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Dexamethasone modulation of *MUC5AC* and *MUC2* gene expression in a generalized model of middle ear inflammation

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Abstract

Objective—To examine the effect of dexamethasone on basal and pro-inflammatory cytokines induced gel forming mucin expression in human middle ear epithelium culture (HMEEC-1).

Methods—HMEEC-1 was exposed to pro-inflammatory cytokines, TNF- α and IL-1 β to identify optimal mucin induction. The HMEEC-1 was incubated with dexamethasone in the steady state and in the presence of pro-inflammatory cytokine stimulation. Expression of *MUC2* and *MUC5AC* was determined by quantitative PCR.

Results—Pro-inflammatory cytokines, TNF- α and IL-1 β , induced *MUC2* and *MUC5AC* expression in HMEEC-1. Dexamethasone reduced steady state mRNA level of *MUC5AC* in a time ($p < 0.05$) and dose dependent manner ($p < 0.0001$). *MUC2* was effectively suppressed at all time-points tested ($p < 0.05$). Temporal difference between dexamethasone suppression of *MUC2* and *MUC5AC* was demonstrated. Dexamethasone inhibits the pro-inflammatory cytokine induced expression of both *MUC2* and *MUC5AC*.

Conclusion—This work provides a conclusive picture of the ability of using glucocorticoids to down-regulate mucin gene expression in human middle ear epithelium using a generalizable model of inflammation which is applicable to multiple potential causes of middle ear epithelium mucosal hypertrophy. This data adds to the promising potential of future interventions for patients with chronic otitis media.

Keywords

mucin; otitis media; cytokines; hearing loss

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INTRODUCTION

Otitis media is a prevalent condition causing frequent pediatric office visits and antibiotic prescriptions. It afflicts most children and, as such, leads to extremely high expenditures of health care dollars globally.¹⁻⁴ In countries with limited health care access, OM is the leading cause of preventable conductive hearing loss and is responsible for mortality due to otitis associated complications⁵. In these areas in which populations are challenged with treatment of OM there exists an increased incidence of chronic ear conditions which lead to permanent hearing loss and important lost opportunities for employment or economic advancement.⁶⁻⁷ Hearing loss associated with otitis media with effusion is also a difficulty in developed countries with developmental delays and behavioral changes associated with this hearing loss.⁸⁻⁹ The substantial annual expenditure on OM treatments and its associated complications represents an important health care burden.¹⁰ There is a critical need to understand OM pathogenesis and develop novel interventions aimed at reducing its health and socioeconomic burden.

Increased mucin secretion from the middle ear epithelium (MEE) is an important physiological event in OM. The production and secretion of mucins under normal physiological conditions provides the primary mucosal defense against pathogen incursion in OM. Dysregulation of mucus, or hypersecretion, is stimulated by both microbial and host-derived factors and contributes significantly to OM pathogenesis. When there exists mucin hypersecretion the excessive production of mucin overloads mechanisms of the host mucociliary clearance resulting in accumulation of viscous fluid in the ME space and subsequent conductive hearing loss. There exists a need for data which examines the regulation of middle ear mucins in an attempt to appropriately design interventions to limit overproduction in cases in which this leads to pathophysiology.

Secretory gel forming mucin (GFM) genes are of particular interest in middle ear epithelial pathology. These are the major components contributing to the viscoelastic property of mucous secretion, the characteristic that can compromise the normal mucociliary clearance and epithelial defense mechanisms. The GFMs identified in human middle ear includes *MUC2*, *MUC5AC*, *MUC5B* and *MUC19*.¹¹ *MUC2* and *MUC5AC* are produced by goblet and basal cells of surface epithelium, while *MUC5B* and *MUC19* are glandular mucins detected mainly in submucosal glands. Studies have demonstrated each of these mucins to be upregulated by proinflammatory cytokines and major pathogenic middle ear bacteria.¹²⁻¹⁴ Among these, *MUC2*, *MUC5AC* and *MUC5B* upregulation were demonstrated in middle ear mucosa of children with OM suggesting their critical roles in OM pathogenesis.¹⁵⁻¹⁷

This current investigation provides a novel data set examining mucin regulation with dexamethasone in human middle ear epithelium. The study design was specifically chosen given the ready availability of this medication already having Food and Drug Association (FDA) approval for use in the middle ear. In particular, recent advances in the production of sustained release preparations of otic steroid medications make steroids a particularly interesting area of study in for patients with difficulties from chronic OM.

MATERIALS AND METHODS

Cell culture

A culture model of human middle ear epithelium (HMEEC-1, initial generous gift by Dr. David J Lim) was an immortalized cell line whose primary characterization regarding transformation and growth properties have been published¹⁸. The culture was maintained in a humidified atmosphere at 37°C with 5% CO₂, in full growth media containing a 1:1 ratio mixture of Dulbecco's Modified Eagle Medium (Life Technologies) and Bronchial Epithelial Cell Basal Medium (Lonza), 10% Fetal Bovine Serum (ATCC), 1% Antibiotic-Antimycotic (containing penicillin, streptomycin and Fungizone by Life Technologies), and supplemented with one bag of BEGM Singlequots (Lonza) for every 500ml portion of the media.

Pro-inflammatory cytokine-induced mucin expression

The culture maintained in full growth media was serum starved overnight and subsequently exposed to 200ng/ml TNF- α (R&D Systems) or 100ng/ml IL-1 β (R&D Systems) for 1, 2, 4, and 8 hours. The optimal time point provided the highest response of mucin gene was tested with culture exposed to various concentrations of the cytokines in basal media containing supplements. Cell lysate was harvested at the end of exposure time.

Effect of dexamethasone on mucin expression

HMEEC-1 in full growth media generally produced mucin at about the level of detection by qPCR. The examination of steroid inhibiting effect will result in greater threshold cycles than the qPCR detection limit (>35 cycles) hence the cells were grown in the presence of 1 μ M retinoic acid (RA, Sigma) for 5 days prior to treatment to enhance the overall level of transcriptional mucin gene expression. To determine a steroid impact on mucin expression, hydrocortisone (as part of BEGM Singlequots supplement) was removed from the culture media 48 hours prior to treatment with dexamethasone to ensure there were no confounding impacts from the culture media. To examine the effect of dexamethasone on the steady state level of *MUC5AC*, the culture was serum starved overnight and subsequently treated with 10 μ M dexamethasone for 2, 4, 8, 16, and 24 hours in basal media (containing supplements and 1 μ M RA) for a time course exposure. For dose dependent experiment, the culture was treated with dexamethasone at 0.00001, 0.001, 0.1, and 10 μ M final concentrations in the basal media. To examine the effect of dexamethasone on pro-inflammatory cytokines induced mucin expression, the optimal condition for each mucin gene response to the specific cytokine was tested with culture in the presence of 10 μ M dexamethasone. Nontreated control cells for both time and dose dependent experiments were handled in the same manner as treated cells. Cell lysate was harvested at the end of exposure time.

Quantitative PCR

Total RNA from experimental cultures was prepared after the indicated treatments using TriZol reagent. Complementary DNA was synthesized using the SuperScript III First-Strand Synthesis System (Life Technologies). Each cDNA reaction prepared for pro-inflammatory cytokines induced mucin experiments required 2 μ g starting total RNA to detect each

specific mucin. For the study of dexamethasone effect on mucin expression, a 0.4 µg of starting total RNA was sufficient for cDNA preparation. Quantitative PCR (qPCR) was carried out on a ViiA7 by Life Technologies using their commercially available TaqMan primer-probes for *MUC2* [part number: Hs00159374_m1], *MUC5AC* [part number: Hs00873651_mH] and *HPRT1* [part number: Hs99999909_m1]. Samples were analyzed in triplicates and a no template control was included for each gene. The expression levels of each of the targeted mucin genes detected by qPCR were normalized to the mRNA level of the house keeping gene *HPRT1*. The relative fold change of mucin gene was calculated using 2^{-Ct} methodology¹⁹.

Statistics

All experiments were performed in triplicate. The results were presented as mean ± standard error of means. Data of the time course studies were analyzed by student's t-test. Differences in mucin gene expression to multiple concentrations of various substances were determined by one-way ANOVA followed by Tukey's Post-hoc. All statistical analyses were performed using Prism software version 5.01 (GraphPad Software, Inc).

RESULTS

Pro-inflammatory cytokines induce gel forming mucin gene expression in HMEEC-1

HMEEC-1 exposed to TNF-α displayed a demonstrable increase in *MUC2* expression at 4 and 8 hour time points ($p = 0.005$ and 0.011 , respectively). The culture exposed to TNF-α at various concentrations at the optimal time point of 4 hours demonstrated a significant increase of *MUC2* ($p < 0.0001$) with greater than 3-fold increase in transcript expression compared with control at 200ng/ml of TNF-α (Fig. 1A–B). Higher levels of induction was observed in the level of *MUC5AC* where significant response was observed as early as 2 hours ($p = 0.049$) and continued at 4 and 8 hours with $p = 0.003$ and 0.014 , respectively (Fig. 1C). With the 4 hour time point as a constant variable, HMEEC-1 exposed to TNF-α displayed a significant increase in *MUC5AC* expression ($p = 0.008$). The optimal concentration of 50mg/ml TNF-α increased *MUC5AC* transcript just under 4 times that of the control level samples (Fig. 1D). The response of each GFM to various concentrations of TNF-α was not linear and it failed to increase mucin expression at the higher dosages.

Similar up-regulation for the two gel-forming mucin genes was demonstrated in the HMEEC-1 exposed to IL-1β. Utilizing the time as a dependent variable, the 2 hour exposure time point was significantly different than other time points ($p=0.007$) in stimulating *MUC2* transcript (Fig. 2A). When the culture was exposed to various concentration of IL-1β for 2 hours, the significant increase of *MUC2* level was observed at all concentrations (Fig. 2B, $p=0.0004$). The level of induction was not linear that it plateaued at the higher dosage of IL-1β. *MUC5AC* response was at 2–4 folds greater than the control at 2, 4 and 8 hours with p -value of 0.036, 0.0003, and 0.016, respectively. Non-linear increase of *MUC5AC* was observed when varied IL-1β dosage. The exposure to 50ng/ml IL-1β demonstrated a 3-fold increase of *MUC5AC* expression compared to control ($p=0.019$). The 50ng/ml of IL-1β was the optimal concentration provided the significant increase of both mucin transcripts.

Dexamethasone reduces steady state level of gel forming mucin genes

The effect of dexamethasone on steady state level of GFM was demonstrated in Fig. 3. Dexamethasone significantly inhibited *MUC5AC* transcript in HMEEC-1 in a time-dependent manner. A greater than 50% reduction of *MUC5AC* expression was observed at every time point tested ($p < 0.05$). At 4 hours of exposure the effective DEX concentrations ranged from 0.001 to 10 μ M. *MUC2* was effectively suppressed at all time-points tested, $p < 0.05$ (Fig. 3C). There exist the temporal difference in DEX suppression of basal *MUC2* and *MUC5AC*. The basal levels of *MUC5AC* were reduced by greater than 50% in the first 2 hours whereas *MUC2* transcripts were reduced by half after 16 hours of exposure.

Dexamethasone inhibits pro-inflammatory cytokine induced mucin expression

To validate the effect of dexamethasone on pro-inflammatory cytokine induced mucin expression, it is important to measure impact at the greatest level of mucin production to maximally be able to determine the impact of the dexamethasone intervention. Therefore, HMEEC-1 was exposed to each specific cytokine (TNF- α and IL-1 β) in the presence of dexamethasone at its optimal conditions. Dexamethasone effectively reversed the TNF- α induced *MUC2* and *MUC5AC* expression to basal level (Fig. 4A). Similar significant inhibition effect was obtained with IL-1 β induced mucin expression for both *MUC2* and *MUC5AC* (Fig. 4B).

DISCUSSION

The mucosal epithelium in the middle ear is similar that found in other areas of the human respiratory tract. Common to all anatomic areas where this respiratory epithelium is found is a need to act as a protective barrier to underlying tissues from damage and injury induced by bacterial and viral pathogens. In addition, other causes of middle ear epithelial injury and inflammation have been studied including environmental agents, such as tobacco smoke or internal agents such as pepsin.^{20–22} When confronted with potential injury from such agents this respiratory epithelium undergoes predictable responses regardless of the potential offending agent. This response includes mucosal hypertrophy, goblet cell proliferation and an increase in the production of gel-forming mucins.^{23–25} Our previous investigations, as well as others^{12–13,26–27}, have demonstrated that a generalizable model for inflammation in the middle ear is a model that examines the up-stream inflammatory signaling cascade which leads to production of increased gel-forming mucins. This cascade includes the inflammatory cytokines TNF- α and IL-1 β . Glucocorticoid ability to limit *Muc2* transcripts has been demonstrated in a murine xenograft model of human pseudomyxoma peritonei.²⁸ Others have demonstrated a repression of *MUC5AC* induction by bacteria or chemokines in other respiratory epithelial cell lines with the use of glucocorticoids.^{29–31} This investigation is the first to examine the impact of a glucocorticoid on the production of both gel-forming mucins, *MUC5AC* and *MUC2*, in a human middle ear model and to demonstrate its impact on basal levels of mucin transcripts in a human middle ear model.

The results of this investigation add to the growing literature that glucocorticoids are promising agents for the regulation of mucin production by middle ear mucosa. Importantly, this current data set is also not limited to a specific agent which may underlie MEE mucosal

inflammatory events. Otitis media is known to be a multifactorial process with many infections having multiple bacteria present or mixed bacterial and viral etiologies.^{32–34} Other investigations have also linked other agents such as gastroesophageal reflux²² and tobacco smoke²⁰ to predisposition to OM through inflammatory-mediated events. A number of studies, including those from our laboratory, have examined the signaling pathways of inflammation in OM.^{13,26–27,35–37} Not surprisingly, given the ubiquitous presence of TNF- α and IL-1 β in upstream cascades of mammalian inflammatory pathways, these inflammatory cytokines have been demonstrated to be critical in the signaling pathways of inflammatory events leading to mucin gene up-regulation and mucin production in human middle ear epithelium. Therefore, we examined these two particular inflammatory cytokines as an upstream generalizable model of middle ear inflammation. Using a generalizable model of inflammation creates an ability to surmise that inhibiting the inflammatory process upstream, as we have done in these experiments, will provide information regarding the modulation of mucin production from a large variety of insults which may contribute to mucin hyper-secretion and ultimately hearing loss or other complications in OM.

The effect of TNF- α and IL-1 β on *MUC5AC* expression was not linear for it failed to increase the expression at higher dosage. For these types of *in vitro* experiments it is not uncommon to have some lack of linear relationship to dosage particularly at higher levels.³⁸ The lack of linear response may be due to the cytokine incapacity to access a saturable receptor-mediated transport process and/or response of the target epithelial cells. The bell shaped response curve may be associated with a signal transduction mechanism in which the cells become resistant to the downstream receptor-mediated stimulation. There also exist negative feedback mechanisms in these signaling pathways that may result in nonlinear response.

The treatment times for TNF- α and IL-1 β were chosen related to maximum expression of the mucin genes being examined in relationship to the inflammatory cytokines. From an *in vivo* perspective, it is important to acknowledge that the inflammatory pathways related to any potential middle ear infectious process will be heterogeneous and be dependent upon both host and pathogen specific conditions. Therefore, any specific treatment in the middle ear with an agent such as dexamethasone will likely require an ability to pharmacologically interact with the middle ear mucosa over a period of time; something which could be achieved with sustained release agents.

As we have discussed previously¹¹, it is also important to examine the impact of inflammatory responses on a variety of gel-forming or secretory mucins which include both *MUC5AC* and *MUC2*. In experiments conducted in our laboratory and elsewhere, each of the gel-forming mucins have been demonstrated to have up-regulation in the presence of generalized inflammatory agents TNF- α and IL-1 β as well as in more specific inflammatory experiments using specific pathogens.^{14,39} The precise function and role of these gel-forming mucins in response to various stimuli has yet to be elucidated. However, this will be an important area of investigation in the future. What is clear from this current investigation is that in our generalizable model of HMEEC-1 inflammation the gel-forming mucins of *MUC5AC* and *MUC2* have a differential response to inflammation and regulation by dexamethasone. However, the inflammatory events that lead to increased production of these

gel-forming mucins is reversed by the glucocorticoid dexamethasone in a dose and time-dependent fashion.

In addition, we examined the ability of dexamethasone to reduce the baseline production of the GFMs. In these experiments basal levels of both *MUC2* and *MUC5AC* were reduced by greater than 50%. The temporal difference between dexamethasone suppression of either mucin genes was demonstrated as well. This is an important consideration as basal levels of production of mucins are important in the overall protective mechanisms of respiratory epithelium. As such, any attempt to regulate MEE mucin production must recognize these positive functions of mucins and will need to consider what might be the optimal level of down-regulation of production.

In patients with chronic otitis media, our laboratory has demonstrated significant up-regulation of mucin gene from middle ear biopsies taken at the time of tympanostomy tube insertion.^{15–16} These patients often have middle ear effusions and hearing loss as their predisposing pathology necessitating surgery. Our laboratory has also demonstrated, in clinical trials, that these patients with chronic otitis media with middle ear effusion have an increased risk of complications following their tympanostomy tube insertion if they are not treated post-operatively with middle ear drops containing a steroid and antibiotic.⁴⁰ These complications can be significant as they can lead to plugging of the tympanostomy tube or persistent drainage from the tympanostomy tube. As such, it is common practice to use these otic drops following surgery in children with middle ear effusions. It has been postulated that the glucocorticoid in these otic drops is important. Particularly, the addition of 0.1% dexamethasone to 0.3% ciprofloxacin has been shown to increase treatment success rate and effectiveness in eradication of pathogens than formulations containing antibiotics alone.^{41–42}

Recent advances in sustained released otic preparations hold promise to simplify the beneficial impact of glucocorticoid preparations. These sustained release compounds provide a potential for use intraoperatively, and thereby limit the need for repeated dosing by parents. Importantly, the rate of release of these preparations can be modified to create a specified dosing regimen to modulate middle ear inflammation in a predictable fashion. Perhaps most importantly, these preparations may provide a potential, in the future, for delivery to reverse the detrimental impact of mucosal inflammation and hypertrophy and its associated mucin hypersecretion without the need for surgical placement of tympanostomy tubes.^{43–45} The potential to achieve this is currently being investigated given the difficulty of delivery through an intact tympanic membrane. In patients with chronic otitis media and non-intact tympanic membranes there exists a more ready avenue for this type of therapeutic intervention. With this possibility of modulating the middle ear inflammatory responses, understanding precise dose and MEE responses to intervention and temporal changes will be critical. The data set presented here gives an early indication of some of the parameters which warrant consideration as these experiments are designed and move forward.

In conclusion, the experiments presented in this manuscript provide a conclusive picture of the ability of using glucocorticoids to down-regulate mucin gene expression and production in human middle ear epithelium using a generalizable model of inflammation which is

applicable to multiple potential causes of MEE mucosal hypertrophy. This data adds to the promising potential of future interventions for patients with chronic otitis media.

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Figure 1. TNF- α induce gel forming mucin gene expression in HMEEC-1

Human middle ear epithelium culture was exposed to 200ng/ml TNF- α at various time points. Both *MUC2* (A) and *MUC5AC* (C) were up-regulated. The response to various dosage of TNF- α was conducted at the optimal exposure of 4 hours for both *MUC2* (B) and *MUC5AC* (D). Bar represent mean $\pm$ SEM. *: $p < 0.05$ vs control.

Figure 2. IL-1 β induce gel forming mucin gene expression in HMEEC-1

Human middle ear epithelium culture was exposed to 100ng/ml IL-1 β at various time points. The response to various dosage of IL-1 β was conducted at the optimal exposure of 2 hours for *MUC2* (B) and 4 hours for *MUC5AC* (D). Bar represent mean $\pm$ SEM. *: $p < 0.05$ vs control.

Figure 3. Effect of dexamethasone on the basal expression of gel forming mucin genes
Gel forming mucin transcripts were assessed in HMEEC-1 culture exposed to 10uM dexamethasone (DEX) at various time points. Dexamethasone reduced the basal expression of *MUC5AC* transcripts (A). At 4 hour exposure, DEX effectively reduced *MUC5AC* at concentration range from 0.00001 to 10 μM (B). The temporal difference between DEX suppression of basal *MUC2* and *MUC5AC* was demonstrated (C). Bar represent mean ± SEM. *: $p < 0.05$ vs control.

Figure 4. Effect of dexamethasone on cytokine induced gel forming mucins in HMEEC-1
 A). Relative expression change of *MUC2* and *MUC5AC* transcripts in TNF- α induced HMEEC-1 was assessed at 4 hour exposure. B). *MUC2* and *MUC5AC* transcripts were assessed in IL-1 β induced condition at 2 hour and 4 hours, respectively. The addition of DEX lowered the induced mucin to basal level. Bar represent mean $\pm$ SEM. *: $p < 0.05$ vs control. #: $p < 0.05$ vs TNF- α . &: $p < 0.05$ vs IL-1 β . TNF- α : 200ng/ml Tumor necrosis factor-alpha. IL-1 β : 100ng/ml Interleukin-1 beta. DEX: 10uM dexamethasone.