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Research Paper

Primary human sinonasal epithelial cell culture model for topical drug delivery in patients with chronic rhinosinusitis with nasal polyposis

Benjamin S. Bleier[a], Ryan M. Mulligan[b] and Rodney J. Schlosser[b]

[a]Department of Otology and Laryngology, Massachusetts Eye and Ear Infirmary, Harvard Medical School, Boston, MA and [b]Department of Otolaryngology-Head and Neck Surgery, Medical University of South Carolina, Charleston, SC, USA

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Correspondence
Benjamin S. Bleier, Clinical Instructor, Massachusetts Eye and Ear Infirmary, Harvard Medical School, 243 Charles St, Boston, MA 02114, USA.
E-mail: bleierb@gmail.com

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Abstract

Objectives The primary human sinonasal epithelial cell culture (HSNEC) allows for in-vitro modelling of mucosal responses to topical therapy. Cultures grown from healthy donors may underestimate changes in individuals with chronic sinonasal disease thereby yielding inaccurate results with respect to this large patient population. The purpose of this study was to analyse HSNECs derived from patients with chronic rhinosinusitis with nasal polyposis (CRSwNP) to determine whether expected disease dependent variables salient to topical drug delivery persist in culture.

Methods Cultures were grown from patients with CRSwNP. Ciliary beat frequency (CBF) (basal and stimulated), permeability (*trans* and paracellular), inflammatory response, and glucocorticoid dose response were measured and compared with healthy controls.

Key findings Methylcholine stimulated CBF was greater in CRSwNP versus controls (ΔCBF_{60min} 7.25 ± 1.02 vs 0.89 ± 1.04 Hz, respectively). Paracellular permeability was greater in CRSwNP versus controls (basolateral $dextran_{120min}$ 18.97 ± 3.90 vs 11.31 ± 4.35 μg/ml, respectively). Lipopolysaccharide (0.1 mg/ml) stimulated interleukin-6 (IL-6) and IL-8 secretion was increased in CRSwNP versus controls (IL-6 Δbaseline 1738.72 ± 654.82 vs 1461.61 ± 533.51%, respectively; IL-8 Δbaseline 137.11 ± 0.83 vs 111.27 ± 0.67%, respectively). CRSwNP cultures were more sensitive than controls to dexamethasone (1 μg/ml) dependent IL-6 and IL-8 suppression.

Conclusions HSNECs derived from patients with CRSwNP retained their primary phenotype with respect to ciliary function, epithelial permeability, irritant induced inflammatory cytokine secretion, and glucocorticoid dose response.

Introduction

Topical nasal drug delivery represents a rapidly expanding field with great potential in the management of sinonasal disease. These treatments offer the ability to deliver high concentrations of therapeutic agent directly to the affected area with minimal systemic exposure. Advances in the understanding of disease dependent factors governing therapeutic efficacy including mucosal residence time, epithelial permeability, and dose response have catalysed investigations into novel delivery strategies.[1] While these approaches hold great promise, they require an experimental platform capable of modelling the complex local interactions between the pharmaceutical agent and the primary respiratory pathology. The use of phenotype specific primary human sinonasal epithelial cell cultures (HSNEC) represents one such platform that may be capable of replicating the multivariate mucosal response to novel topical therapies. The goal of this study was to characterize the disease specific physiological behaviour of HSNECs derived from patients with chronic rhinosinusitis with nasal polyposis (CRSwNP) and their response to known topical stimuli.

Upon reaching its site of action, topical therapies have a limited temporal window in which to exert their effect. The principle determinant of this therapeutic contact time is the rate of mucociliary clearance which in turn, is governed by

ciliary beat frequency (CBF).[1,2] CBF may be dynamically regulated in response to a variety of mechanical, hormonal, and neural stimuli.[3,4] Studies in mucosal explants have suggested also that both basal and stimulated CBF are altered in diseased mucosa and may contribute to the more rapid clearance of topical agents in chronic rhinosinusitis.[5] The impact of both mechanical and chemical stimulation on CBF may therefore depend on the underlying disease state and thus the ability to measure these effects *in vitro* would be valuable.

Epithelial drug absorption represents another important factor influencing topical therapeutic efficacy. Permeability is regulated by transcellular and paracellular barriers, which selectively permit the polar transit of a variety of molecules based on multiple factors including size and charge. Modification of epithelial permeability has been the focus of many studies in an effort to enhance both local and systemic drug absorption. Furthermore, previous studies have demonstrated that components of paracellular junctional proteins including desmosomal proteins DSG2 and DSG3 may be reduced in diseased mucosa.[4] These disease dependent alterations in intercellular junctional proteins may significantly impact drug absorption and are poorly represented in current in-vitro models.

These experimental platforms must also be capable of modelling the active epithelial role in the regulation of inflammatory and immunological response to multiple topical stimuli.[5–7] In-vivo mucosal secretion of Th1 and Th2 polarizing cytokines has been demonstrated in response to a variety of infectious and allergic insults, respectively.[8,9] While it has been well established that cytokine secretion is determined by the underlying disease state, those studies have suggested that their sensitivity to glucocorticoid mediated suppression may be disease dependent.[10] As a result, any technique designed to model these effects must be capable of differentiating not only the level and pattern of cytokine secretion across disease phenotype but the differential response to anti-inflammatory therapeutic agents as well.

Epithelial derived cell cultures represent a technique which offer the potential to analyse irritant and drug specific mucosal effects in an in-vitro setting, thereby providing a screening tool for novel topical therapy.[11] Cultures that are derived directly from normal human nasal mucosa tend to display a greater retention of their primary phenotype relative to those grown from immortalized cell lines. These primary epithelial cultures are therefore capable of providing more precise predictions of intranasal drug behaviour. Despite this, HSNECs derived from healthy mucosa may still fail to accurately model the effects of sinonasal disease on topical drug efficacy with respect to ciliary function, permeability, and dose response. The goal of this study was to therefore characterize a HSNEC model derived from patients with CRSwNP in an effort to more accurately make predictions regarding the effects of topical stimuli in pathologic mucosa.

Materials and Methods

Mucosal biopsy procurement

Procurement of the sinus mucosal biopsies utilized to generate primary nasal epithelial cell cultures was approved by the Medical University of South Carolina Institutional Review Board. Control subjects were defined as patients free of rhinosinusitis and undergoing surgery for either cerebrospinal fluid leak repair or tumour removal. Established consensus diagnostic criteria were used to define patients as having CRSwNP.[12] Exclusion criteria included the following: use of oral steroids or immunotherapy within the preceding four weeks, aspirin sensitivity (ASA triad), ciliary dysfunction, autoimmune disease, cystic fibrosis or any known immunodeficiency. All tissue from both groups was derived from Schneiderian mucosa within the middle meatus.

Primary nasal epithelial cell culture

Tissue explants were washed and digested in Pronase for 90 min at 37°C. Cell suspensions were separated from particulate matter by centrifugation and resuspended in basal epithelial growth medium (BEGM) (Lonza, Basel, Switzerland). Cells were plated for 2 h on standard tissue culture plates to remove contaminating fibroblasts. Cells were then expanded for three to five days on collagen-coated 75 cm^2 dishes (Corning Life Sciences, Corning, NY, USA). Once confluent, the HSNECs were trypsinized and re-seeded evenly on human collagen type IV-coated tissue 6-well culture plates (Corning Life Sciences). Submerged cultures were grown to 80% confluence on 6-well plates in BEGM and starved of hydrocortisone for 24 h before lipopolysaccharide (LPS) or cigarette smoke extract (CSE) exposure. Air–liquid interface cultures were grown on 6-well Transwell permeable supports (Corning Life Sciences) in Dulbecco's modified Eagle media (DMEM) (Invitrogen, Carlsbad, CA, USA) until fully confluent and differentiated with evidence of ciliation and active beating.

Cigarette smoke extract preparation

CSE was made by bubbling smoke from four 3R4F cigarettes (University of Kentucky, Lexington, KY, USA) through 50 ml DMEM using a Shapiro cigarette smoke machine (Washington University, St Louis, MO, USA). The solution was filtered through a 0.22-μm filter (Corning Life Sciences) and used immediately. CSE was diluted with fresh DMEM to create a 5% solution.

Ciliary beat frequency determination

Ciliary response to exogenous stimuli was measured on air–liquid interface cultures using a VWR Vista Vision (West Chester, PA, USA) microscope at a magnification of

160x. Images were captured using a high-speed monochromatic digital video camera (Basler AG, Ahrensburg, Germany) and analysed using the Sisson-Ammons Video Analysis (SAVA) system version 2.1.[13] Five regions of interest were selected within each culture dish to ensure that CBF was recorded from the same area throughout the experiment. The reported frequencies represent the arithmetic means of these values. Baseline CBF was determined before treatment. Following stimulation, recordings were made at time zero and at subsequent 15-min intervals for a total of 60 min. Mechanical stimulation consisted of a shear force resulting from the addition of DMEM. Chemical stimulation consisted of the addition of DMEM containing 10^{-6} mol/l acetyl-β-methylcholine chloride, a β-methyl ester of acetylcholine and known dose-dependent stimulant of CBF.[14]

Measurement of epithelial permeability

Transcellular permeability was determined in air–liquid interface cultures using dexamethasone (Sigma), a known transcellular marker.

Paracellular permeability was similarly determined using fluoresecein isothiocyanate (FITC)-labelled 40 kDa dextran (Invitrogen). Before exposure, cultures were tested for confluence and barrier maturity by measuring the transepithelial electrical resistance (TEER) using a Millicell ERS-2 Volt-Ohm meter (Millipore, Billerica, MA, USA). Only cultures with a TEER between 400–500 Ω/cm^2 were considered confluent and utilized.[15] There were no significant differences between cultures with respect to the time required to achieve confluency. Both of the transport markers were dissolved in phosphate buffered saline (PBS, pH 7.4) and added to the apical surface of the air–liquid interface in 1-ml samples. Degree of transport was determined by the time-dependent accumulation of marker in 2 ml PBS in the basolateral chamber at 30-min intervals for 120 min. Dexamethasone concentration was tested by ELISA (Neogen, Lansing, MI, USA). FITC-labelled dextran concentration was determined based on fluorescence using a FLUOstar Optima microplate fluorometer (BMG Labtech, Cary, NC, USA).

The impact of exogenous irritant exposure on epithelial barrier function was determined by measuring TEER and paracellular permeability using the described methods following a 15-min apical exposure to 5% CSE. Subsequent to this exposure the CSE solution was aspirated and replaced with fresh media for the remainder of the experiment.

Determination of irritant induced cytokine secretion

Secretion of irritant induced pro-inflammatory cytokines was determined in submerged HSNECs grown to 80% confluence in BEGM. Cultures were starved of hydrocortisone for 24 h before irritant exposure. The irritant consisted of a 24-h exposure to 1 ml LPS samples derived from *Pseudomonas aeruginosa* and diluted in DMEM (0.01 or 0.1 mg/ml; Sigma-Aldrich, St Louis, MO, USA). The concentration ranges were derived from dose responses reported previously.[16] Following irritant exposure, the supernatant in each well was collected and centrifuged at 10^4 rev/min for 10 min to remove any cellular or particulate debris. Determination of interleukin-6 (IL-6) and IL-8 concentrations was performed by ELISA (BD Biosciences, San Diego, CA, USA).

Determination of glucocorticoid dose response

The phenotype specific reduction of inflammatory cytokine secretion was determined in control and CRSwNP cultures exposed to 0.1 mg/ml LPS. During their 24-h exposure, cultures were co-incubated with dexamethasone diluted in media (0.001 or 1 mg/ml). Determination of concentrations of IL-6 and IL-8 were performed as described above.

Statistical analysis

All statistical analyses were performed using Stata version 6.0 (College Station, TX, USA, 1999). Ciliary beat frequency, permeability, and cytokine levels were compared using a Kruskal–Wallis non-parametric analysis of variance with post-hoc multiple comparison testing using the Bonferroni procedure. The difference in conditions between test and control was considered to be statistically significant when $P < 0.05$.

Results

Ciliary response to mechanical and chemical stimuli

No significant difference was found between the baseline CBF in control and CRSwNP derived tissue (7.34 $\pm$ 0.63 vs 5.31 $\pm$ 0.19 Hz, respectively). Following application of acetyl-β-methylcholine (10^{-6} mol/l), both groups demonstrated a significant upregulation in CBF across all time points measured relative to the mechanical stimulus resulting from the media application alone (Figure 1a and b). The change from baseline CBF following exposure to acetyl-β-methylcholine was significantly greater in cultures derived from CRSwNP than control across all time points (Figure 1c).

Phenotype specific transcellular and paracellular permeability

No significant difference in apparent permeability (P_{app}) or basolateral accumulation was found between control and CRSwNP groups at any time point with respect to the transcellular transit marker dexamethasone (1 mg/ml)

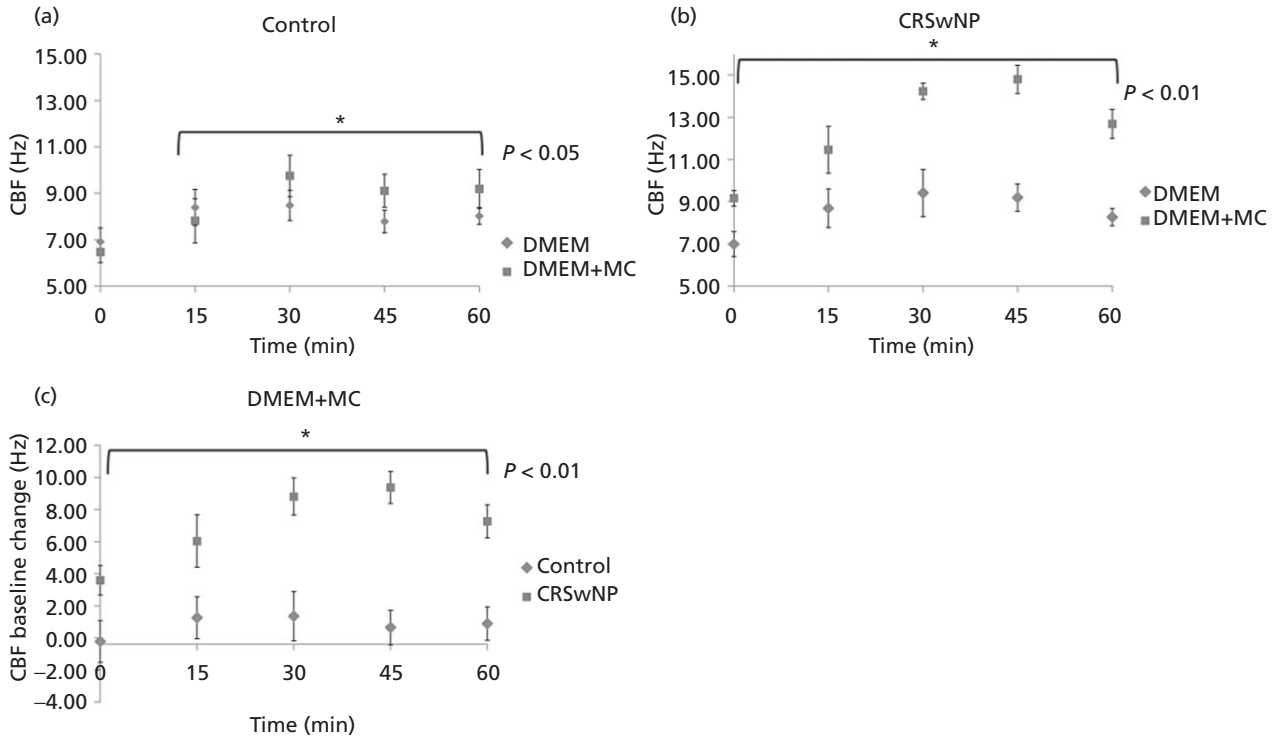

Figure 1 Ciliary beat frequency in air–liquid interface culture derived from control mucosa over time. (a) Following exposure to acetyl-β-methylcholine (MC; 10^{-6} mol/l) in Dulbecco's' modified Eagle medium (DMEM), ciliary beat frequency (CBF; Hz) was significantly upregulated relative to cultures exposed to DMEM alone ($n = 10$). (b) Following exposure to acetyl-β-methylcholine (10^{-6} mol/l) in DMEM, CBF was significantly upregulated relative to cultures exposed to DMEM alone ($n = 10$). (c) Following exposure to acetyl-β-methylcholine (10^{-6} mol/l) in DMEM, the increase from baseline CBF was significantly greater in CRSwNP than control mucosa at all time points ($n = 10$).

(Figure 2a). With respect to the paracellular transit marker FITC-labelled 40 kDa dextran (1 mg/ml), baseline permeability was significantly greater in CRSwNP than control groups beyond 60 min of apical exposure (basolateral concentration at 120 min: 18.97 ± 3.90 vs 11.31 +/− 4.35 μg/ml, $P < 0.05$; respectively) (Figure 2b).

Impact of irritant exposure on paracellular permeability and TEER

In addition to the presence of the CRSwNP phenotype, exposure of the culture to 5% CSE further increased paracellular permeability to FITC-labelled 40 kDa dextran. While the basolateral concentration in the 5% CSE group demonstrated an increasing trend relative to control at all time points, the difference was statistically significant only at 60 min post-exposure (Figure 2c). This increase in permeability corresponded with a relative decrement in TEER in the 5% CSE vs control groups, which was observed at all time points and was significant at 90 min post-exposure (166.7 ± 19.59 vs 176.5 ± 22.9 Ω/cm^2, $P < 0.05$, respectively) (Figure 2d).

Dose-dependent lipopolysaccharide-induced inflammatory cytokine secretion

A dose-dependent secretion of both IL-6 and IL-8 was observed in the control and CRSwNP groups following exposure to escalating doses of LPS. With respect to IL-6, the percent increase in baseline expression was significantly greater in CRSwNP than control in response to 0.1 mg/ml LPS (1738.72 ± 654.82 vs 1416.61 ± 533.51%, $P < 0.05$, respectively) (Figure 3a). With respect to IL-8, the percent increase in baseline expression was significantly greater in CRSwNP than control in response to both 0.01 and 0.1 mg/ml LPS (Figure 3b).

Dose response to glucocorticoid exposure

Both control and CRSwNP cultures demonstrated a trend towards a dose-dependent suppression of LPS (0.1 mg/ml)-induced IL-6 and IL-8 cytokine secretion following exposure to escalating doses of dexamethasone. Significantly greater reduction was seen in both IL-6 and IL-8 secretion following treatment with 1 mg/ml dexamethasone compared with 0.001 mg/ml (Figure 4a and b). Cultures derived from

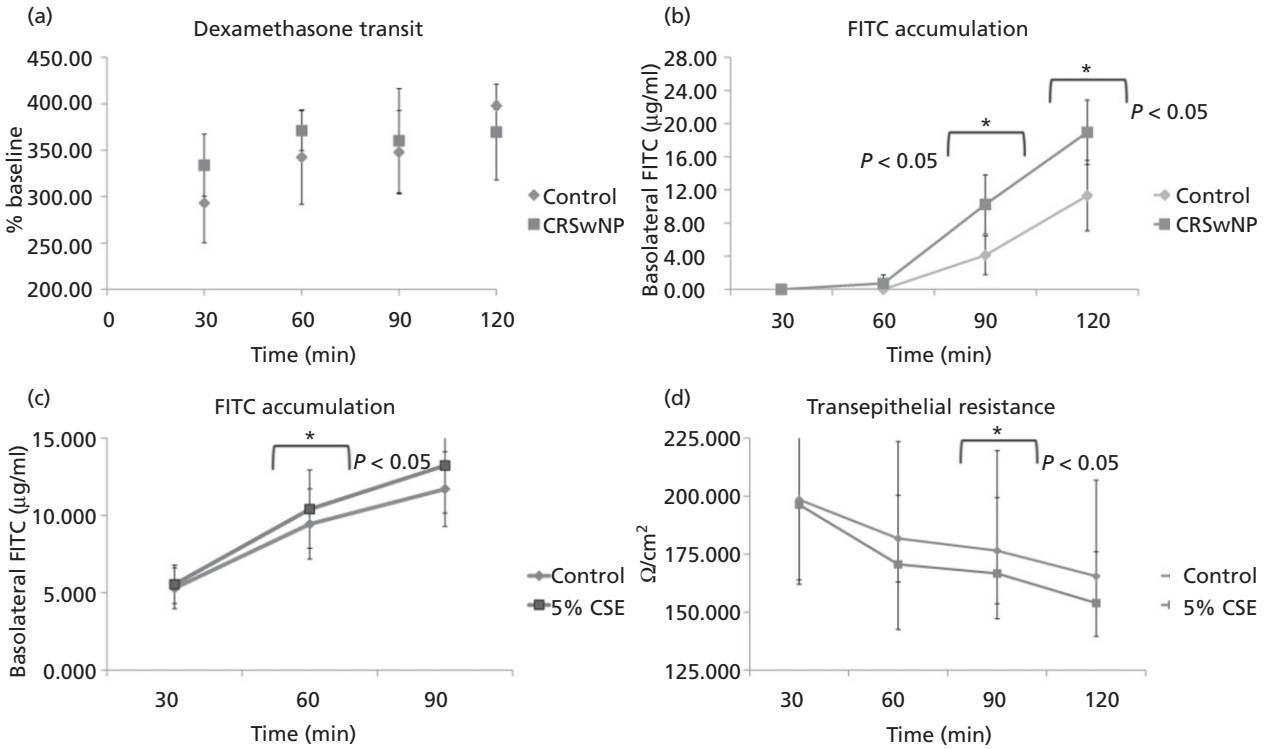

Figure 2 Phenotype specific transcellular and paracellular permeability in cultures derived from control and chronic rhinosinusitis with nasal polyposis mucosa. (a) Basolateral accumulation of the transcellular marker dexamethasone in air–liquid interface (ALI) cultures derived from both control and CRSwNP (chronic rhinosinusitis with nasal polyposis) mucosa following apical exposure to a 1 mg/ml dexamethasone solution. No difference between groups was seen in the percent increase from baseline basolateral concentration ($n = 4$). (b) Basolateral accumulation of the paracellular marker FITC-labelled 40 kDa dextran in ALI cultures derived from both control and CRSwNP mucosa following apical exposure to a 1 mg/ml FITC-labelled 40 kDa dextran solution. Basolateral concentration was significantly greater in CRSwNP than control mucosa at 90 and 120 min post-exposure ($n = 4$). (c) Basolateral accumulation of the paracellular marker FITC-labelled 40 kDa dextran in ALI cultures derived from CRSwNP mucosa following a 15-min apical exposure to phosphate buffered saline (PBS; control) or 5% cigarette smoke extract (CSE) in PBS. Basolateral concentration was significantly greater in the 5%CSE group at 60 min post-exposure than control ($n = 4$). (d) Transepithelial electrical resistance (TEER) in ALI cultures derived from CRSwNP mucosa following a 15-min apical exposure to PBS (control) or 5%CSE in PBS. TEER was significantly reduced in the 5%CSE group at 90 min post-exposure than control ($n = 4$).

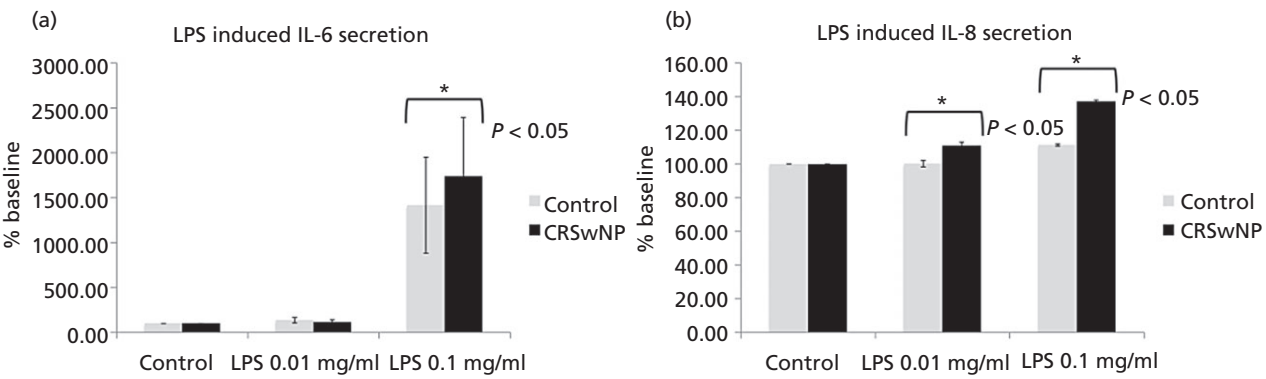

Figure 3 Dose response in interleukin-6 and interleukin-8 secretion in cultures derived from control and chronic rhinosinusitis with nasal polyposis mucosa following exposure to escalating doses of lipopolysaccharide. (a) At the highest dose of lipopolysaccharide (LPS), CRSwNP (chronic rhinosinusitis with nasal polyposis) cultures demonstrated a significantly greater percent increase in baseline interleukin-6 (IL-6) secretion relative to control cultures ($n = 6$). (b) At both doses of LPS, CRSwNP cultures demonstrated a significantly greater percent increase in baseline IL-8 secretion relative to control cultures ($n = 6$).

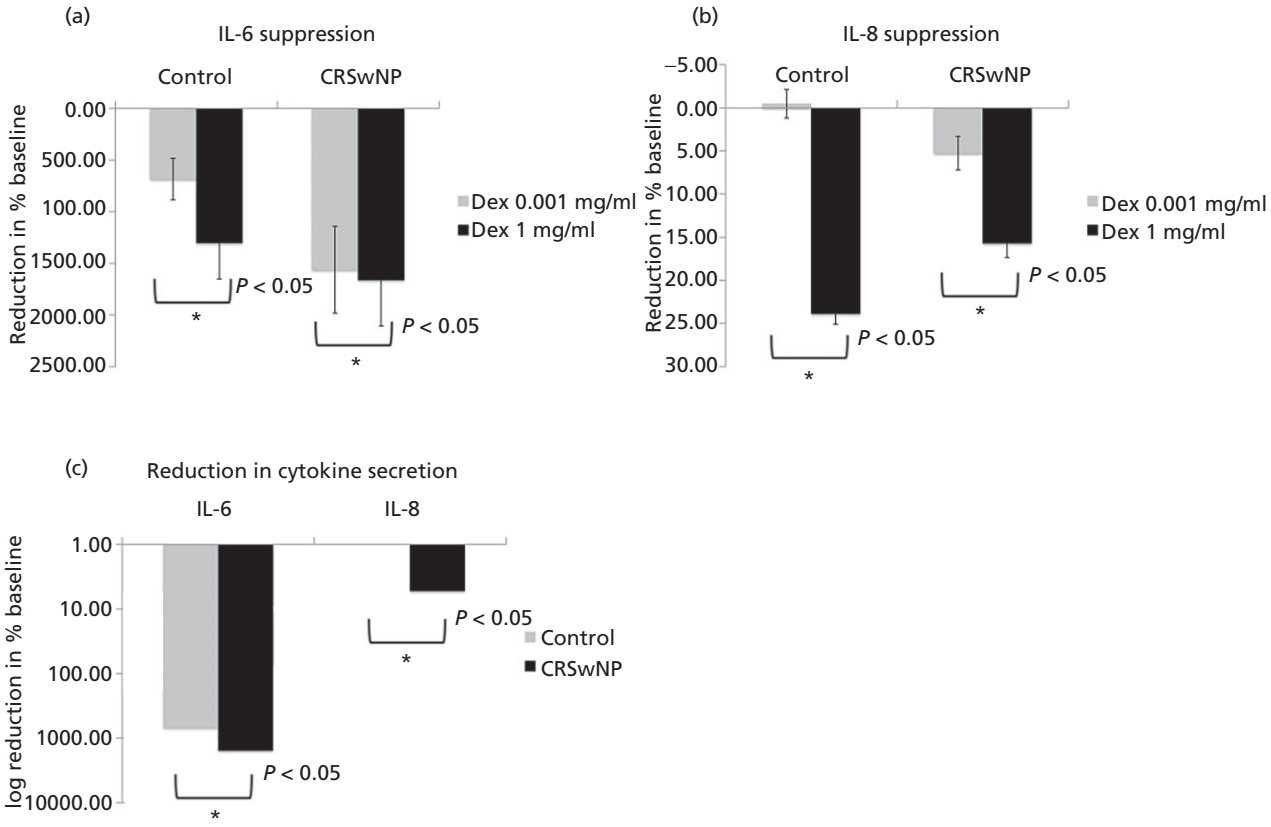

Figure 4 Dose-dependent lipopolysaccharide-induced inflammatory cytokine section in control and chronic rhinosinusitis with nasal polyposis mucosa. Reduction in percent baseline interleukin-6 (IL-6) and IL-8 secretion demonstrated a dose response trend when treated with escalating doses of dexamethasone (Dex) in lipopolysaccharide (LPS; 0.1 mg/ml)-exposed cultures derived from both control and CRSwNP (chronic rhinosinusitis with nasal polyposis) mucosa. (a) In both groups, reduction in IL-6 in response to 1 mg/ml dexamethasone was significantly greater than in response to 0.001 mg/ml ($n = 6$). (b) In both groups, reduction in IL-8 in response to 1 mg/ml dexamethasone was significantly greater than in response to 0.001 mg/m ($n = 6$). (c) The percent reduction in both IL-6 and IL-8 secretion in LPS (0.1 mg/ml)-exposed cultures in response to 0.001 mg/ml dexamethasone was significantly greater in the CRSwNP group than the control group ($n = 6$).

patients with CRSwNP demonstrated a significantly greater percent reduction in both IL-6 and IL-8 expression relative to control patients following exposure to 0.001 mg/ml dexamethasone (Figure 4c).

Discussion

The HSNEC model represents a promising approach for in-vitro epithelial modelling in which mucosa is harvested directly from an individual patient and induced to form confluent monolayers in culture. HSNECs are a valuable research tool as they tend to display greater primary phenotypic retention than immortalized cell lines. In spite of this, disease specific mucosal attributes such as CBF, phospholipid secretion, xenobiotic metabolism, extracellular matrix interaction, and expression of inflammatory cytokines may not be accurately represented by cultures derived from healthy mucosa.[17] The purpose of this study was to critically analyse primary cultures derived from pathologic mucosa to determine whether known disease dependent variables salient to drug delivery persist in culture.

The first variable examined was basal and stimulated ciliary beat frequency. Previous clinical studies had demonstrated that basal CBF was diminished in patients with chronic sinus disease.[2] Our in-vitro data was consistent with these findings and demonstrated a trend towards reduced baseline CBF in the CRSwNP cultures. Both cultures demonstrated a preserved capacity for cholinergic-dependent stimulation also found in previous explant studies. The fact that CRSwNP cultures exhibited a significantly greater upregulation lends support to the theory suggested by Chen *et al*.[3] that these cells develop a differential capacity to accelerate CBF to compensate for the obstructive nature of the polyps.

The ability to accurately measure epithelial permeability to both ionic and macromolecular transit represents a particular strength of the air–liquid interface platform. Previous studies have confirmed that these cultures develop mature paracellular junctions providing the opportunity to measure the impact of local agents on barrier function.[11] The lack of difference in transcellular dexamethasone transit between control and CRSwNP cultures would be expected given the lack of evidence of any cell membrane ultrastructural differences between the two groups. In contrast, our finding of significantly increased FITC-labelled dextran transit in CRSwNP cultures relative to control suggested that the reduced intercellular junctional proteins noted in polyp biopsy specimens may have persisted in culture.[4] The further decrement in barrier function following cigarette exposure was also consistent with previous studies and may provide evidence for the preservation of phosphorylation-dependent paracellular transit pathways within these monolayers.[18]

The retention of disease specific phenotype was supported by our irritant-induced cytokine assays. Confluent air–liquid interface cultures are known to have blunted inflammatory responses with respect to cytokine secretion and thus submerged cultures were used in these studies. IL-6 and IL-8 represent components of independent inflammatory pathways that are known to be both overexpressed in CRSwNP and secreted by epithelial cells.[19] Our findings were consistent with this and correlated with previous mucosal biopsy studies, suggesting that disease mediated inflammatory responses to environmental irritants remain intact.[9,10] Additionally, these pathways were found to be subject to dose-dependent glucocorticoid-mediated suppression, which was consistent with clinical findings.

Conclusions

Topical drug delivery offers multiple benefits in the treatment of sinonasal disease. Previous studies suggested that the underlying disease phenotype significantly influenced many of the physiologic factors impacting the efficacy of these therapies. The development of an experimental platform capable of reproducing these variables has the potential to greatly enhance our understanding of the local impact of both respiratory irritants and topical therapeutic agents on the pathophysiology of sinonasal disease. Our findings suggested that HSNECs derived from patients with CRSwNP retained their disease specific primary phenotype with respect to ciliary function, epithelial permeability, irritant-induced inflammatory cytokine secretion, and glucocorticoid dose response. Future studies with other primary inflammatory phenotypes are required to further elucidate this model.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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