Knockdown of interleukin-8 in airway epithelial cells

Abstract

Introduction: Cystic fibrosis (CF) is a neutrophil-dominated lung disease. The neutrophil chemokine interleukin-8 (IL-8) is present at higher than normal levels in the lungs of individuals with CF and is a key factor responsible for neutrophil infiltration into the lungs. Lipopolysaccharide (LPS) expressed by Pseudomonas aeruginosa in the CF lung can stimulate IL-8 expression by airway epithelial cells. Inhibiting the expression of IL-8 using small-interfering RNA (siRNA) represents a potential gene therapy approach for CF. Here, the efficacy of an IL-8-directed siRNA at inhibiting IL-8 gene and protein expression is tested in basal and LPS-stimulated human bronchial epithelial cells.

Methods: 16HBE14O- cells were transfected with either scrambled, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or IL-8-specific siRNAs. RNA was then isolated and used in quantitative real-time PCR to measure GAPDH, IL-8 or beta-actin (housekeeping) gene expression. Lastly, IL-8 protein production was quantified in cell supernatants by IL-8 ELISA.

Results: Transfection of 16HBE14O- cells with 30nM IL-8 siRNA for 48 hours led to an 84% knockdown in IL-8 mRNA (p<0.05). Treatment with LPS (10µg/ml, 6h) increased IL-8 gene expression 1.5-fold (p<0.05) and IL-8 siRNA caused a significant knockdown of IL-8 protein in LPS-treated cells (p<0.05). Furthermore, basal IL-8 protein production was decreased by IL-8 siRNA. LPS treatment increased IL-8 protein expression in 16HBE14O- cells. IL-8 siRNA failed to decrease this effect.

Conclusions: The data show that IL-8 siRNA can inhibit basal and LPS-induced IL-8 gene expression in 16HBE14O- cells. Further investigation is required to optimise the conditions for inhibition of IL-8 protein production, and to study its potential role in CF therapy.
Studies have since proven that A1AT replacement therapy (augmentation therapy) causes the inhibition of proteases and improves bactericidal processes in CF patients.\(^4\)

Naturally occurring anti-proteases and NE inhibitors, such as secretory leucoprotease inhibitor (SLPI), Elafin, monocyte neutrophil elastase inhibitor (MNEI) and eglin-c, also represent potential therapies that may suppress IL-8 expression in CF lungs.\(^5\) However, the effect of inhibiting the expression of IL-8 in human bronchial epithelial cells using small-interfering RNA (siRNA) remains unknown. siRNA can reduce gene expression through RNA interference (RNAi), an enzyme-mediated process that involves RNA-protein interactions characterised by four major steps: assembly of siRNA with RNA-induced silencing complex; activation of the complex; target recognition; and, target cleavage.\(^7\) The manipulation of IL-8 expression using siRNA could represent a new gene therapy approach for CF.

The aims of this project are: (1) to grow and transfect 16HBE14O\(^{-}\) cells with siRNA lengths designed to knockdown IL-8 gene expression; (2) to quantify the IL-8 expression; and, (3) to compare the extent of IL-8 knockdown in normal and LPS-stimulated bronchial epithelial cells. The effect of siRNA on IL-8 expression will be tested in time-course, dose response and combination experiments.

**Methods**

**Cell culture**

**Cell line**

16HBE14O\(^{-}\) cells were maintained at 37°C in a 5% CO\(_2\) incubator and supplemented with MEM-GlutaMax (Gibco, 1x) mixed with 10% foetal calf serum (FCS) and 1% penicillin-streptomycin (P/S).

**Splitting HBE cells**

Cells were grown in a 25cm\(^2\) Corning flask pre-coated with 3mL fibronectin until 50-80% confluent. They were split using Dulbecco’s phosphate buffered saline (DBPS, Gibco, 1x) and polyvinylpyrrolidone-EGTA-trypsin (PET) solution. The PET solution contained Hepes buffered saline solution (HBSS), 10% polyvinylpyrrolidone solution (PVP), 0.2% EGTA in HBSS and 0.25% trypsin in 0.02% EDTA. Cells were counted using Glastic (KOVA) haemocytometers and placed onto a cell culture cluster plate (Costar) at a concentration of 1.5 x 10\(^5\) cells/well.

**Cell treatment**

**siRNA transfection**

Each well containing a 16HBE14O\(^{-}\) cells was treated with 250µl of MEM-GlutaMax +FCS +P/S and 50µl of siRNA transfection reagents. The siRNA reagents consisted of Opti-MEM (Gibco) mixed in solution with Ribozinc (Novagen) and the siRNA of interest at a concentration of 30nm/well. Either silencer negative control siRNA, silencer GAPDH siRNA or silencer IL-8 select validated siRNA (Ambion) was used. Each siRNA transfection was carried out in triplicate. Once transfected, the cells were incubated for 24-48 hours in an incubator at 37°C in 5% CO\(_2\). In some experiments, supernatants were removed and stored at -4°C after incubation was complete.
LPS treatment

Cells were further stimulated with LPS from *Pseudomonas aeruginosa* (Sigma-Aldrich). Cells were seeded into 18 wells of a cell cluster plate (Costar) and transfected with the siRNA of interest. Cells were incubated at 37°C in 5% CO₂ for 24 hours, following which supernatants were removed and stored at -20°C. Of the 18 wells, nine were supplemented with 1mL 1% FCS-MEM-GlutaMax. The other nine were treated with 1mL LPS (10µg/mL) in solution with 1% FCS-MEM-GlutaMax. The cells were incubated for six hours at 37°C in 5% CO₂. After incubation, supernatants were removed and stored at -20°C.

RNA isolation

RNA was isolated from the 16HBE14O- cells using 500µl/well TRI reagent (Sigma-Aldrich) as per the manufacturer’s protocol. This was followed by phase separation using chloroform (100µl/sample, Sigma-Aldrich), RNA isolation using isopropanol (250µl/sample, Sigma), washing of the RNA pellet in 75% ethanol and dissolution in 0.1% diethyl pyrocarbonate (Sigma-Aldrich) H₂O as per the manufacturer’s protocol.

cDNA synthesis and qRT-PCR

RNA quantification was carried out using a Nanodrop and equal quantities of RNA were reverse transcribed into cDNA using the Quantitect Reverse Transcription kit (Qiagen). The resulting cDNA was used for quantitative real-time (qRT) PCR.

A 1x qRT-PCR mastermix was made up to 20µl and added to each well of the 96-well PCR plate. The 1x mix consisted of 2µl template cDNA, 10µl of SyBr Green (Roche), 6µl of PCR H₂O (Roche) and 1µl each of a forward and reverse primer (10pmol/µl β-actin, GAPDH or IL-8; Roche). Amplification was performed using the LightCycler 480 PCR system (Roche) at an annealing temperature of 56°C for 45 cycles. Quantitative expression of the target gene was determined using the 2(-ΔΔCt) method.8,9 Statistical analysis was conducted using two different algorithms – one generating a single value from triplicate data and the other comparing individual values to a single standard to generate triplicate data.

Enzyme-linked immunosorbent assay (ELISA)

The supernatants acquired from the cell culture plates after siRNA transfection/LPS treatment were measured for the quantity of IL-8 protein (pg/mL) produced using the Sandwich ELISA protocol.10 Spectrophotometry was used to measure IL-8 at an absorbance of 405nm at 0.1 seconds.
Statistical analysis

Assays were performed in triplicate and the data was analysed by Prism 4.0 using student t-test.

Results

The objective of these experiments was to observe a knockdown of IL-8 gene expression in 16HBE14O cells using siRNA. The quantitative gene expression of IL-8 and the production of IL-8 protein were measured by RT-PCR and ELISA, respectively. The effects of siRNA on IL-8 gene and protein expression were investigated in both basal and LPS-stimulated 16HBE14O cells.

Knockdown of IL-8 in basal HBE cells

Duplicate experiments were carried out to measure the knockdown of basal IL-8 expression in 16HBE14O cells that were transfected in triplicate with a negative control scrambled β-actin siRNA (SCR), GAPDH siRNA or IL-8 siRNA. Figure 2a shows a significant 80% knockdown in IL-8 (0.20) with β-actin as the control (p<0.05). Because of the similarity in expression of the β-actin (1.00) and GAPDH (1.06) reference genes (Figure 2a), GAPDH was subsequently chosen as the control to compare the relative knockdown of IL-8 (Figure 2b). Figure 2b demonstrates a 50% IL-8 siRNA-mediated knockdown in IL-8 (p<0.05). Furthermore, the optimal cell density of 16HBE14O cells was found to be 1.5x10⁵ cells/well (data not shown); this cell density was used in further experiments.

IL-8 protein production in basal HBE cells

After the 16HBE14O cells were transfected with siRNA and incubated for 48 hours, the supernatants were isolated and IL-8 protein production was assessed using ELISA (Figure 3). IL-8 protein was increased (824pg/mL (95% CI 725-923)) in the supernatants of 16HBE14O cells transfected with IL-8 siRNA compared to cells transfected with SCR siRNA (618pg/mL (95% CI 544-690)), but not to those transfected with GAPDH siRNA (698pg/mL (95% CI 614-782)).

Knockdown of IL-8 in HBE cells treated with LPS

Further experiments were carried out to compare the knockdown of IL-8 in untreated 16HBE14O and LPS-treated 16HBE14O cells (Figure 4). An 84% knockdown in IL-8 in basal 16HBE14O cells was documented (p<0.05), as seen previously. No statistically significant knockdown in IL-8 was witnessed in 16HBE14O cells treated with LPS (p=1.0).

A duplicate experiment was carried out (Figure 5) comparing the effect of IL-8 siRNA against the GAPDH control siRNA on knockdown...
of IL-8 gene expression. IL-8 siRNA did not significantly knock down basal IL-8 expression (30% reduction; *p*=1.0). There was a significant increase in the expression of IL-8 in LPS-treated cells (1.55) as compared to basal cells (0.90) transfected with GAPDH siRNA (*p*=0.05). There was also a significant knockdown of IL-8 in LPS-treated cells (0.4) (*p*=0.05).

**IL-8 protein production in LPS-treated HBE cells**

The supernatants acquired from both basal and LPS-treated 16HBE14O- cells at 24 hours post siRNA transfection. Supernatants were acquired from 16HBE14O- cells 30 hours post siRNA transfection. Amount of IL-8 was measured using ELISA at an absorbance of 405nm for 0.1 seconds (*p*=0.14).

Furthermore, a statistically significant knockdown in IL-8 gene expression was seen in LPS-induced HBE cells transfected with IL-8 siRNA (*p*<0.05) (**Figure 5**). These positive results reiterate the efficacy of siRNA, and put it forth for gene therapy for CF patients in the future.

The results of IL-8 inhibition are consistent with findings demonstrated in a study conducted by Tsivkovskii et al. in which the effect of a quinolone on IL-8 expression was investigated. The effect of levofloxacin on pro-inflammatory cytokine secretion by human airway epithelial cells *in vitro* was examined, and results demonstrated a dose-dependent reduction in IL-8 in both tumour necrosis factor α (TNFα) and LPS-induced human airway epithelial cells. This suggests that levofloxacin could also be of therapeutic benefit in CF. Further studies demonstrated that IL-8 suppression can be successfully accomplished by several techniques. An extensive knockdown of IL-8 gene expression from 1.55 (95% CI 1.3-1.8) to 0.19 (95% CI 0.19-0.20) (*p*<0.05) was seen in LPS-induced cells (**Figure 5**). The magnitude of knockdown in LPS-induced cells exceeded that witnessed in basal cells; this result was unexpected, as LPS has been documented to stimulate the production of IL-8. It must be noted that these results were not reproduced. Further experiments should be carried out under the same conditions to attain a truer representative of the IL-8 knockdown value in LPS-induced HBE cells.

The amount of basal IL-8 protein production detected using ELISA was significantly lower in LPS-induced HBE cells transfected with GAPDH siRNA (*p*<0.05) (**Figure 6a**). The supernatants acquired from 16HBE14O- cells 24 hours post siRNA transfection. Supernatants were acquired from both basal and LPS-treated 16HBE14O- cells at 30 hours post siRNA transfection. Amount of IL-8 protein was measured using ELISA at an absorbance of 405nm for 0.1 seconds (without LPS, *p*=0.5; with LPS, *p*=0.9).

**Figure 6a:** IL-8 protein production in supernatants of basal 16HBE14O- cells at 24 hours post siRNA transfection. Supernatants were acquired from 16HBE14O- cells 24 hours post siRNA transfection. Amount of IL-8 was measured using ELISA at an absorbance of 405nm for 0.1 seconds (*p*=0.14).

**Figure 6b:** IL-8 protein production in supernatants of basal 16HBE14O- cells and treated 16HBE14O- cells at 30 hours post siRNA transfection. Supernatants were acquired from both basal and LPS-treated 16HBE14O- cells 30 hours post siRNA transfection. Amount of IL-8 protein was measured using ELISA at an absorbance of 405nm for 0.1 seconds (without LPS, *p*=0.5; with LPS, *p*=0.9).

Discussion

The inflammatory chemokine IL-8 is a key factor responsible for the infiltration of neutrophils in the CF lung. In this project, the efficacy of siRNA in knocking down basal and LPS-induced IL-8 gene and protein expression in human bronchial epithelial cells was explored. A knockdown in basal IL-8 gene expression between 50% and 84% was seen in basal HBE cells transfected with IL-8 siRNA (**Figures 2a, 2b** and **4a**). These results were reproducible and statistically significant (*p*<0.05). This indicates that IL-8 siRNA is very effective in inhibiting IL-8 gene expression in normal human bronchial epithelial cells.
The change in IL-8 (ΔIL-8)

![Graph showing the change in IL-8](image)

FIGURE 6c: The change in IL-8 (ΔIL-8) protein production between supernatants of basal 16HBE14O- cells and treated 16HBE14O- cells. The change in IL-8 protein production between basal 16HBE14O- cells and LPS-treated 16HBE14O- cells (as measured in Figure 3b) was calculated (p=0.4).

in technique; although the sandwich ELISA is quite sensitive, it involves indirect antibody detection and there is a risk of cross-reactivity resulting in a non-specific signal. As such, it is unclear whether the IL-8 siRNA has a significant effect on basal or LPS-induced IL-8 protein production.

IL-8 protein expression patterns detected in the study differ from previous findings. Von Scheele et al. tested the efficacy of budesonide in inhibiting IL-8 in bronchial epithelial cells treated with organic dust, TNF or LPS using ELISA. Results showed that budesonide suppressed the secretion of IL-8 in cells treated with all three stimuli.

Conclusions and future recommendations

A successful knockdown in basal and LPS-induced IL-8 gene expression was seen in HBE cells. The effect of IL-8 siRNA on basal and LPS-induced IL-8 protein production remains unclear and should be further investigated using varying doses of siRNA and longer transfection times. Future work should aim to explore the efficacy of IL-8 siRNA in different types of cells and growth environments. The next steps could be investigating the knockdown of IL-8 using siRNA in an air-liquid interface culture or the use of animal models. Furthermore, the effect of siRNA in CF airway epithelial cells and human monocytes should be explored to gauge the suitability of siRNA as a therapeutic modality in the CF patient.

Acknowledgements

I would like to thank the Health Research Board for funding this project. I would also like to thank Irene Oglesby, Tomás Carroll, Catherine Coughlan and Isabel Vega-Carrascal for their support, encouragement and invaluable teaching. Finally, I wish to thank Dr Catherine Greene for giving me the opportunity to work on this project and for her extensive guidance throughout.

References