antisense *CFTR* S-ODNs were anticipated to reduce the expression of cellular CFTR protein, and the level of CFTR function. Antisense, but not sense or missense, *CFTR* S-ODN significantly increased *TBM* mRNA expression. These data suggest that mucin hypersecretion in CF may be a direct consequence of *CFTR* dysfunction; the specific mechanism through which this effect is mediated is not known.

Keywords: **antisense oligodeoxynucleotides, cystic fibrosis, epithelial cells, gene regulation, glycoprotein, immortalization, mucin, RT-PCR (reverse transcriptase-polymerase chain reaction)**

Cystic fibrosis (CF) is characterized by airway, pancreatic and gastric mucous hypersecretion with concurrent pulmonary infection by *Pseudomonas, Staphylococcus aureus* and other bacteria, and a neutrophilic inflammatory response [1]. The copious mucus is composed of sialylated and sulfated mucins exocytosed from epithelial goblet and sub-mucosal gland mucous cells (Alcian Blue staining material), sub-mucosal gland serous cell products (lysozyme, lactoferrin, sIgA and hyaluronan), plasma transudate, neutrophil elastase, DNA and other cellular debris [2,3]. Mucins have a high molecular weight polypeptide core which contains high proportions of serine and threonine that are the attachment sites for branched oligosaccharides. Current cloned human and other mucin genes include: *MUC1* (mammary gland), *MUC2* (intestinal, basic), *MUC3* (intestinal, neutral), *MUC4* (tracheo-bronchial), *MUC5*AC and MUC5B (tracheo-bronchial), *MUC6* (stomach), *MUC7* (salivary), *MUC8* (tracheo-bronchial); *RIM* (rat intestinal mucin); *Muc1* (mouse homolog of *MUC1*), *FIM* (frog integumentary mucin); *TBM* (canine tracheo-bronchial mucin); *BSM* (bovine submaxillary gland mucin); and *PSM* (porcine submaxillary mucin). *MUC4, MUC5, MUC8* and a potential human analog to *TBM* (MUC5B) have been identified in human airway mucosa [4–17]. The human -canine similarity is shown by the ability of nucleic acid probes to cross-detect, a property also exhibited by antibodies to the polypeptide. Excessive exocytosis of mucin components ("mucin hypersecretion") may be due to neutrophil elastase [18,19], altered mucin glycosylation [20,21] or intrinsic alterations of mucin gene expression induced by dysfunction of the *CFTR* [22,23].

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To assess the latter hypothesis, we examined the expression of the human analog for the canine tracheobronchial mucin gene (*TBM*) mRNA in a human bronchial epithelial cell line (HBE1) and human nasal mucosal primary explants *in vitro.* HBE1 cells are prototypic mucous cells that secrete Alcian-Blue staining material and TBM-immunoreactive material [24–28]. Antisense phosphorothioate oligodeoxynucleotides (S-ODN) are now routinely used to inhibit expression of specific genes [29–33]. S-ODNs to *TBM* were tested for their abilities to inhibit *TBM* mRNA expression in these systems. Increased production of *TBM* mRNA was demonstrated using the secretagogues 8-Br-cAMP and PGE1. Previously we have reported the transcriptional induction of the *TBM* gene by prostaglandins (PGE1, PGE2) and cAMP and identified two functional cAMP response elements (CREs) in the *TBM* promoter [34]. To generate a CF-like state, the human nasal mucosal tissue was incubated with antisense S-ODN to *CFTR* and then *TBM* mRNA expression assessed. We describe a series of S-ODNs that can inhibit *TBM* mRNA expression *in vitro,* and demonstrate that mucus secretagogues and antisense S-ODN to *CFTR* can both induce *TBM* mRNA expression. Complexes of biotin-labelled AS3 (Table 1) S-ODN and fluorescein-avidin were visualized by fluorescence microscopy in the nucleus of HBE1 cells after 24 hr incubation (data not shown) indicating successful uptake. In concentrations of up to 10 mM none of the seven S-ODNs demonstrated cellular toxicity by Trypan Blue exclusion.

Methods

Human inferior turbinate mucosa was obtained at surgery for subjects with nasal reconstruction, trauma, hypertrophy with intractible nasal obstruction, sleep apnea, and sphenopalatine procedures. Tissue was transported to the laboratory in L-15 transport medium on ice. Mucosa was dissected from the turbinate bone, and 3mm x 3mm fragments cultured in sterile 24 well polystyrene culture plates with 2 ml CMRL 1066 media in a humidified 5% CO_2 atmosphere [35].

S-ODNs, 1 mM for cells and 5 mM for tissues, were mixed with Lipofectin (Life Technologies, Inc., MD) and transfection was done according to the manufacturer's instructions. In experiments where different final concentrations of S-ODNs were used, the amount of lipofectin was adjusted proportionately. Medium was changed each day with fresh medium containing the S-ODN. The controls used were: no addition (medium control), lipofectin without any S-ODN, sense S-ODN, and scrambled (or mixed) S-ODN with the same GC content as that for antisense S-ODNs. The specific anti-sense nucleotide sequences employed are listed in Table 1.

After three days of culture, explants were removed and frozen at -70°C. Frozen explants were homogenized in 1 ml Tri-Reagent and RNA extracted as per manufacturer's instructions. Reverse transcriptase polymerase chain reaction and gel electrophoresis were carried out as follows [36]:

RNA isolated from the cells or explants (generally one microgram) was reverse transcribed into DNA by incubation with Moloney leukemia virus reverse transcriptase (150 units) in 50mM Tris buffer, pH 8.1 in the presence of 1mM dithiothreitol, 15mM NaCl, 3mM magnesium chloride, 10 units of RNAsin, 0.2 micrograms of random hexamers and 0.8mM dNTP's; incubation was performed for one hour at 37 degrees. The reaction was stopped by heating to 95 degrees for 10 minutes and the products used for PCR amplification with primers specific for either tracheal mucin or GAPDH. The primers employed were:

for TBM (sense), 5′ - GTGTTCAAGAATCCATTCTCA AGATT-3′ (;
for TBM (antisense), 5′ -CGATAAGCTTGAATATCGA ATTCC-3′;
for GAPDH (sense): 5′ -CCACCCATGGCAAATTCCA TGGCA-3′ and,
(antisense): 5′ -TCTAGACGGCAGGTCAGGTCCACC-3′.

The expected size of the PCR fragment for the mucin is 780 base pairs.

Table 1. S-ODNs used to suppress mucin transcription. Positions correspond to the sequence of the canine tracheal mucin coding region (15); +1 is the initial nucleotide at the start of transcription. Numbers in the coding region correspond to distance from the A of the first ATG.

A. Pre-mRNA (Splicing) Region	
AS1 (−22/+4)	CTCACATCCTTGTTTTAAGAACAGTT
AS2 (−1/+24)	CAGTTTGTAACCATAAACTTATTTA
B. Transcription Initiation Region	
AS3 (+43/+65)	GTGGGCAAACAAATAAAGAACCCC
AS4 (+15/+34)	AACTTATTTAAAACCCTGGGA
C. Translation Region	
AS5 (901/932)	ACCACCACTACGATGCCAACCCCAACACCCA
AS6 (3360/3390)	AACTTCAGGAACCCCCGTATCAACTAAACT
AS7 (40/60)	TTCACGGGCATGGATCTTGAG

For all experiments, values were corrected for the *GAPDH* mRNA levels present in the same sample [36]. The integrity of the RNA preparation (from HBE1 cells) was tested by gel electrophoresis. Pre-hybridization was for 1–4 h at 42°C in a solution containing 50% formamide, 5 X Denhardt's solution, 5 X SSC [7], 50 mM sodium phosphate, pH 7.0, 250 mg/ml sonicated salmon sperm DNA, 250 mg/ml yeast tRNA, 2 mg/ml poly (A), 2 mg/ml poly (C), and 0.1% SDS. Probes were either ^{32}P-labeled or biotin labeled according to the suppliers' protocol (Life Technologies, Inc., Gaithersburg, MD). The mucin probes used were: 5′-GACATTCTTGCTAATCAGTGCGGG-3′ AND 5′-TGGGGGGCACCCCCGTAGTAGGGG-3′ - these correspond to loci about 275 and 1200 bases from the initial ATG. The specific size of the mRNA for tracheobronchial mucin is difficult to determine since Northern blots are characteristically smeared with a range of 3 to 9 kb. Samples were hybridized in the same buffer without tRNA at 42°C for 18 h. After hybridization, membranes were washed twice for 15 min in 2 X SSC at room temperature, once for 30 min in 40% formamide/ 2 X SSC at 37°C, twice for 10 min in 2 X SSC/ 0.1% SDS at room temperature, and once for 10 min in 0.1 X SSC/0.1% SDS at room temperature. Finally the membranes were washed for 10 min at room temperature in 0.05 M Tris-HCl, pH 7.5 containing 0.075% (w/v) BSA. The blocking agent was Denhardt's solution containing Ficoll, polyvinyl pyrrolidone and bovine serum albumin (fraction V). Salmon sperm was added as a carrier molecule for the cRNA probe to improve hybridization. The Ca^{++}-chelating agent EGTA (0.2M) was added to minimize the destabilizing effects of Ca^{++} on the hybridization of the cRNA molecule to its target. Dextran sulfate (25%, w/v) was added to improve the contact between the cRNA molecule and the target RNA molecules. The concentration of probe was 50–150 ng/ml during the hybridization reaction. Results were analyzed by densitometry analysis or direct counting of hybridizing membranes. S-ODNs were 5′ labeled with [gamma-^{32}P] ATP by use of T4 polynucleotide kinase and further purified by dialysis (specific activity 8×10^8 dpm/ug). The transfection medium containing 1×10^6 dpm/ml ^{32}P ODN and 1 mM unlabeled S- ODN was added to the cells and stability of S-ODNs was determined according to (14). Messenger profiles were analogous to those previously reported [15].

Results and discussion

PGE1 significantly increased the ratio of *TBM /GAPDH* RT-PCR products (4.39+/− 0.78, n=3) above control levels in HBE1 cells, indicating increases in steady state *TBM* mRNA expression ($p < 0.05$, paired t-test for statistics) (Fig. 1). AS7 S-ODN to *TBM* significantly suppressed PGE1-induced *TBM* mRNA ($p < 0.00025$ vs. PGE1 treated samples). This effect was apparent with 0.1 mM AS7 (94% suppression toward baseline levels, $p < 0.05$ vs.

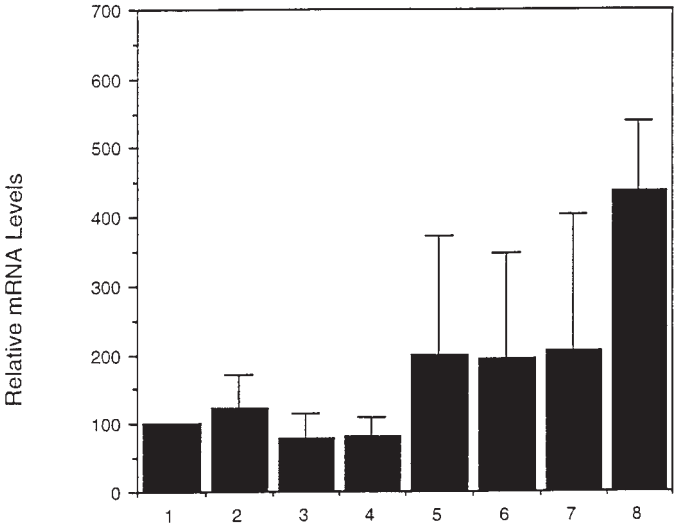

Figure 1. Suppression of PGE1 induced ***TBM*** mRNA expression by antisense ODN in HBE1 cells. Cells were treated with PGE1 and/or antisense S-ODNs (n = 3 per treatment). *TBM* and *GAPDH* expression was determined by quantitative RT PCR (7). 1, Control; 2, 1mM PGE-1 + 0.1mM AS-7; 3, 1mM PGE-1 + 1mM AS-7; 4,1mM PGE-1 + 10mM AS-7; 5, 1mM PGE-1 + 0.1mM AS-3; 6, 1mM PGE-1 + 1mM AS-3; 7, 1mM PGE-1 + 10mM AS-3; 8, 1mM PGE-1. Sequences were selected based on the nucleotides sequence of the canine gene [5].

PGE1 alone). Higher concentrations of AS7 reduced *TBM* mRNA expression to less than baseline (media treatment), but did not totally obliterate *TBM* mRNA expression. AS3 had intermediate, and highly variable effects that did not reach statistical significance. The other S-ODNs (Table 1) had no effect on *TBM* mRNA expression (data not shown). AS7 antisense (n=2) and sense S-ODNs (n=2) alone had no significant effect on *TBM/GAPDH* ratios.

In explants, 8-Br-cAMP increased the *TBM/GAPDH* ration (4.09+/− 0.58, n=10) compared to medium treatment (1.04+/− 0.12, n=17; $p < 0.000001$) (Fig. 2); there was no effect on *GAPDH* expression based on densitometry measurements. AS7 *TBM* S-ODNs reduced 8-Br-cAMP induced *TBM/GAPDH* levels (1.39+/− 0.09, n=8) to those of control explants ($p = 0.00005$ vs. 8-Br-cAMP treatment). Addition of antisense *CFTR* to 8-Br-cAMP treatment (4.20 +/− 0.64, n= 9) had no additive effect. These data indicate that 8-Br-cAMP induced *TBM* mRNA. Analogous effects were seen in explants treated with PGE-1 - increased *TBM/GAPDH* ratio (3.92 +/− 0.50, n= 10) compared to medium treatment ($p < 0.000001$) (data not shown); there was no effect on *GAPDH* expression. AS7 antisense *TBM* S-ODNs reduced PGE1-induced *TBM/GAPDH* levels (1.01 +/− 0.13, n = 6) to those of control, medium-treated explants ($p = 0.00025$ vs. PGE1 treatment).

Antisense S-ODN to *CFTR* significantly increased the *TBM/GAPDH* ratio (4.70 +/− 0.48, n = 13) compared to medium treated explants (1.19 +/− 0.13, n=13, p <

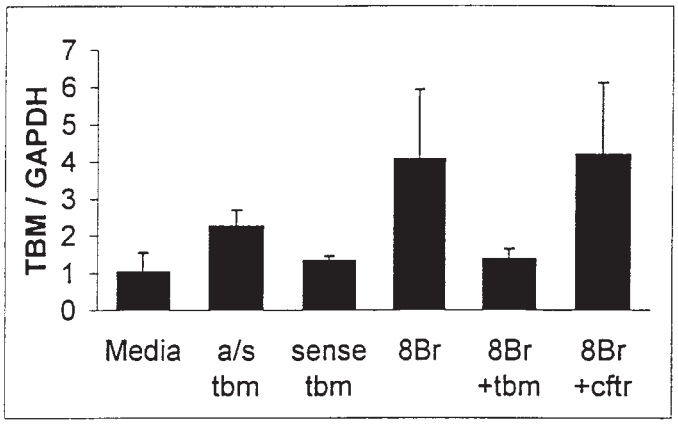

Figure 2. Effects of 1 mM 8-Br-cAMP and AS7 ***TBM*** antisense S-ODN on ***TBM/GAPDH*** ratios from human nasal mucosal explants. AS-7 antisense (a/s tbm) or sense oligodeoxynucleotide was utilized at a concentration of 1mM; 8-Br-cAMP (8 Br) was at 1mM; antisense to CFTR (cftr) was at 1mM. The + symbol indicates addition of the antisense nucleotide indicated. Incubation conditions were as described in [34].

0.000001) (Fig. 3). Lipofectin, sense and missense S-ODNs had no effect. There was no difference in the *TBM/GAPDH* ratio for RNA extracted from fresh tissue frozen immediately after surgery, and explants cultured in medium alone. Addition of antisense S-ODN to CFTR did not lead to further significant augmentation of *TBM/GAPDH* ratios. Previous studies using antisense to CFTR in kidney epithelial cells have documented the efficacy of this approach in reducing CFTR gene expression. In addition, a reduction of transepithelial fluid secretion was shown in conditions where CFTR was inhibited [37]. The data indicate that antisense *CFTR* induced *TBM* mucin mRNA expression in human nasal explants *in vitro*. This suggests that reductions in *CFTR* levels or activity may directly lead to mucus hypersecretion through an effect on expression of the mucin gene. The concurrent effect on the physical properties of the secreted mucus may also be related to a possible diminution in associated fluid secretion.

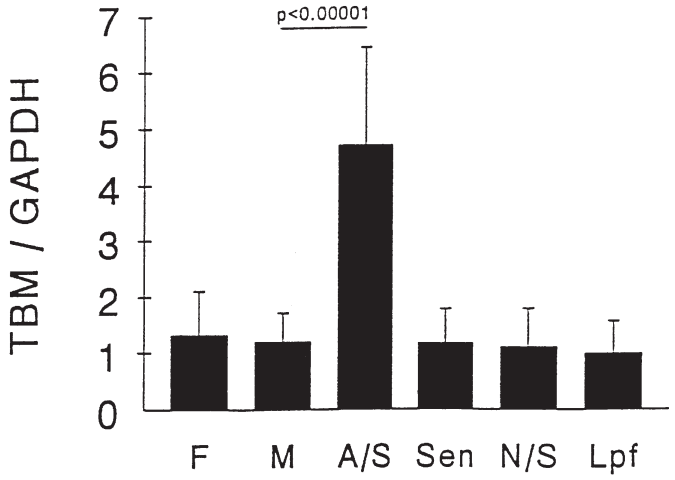

Figure 3. Effect of ***CFTR*** antisense S-ODN on ***TBM/GAPDH*** ratios from human nasal mucosal explants. Antisense to *CFTR* (A/S) significantly increased *TBM* expression. *TBM/GAPDH* ratios were not significantly different between freshly frozen (F), medium (M), sense S-ODN (Sen), nonsense S-ODN (N/S), and lipofectin (Lpf) treated explant.

The cAMP system is of particular importance in the *TBM* system since the *TBM* promoter has been shown to contain two cAMP response elements (CREs) [34]. It is not yet documented if the effects seen in these studies on treatment with antisense to CFTR are mediated by cAMP although it is known that PK-A mediated phosphorylation is one means of CFTR regulation.

These data demonstrate that antisense S-ODNs to CFTR significantly increase *TBM/GAPDH* ratios suggesting that CFTR dysfunction may directly lead to mucin hypersecretion. This effect would be independent of bacterial infection or neutrophilic inflammation and indicates that induction of mucin expression and secretion of increased amounts of mucoglycoconjugates into fetal and neonatal CF airways could offer novel ecological niches for colonization by opportunistic organisms. Prior work by other investigators has shown effects on mucin secretion in cystic fibrosis for salivary and pancreatic cells as well [38,39]. Modulation of gene expression *via* nucleic acid sequence-specific intervention represents a new paradigm for drug discovery and development. Antisense S-ODN can successfully interfere with viral replication, oncogene expression, and other processes. Cellular uptake of ODNs, either as such or as conjugates, and selective inhibition of gene expression are well established. AS7 antisense to *TBM* blocked the increase in *TBM* mRNA after PGE1 or 8-Br-cAMP treatment, suggesting that AS7 or analogous antisense molecules may offer a more direct approach for the treatment of mucin hypersecretion in cystic fibrosis or chronic asthmatic disease.

Liposomes (Lipofectin) provided effective delivery of S-ODNs into cells in culture, and into blocks of mucosal tissue *in vitro* and have the advantages of being non-toxic, non-immunogenic and, unlike retroviral vectors, do not require dividing cells. Our data indicate that antisense S-ODNs to *CFTR* induce *TBM* mRNA in human mucous cells, and that AS7 antisense to *TBM* delivered in liposomes specifically reduces expression of this mucin.

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