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IL-8 Regulates Mucin Gene Expression at the Posttranscriptional Level in Lung Epithelial Cells1,2

Maria V. Bautista,† Yajun Chen,* Vessela S. Ivanova,* Michael K. Rahimi,* Alan M. Watson,* and Mary C. Rose4,*†‡§

Airway inflammation and mucus hypersecretion/overproduction/obstruction are pathophysiological characteristics of cystic fibrosis, asthma, and chronic obstructive pulmonary disease. Up-regulation of airway mucin genes by inflammatory/immune response mediators is one of the major contributors to mucin overproduction. IL-8, a potent proinflammatory mediator and neutrophil chemoattractant, is present at high levels in the airway secretions of such patients. In this study, the effects of IL-8 on expression of two major airway mucin genes, MUC5AC and MUC5B, were evaluated. IL-8 increased the mRNA abundance of both mucin genes in two human respiratory tract-derived cell lines (A549 and NCI-H292) in a time- and concentration-dependent manner. IL-8 also increased MUC5AC and MUC5B mRNA levels in primary normal differentiated human bronchial epithelial cells, with a high concentration of IL-8 required to increase MUC5B mRNA levels. IL-8 did not transcriptionally up-regulate MUC5AC gene expression, but rather increased the stability of the MUC5AC transcript, suggesting regulation at the posttranscriptional level. In addition, IL-8 altered the levels of RNA-binding proteins to specific domains in the 3′-untranslated region of the MUC5AC transcript. Taken together, these data indicate that the IL-8-induced binding of RNA-binding proteins to the 3′-untranslated region of MUC5AC is a potential mechanism for regulating MUC5AC gene expression at the posttranscriptional level, thus suggesting a new role whereby IL-8 sustains mucin gene expression in inflamed airways. The Journal of Immunology, 2009, 183: 2159–2166.

Mucin glycoproteins (mucins), the major macromolecular components of the mucosal layer that covers and protects the respiratory tract against pathogens and environmental toxins, are now recognized as part of the innate immune response system (1–3). Mucins are overproduced and hypersecreted in pulmonary diseases such as asthma, cystic fibrosis (CF), and chronic obstructive pulmonary disease (COPD)/bronchiolitis, where airway obstruction by mucins/mucus is a major contributor to morbidity and mortality (4–9). Mucins are large, highly O-glycosylated macromolecules with a protein backbone encoded by a specific MUC gene and characterized by tandem repeats. Eighteen human MUC genes have now been identified and at least 12 are expressed at the mRNA level in the respiratory tract (reviewed in Ref. 10). MUC5AC and MUC5B mucins are the predominant secretory mucin gene products expressed in human airway secretions in healthy individuals, and their levels are increased in patients with lung diseases (11).

Mucin gene expression is regulated by inflammatory/immune response mediators and byproducts. Various mediators regulate specific mucin genes in vitro at the transcriptional (10, 12) and/or posttranscriptional level (reviewed in Ref. 10). The MUC5AC gene is transcriptionally up-regulated by several inflammatory byproducts or mediators, including LPS (13), IL-9 (14), neutrophil elastase (NE) (15), TNF-α, and IL-1β (16). The MUC5AC gene is also regulated at the posttranscriptional level by TNF-α (17) and NE (18), which mediators increase the stability of the MUC5AC transcript. Less information is known about the regulation of MUC5B gene expression. In lung epithelial cells, MUC5B mRNA abundance is not altered by TNF-α or by PG metabolites (17), whereas MUC5B is transcriptionally up-regulated by IL-6/IL-17 (19).

Many of the inflammatory mediators and byproducts that increase mucin gene expression in vitro are elevated in vivo in the airway secretions of patients with asthma, CF, or COPD. IL-8, a chemokine and proinflammatory mediator (20), is also present at high levels (1–21 nM) in the bronchoalveolar lavage fluid of infants and young children with CF (21) and in the tracheal aspirates or sputum of CF, COPD, and asthmatic patients (22–27). IL-8 is secreted into lung fluid by macrophages and by lung epithelial cells (28) and recruits neutrophils to the sites of inflammation (29). It likely accounts for most of the neutrophil-predominant chronic inflammation in CF airways (30) and acute inflammation in COPD airways (31). Epithelial cells in the lower and upper respiratory tracts of CF patients have an augmented production of IL-8 (23, 32–34), which may account for the proinflammatory phenotype in CF lungs (35). We hypothesized that IL-8, as with other inflammatory/immune response mediators, might increase expression of mucin genes and thereby contribute to excessive airway mucus production in patients with chronic lung diseases. Thus, we investigated whether IL-8 altered the expression of MUC5AC and/or...
MUC5B mucin genes in human lung epithelial cells. Our findings demonstrate that IL-8 increases the abundance of MUC5AC and MUC5B mRNA in a time- and concentration-dependent manner in two respiratory tract-derived cancer cell lines, as well as in differentiated normal human bronchial epithelial (HBE) cells. Additionally, data show that IL-8 does not transcriptionally regulate MUC5AC gene expression, but rather increases transcript stability by altering the levels of RNA-binding proteins (RBP), which classically regulate gene expression posttranscriptionally by binding to cis-sequences in the 3'-untranslated region (UTR) of target transcripts (36, 37). This study suggests that IL-8, which recruits neutrophils to inflamed airways, may also directly contribute to mucin overproduction by regulating MUC5AC gene expression at the posttranscriptional level to increase mucin mRNA stability in lung epithelial cells.

Materials and Methods

Cell culture

Respiratory tract-derived carcinoma cell lines (A549 and NCI-H292) obtained from the American Type Culture Collection were grown in complete medium (Ham’s F-12K and RPMI 1640, respectively), supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), and glutamine (2 mM) and maintained at 37°C in a humidified 5% CO₂ atmosphere, as described previously (38). Normal primary HBE cells (Cambrex) were cultured as described by Krunkosky et al. (39). HBE cells were seeded at 1 x 10⁶ cells/well on Transwell clear culture inserts (Costar). When 75% confluent (5-7 days after plating), HBE cells were induced to differentiate by removing the apical medium to establish an air-liquid interface. Basal medium was changed daily thereafter. Experiments were performed on differentiated cells at day 17 after plating.

Exposure of cells to IL-8

A549 (2 x 10⁶ cells/well) and NCI-H292 (3 x 10⁶ cells/well) cells were seeded into 6-well plates (Costar) and grown overnight in complete medium. Cells were washed twice with serum-free medium, then incubated with human rIL-8 (R&D Systems) at the concentrations and times indicated in the figure legends. For HBE cells, medium was removed, and the apical and basal compartments were washed with PBS 17 days after an air-liquid interface had been established. IL-8 was added to the basal medium and to the apical surface at the concentrations and times indicated in the figure legends.

RNA extraction and Northern blot analysis

Total cellular RNA was extracted using TRIzol (40) and Northern blot analyses were performed (41) as described previously. The 541-bp EcoRI BamHI fragment of cDNA clone NPa encoding the 3'-end of MUC5AC (42), 984-bp EcoRI insert of cDNA clone HGBM1-1 encoding a tandem repeat region of MUC5B (43) (provided by Dr. G. O’affer, Boston University School of Medicine, Boston, MA), and 1100-bp NotI/XhoI cyclophilin insert (American Type Culture Collection) were labeled to a specific activity of 15–20 x 10⁶ cpm/μg with [α-32P]deoxycytidine triphosphate (New England Nuclear) using a random primer labeling kit (Stratagene).

Real-time PCR analysis of gene expression

cDNA libraries were generated from 5 μg of total RNA using SuperScript II reverse transcriptase (Invitrogen) and random primers. Real-time PCR analyses of MUC5AC and GAPDH mRNA were performed on the cDNA libraries using the primers and ABI PRISM 7700 sequence detection system described previously (40). The sequences used for MUC5B mRNA analyses (44) were as follows: forward, 5'-CG ATCCCACAGGTCCCTC-3' and reverse, 5'-CCTCCTCCCGTCTA CAGT-3' and primers and fluorescence probe, FAM-CAACCCCAAGC CTTTCACTCAG-TAMRA. GAPDH was unchanged by IL-8 exposure and used as an internal control for normalizing MUC5AC and MUC5B mRNA levels in control and experimental samples. A series of dilutions was performed to determine the linear dependence of the threshold cycles on the concentration of template RNA. Relative quantification of MUC5AC and MUC5B mRNA in control and experimental samples was obtained using the standard curve method.

Western blot analyses

Mucin protein levels in cellular secretions and lysates were evaluated by Western blot analyses as described previously (38). Apical secretions (500 μl) were collected at 24 h after exposure of primary-differentiated HBE cells to IL-8 or control vehicle. Cells were washed with 500 μl of PBS, which was added to the apical secretions. Cells were lysed in cell lysis reagent (Promega). Five hundred microliters of the combined apical secretions or 40 μg of protein lysates was lyophilized, resolubilized in 40 μl of electrophoresis sample buffer (2.5% SDS/4.5 M urea/5% β-mercaptoethanol/25% glycerol/0.005% bromophenol blue/0.08 M Tris HCL (pH 7.5)), and incubated for 15 min at 95°C. Samples were loaded on a 1% SDS/Tris-acetate buffer/EDTA agarose gel and electrophoresed for 1 h at 35 V, then stained at 15 V. Proteins were transferred to a polyvinylidene difluoride membrane (Osmonics) by a positive pressure of 75 mm Hg for 2.5 h. The membrane was blocked in 5% nonfat milk (Bio-Rad) solution and immunostained for MUC5AC and MUC5B mucins, respectively, using affinity-purified anti-MUC5AC-TR3A (38) and anti-MG1 (45) Abs at a dilution of 1/500. After incubation with a HRP-conjugated secondary Ab (Kirkegaard & Perry Laboratories) and Supersignal West Dura Extended Substrate (Pierce), mucin bands were visualized on a Chemi-Doc universal bio (Bio-Rad). The developed blots were digitized in monochromegne with a charge-coupled device imaging documentation station (Bio-Rad). The respective intensities of each MUC5AC-positive band in the resulting TIFF densitometrically semiquantitated using the Quantity One 4.3.1 image processing software (Bio-Rad) and box-shaped markers. Mucin levels were evaluated by densitometry and normalized to mucin levels in control (vehicle-exposed) cells.

MUC5AC promoter analyses

Transient transfection with the MUC5AC-luc promoter was performed as described previously (40). Briefly, A549 cells were seeded into 12-well plates (Costar) and grown to 60% confluence in complete media. Cells were washed twice with OPTIMEM (Invitrogen) reduced-serum medium, then incubated with 0.33 μg of pGL3 or the 1.5-kbp MUC5AC-luc plasmid (40). 1 μl of Lipofectamine, and 0.5 ml of OPTIMEM for 6 h. Transfected cells were washed and incubated with complete medium overnight, then washed and maintained in serum-free medium, before incubation with IL-8 for 24 h. Cell lysates were assayed for protein and for luc activity using the Luciferase assay kit (Promega). The resulting photon production was quantified using the Single Photon Monitor on a Beckman LS6500 scintillation counter.

RNA stability assays

A549 and NCI-H292 cells were plated on 6-well plates as described above, grown overnight, washed in serum-free medium, and incubated in serum-free medium with or without IL-8 (20 nM) for 24 h. Transcription was stopped by addition of 5 μg/ml actinomycin D (Sigma-Aldrich), and cells were harvested at 4, 8, or 16 h. Total cellular RNA was extracted and Northern blot analysis was performed.

RNA electrophoretic mobility shift assays (REMSA)

Nuclear and cytoplasmic protein extracts for use in REMSA were isolated from A549 cells using a procedure based on standard protocols (46). Briefly, cells were trypsinized at 95% confluence and washed with PBS three times. Buffer A (10 mM HEPES (pH 7.65), 1.5 mM MgCl₂, 10 mM KCl, 2 mM DTT, 0.2 mM PMSF, and 1/100 volume Sigma-Alrich protose inhibitor mixture) was added to the estimated packed cell volume at a ratio of 1:5. Cells were incubated on ice for 10 min, homogenized until lysed, rocked for 30 min at 4°C, then centrifuged for 10 min at 27,000 g. The supernatant was aliquoted and stored at ~80°C as cytoplasmic extract. The remainder of the samples was pelleted by centrifugation at 21,920 x g, 4°C, 15 min. Two volumes of buffer C (20 mM HEPES (pH 7.65), 25% glycerol, 42 mM NaCl, 1.5 mM MgCl₂, 200 mM EDTA, 2 mM DTT, 0.2 mM PMSF, and 1/100 volume Sigma-Aldrich protose inhibitor mixture) was added to the estimated pellet volume, and the nuclei were homogenized until lysed, rocked for 30 min at 4°C, then centrifuged for 10 min at 27,920 x g at 4°C. The supernatant was collected and stored as a nuclear extract at ~80°C. The protein content of the cytoplasmic and nuclear extracts was determined by the Bio-Rad protein assay kit using a BSA standard.

For REMSA, RNA oligonucleotides of 45 bases were synthesized and biotinylated at the 5'-end (Integrated DNA Technologies). The RNA-EMSA reaction mix (20 μl), which contained 60 mM KCl, 10 mM HEPES (pH 7.6), 3 mM MgCl₂, 1 mM DTT, 2.5% glycerol, 0.5 mM PMSF, 1/6 volume proteinase inhibitor mixture, 50 μg/ml RNA, 1/40 volume poly(I):
poly(C), 1/40 volume SUPERase-H18528 (Ambion), 25–30 μg of protein extract, and 25 fmol RNA oligonucleotides, was incubated for 10 min at room temperature and for 20 min at 4°C, then loaded onto a 5% native polyacrylamide gel that had been pre-electrophoresed in 0.5× Tris-borate EDTA (TBE) at 25 mA constant current for several hours. Electrophoresis was performed at 25 mA constant current in 0.5× TBE (pH 7.5) for 45 min. The complexes were transferred to a positively charged nylon membrane BrightStar-Plus (Ambion) with 1× TBE (pH 8.3) at 200 mA for 30 min. The visualization of the complexes was performed with BrightStar BioDetect kit according to the manufacturer’s instructions.

Statistical analysis

Analysis of IL-8 exposure data for each mucin gene and cell line was performed using an ANOVA and F-test for linear trends to evaluate differences between four concentration groups (0, 0.25, 2.5, and 25 nM IL-8). Values with p < 0.01 were considered significant. Mucin protein expression was analyzed using a nonpaired two-sample t-test to compare the density of the individual mucin protein band in the control secretions to that at each IL-8 concentration.

Results

IL-8 increases MUC5AC mRNA abundance in lung epithelial cells

Two respiratory tract-derived cell lines, NCI-H292 and A549, which express low to moderate levels of MUC5AC and MUC5B mRNA levels at or near confluency (38), were used to evaluate whether IL-8 altered mucin gene regulation in lung epithelial cells. Cells exposed to low levels of IL-8 (2.5 nM) increased MUC5AC mRNA abundance in a time-dependent manner (Fig. 1) but did not alter cyclophilin levels, which were used for standardization. IL-8 increased the abundance of MUC5AC mRNA 4-fold in A549 (A) and 3-fold in H292 cell lines (B) within 24 h, compared with cells exposed only to vehicle (p < 0.001).

Both cell lines were exposed to increasing concentrations of IL-8 (0.25, 2.5, or 25 nM) for 20 h, and steady-state mRNA levels of MUC5AC and cyclophilin were analyzed by Northern blot analysis. Increased abundance of MUC5AC mRNA, in contrast to a constant level of cyclophilin mRNA, was observed following exposure of H292 cells to IL-8 (Fig. 2A). Similar results were obtained with A549 cells (data not shown). Ethidium bromide staining of Northern gel demonstrates integrity of RNA and equivalent loading of each sample per lane. C, Concentration effect of IL-8 stimulation on MUC5AC mRNA expression in A549 (A) and H292 (B) cells. Band densities of MUC5AC and cyclophilin transcripts were quantified and normalized to cyclophilin levels. Values are means of the densitometric reading ± SE for four experiments. Statistically significant differences are indicated by asterisks: *, p < 0.0001.

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IL-8 in both cell lines (Fig. 2C). IL-8 (25 nM) resulted in a 4.1-fold increase in MUC5AC mRNA expression in A549 cells and a 3.0-fold increase in H292 cells, as compared with vehicle-treated controls.

IL-8 increases MUC5B mRNA abundance in lung epithelial cell lines

The effect of IL-8 on MUC5B mRNA expression in lung epithelial cell lines was likewise investigated. After exposure to IL-8 (2.5 nM), MUC5B mRNA levels in A549 (Fig. 3A) and H292 cell lines (Fig. 3B) were increased significantly at 24 h. Cells exposed to increasing concentrations of IL-8 (0.25, 2.5, or 25 nM) for 20 h exhibited a concentration-dependent increase in MUC5B mRNA levels in A549 and in H292 cells (Fig. 3C). At the highest IL-8 concentration (25 nM) used in these experiments, a 3.6- or 3.2-fold increase, as compared with controls, was observed in A549 or H292 cell lines, respectively. These data demonstrated that IL-8 exposure increased both MUC5AC and MUC5B mRNA in a concentration- and time-dependent manner in two lung epithelial cell lines.

IL-8 increases MUC5AC and MUC5B mRNA expression in primary-differentiated HBE cells

To determine whether the effect of IL-8 on mucin gene expression in lung cancer cell lines was relevant to what occurs in primary lung cells, we evaluated the effect of IL-8 on MUC5AC and MUC5B mRNA and protein expression in normal primary differentiated HBE cells. These cells, when grown and maintained at an air liquid interface (reviewed in Ref. 47), differentiate to histologically mimic an airway epithelium with ciliated and basal cells and goblet cells that express MUC5AC and MUC5B mRNA (48). Increasing concentrations of IL-8 resulted in a significant increase of MUC5AC mRNA (Fig. 4A). That the increase was lower than that observed in the lung cancer cell lines presumably reflects the lower number of goblet cells that express MUC5AC in the heterogeneous HBE model system. A considerably higher IL-8 concentration (75 nM) was required to significantly increase MUC5B mRNA abundance (Fig. 4B) in the HBE model system, suggesting that IL-8 regulation of the MUC5B gene may not be similar in primary cells and cell lines. The ability of IL-8 to significantly increase MUC5AC mRNA expression at similar IL-8 concentrations in primary-differentiated HBE cells and in two cancer cell lines suggests a common mechanism whereby IL-8 regulates MUC5AC mucin gene expression in human lung epithelial cells.

To evaluate whether the above-observed increases in mucin mRNA abundance translates into increased expression of mucin protein, the levels of MUC5AC and MUC5B mucins in cell secretions were evaluated by Western blot analyses. Exposure of cells to IL-8 resulted in increased levels of MUC5AC mucin in primary-differentiated HBE cell secretions (Fig. 5A) and in...
A549 cell secretions (data not shown), although the fold increase was not as high as observed with MUC5AC mRNA at similar IL-8 concentrations. Analysis of lysates of HBE cells demonstrated that IL-8 likewise increased expression of MUC5AC protein (data not shown). IL-8 had minimal effect on MUC5B mucin levels in differentiated HBE cell secretions at 25 nM (Fig. 5B) or in cell lysates (data not shown) in accordance with the effect of IL-8 on MUC5B mRNA abundance at that concentration (Fig. 4B). These data suggest that in the more analogous physiological model, IL-8 has a greater impact on MUC5AC than on MUC5B gene expression. Thus, our studies focused on the IL-8-induced regulation of MUC5AC gene expression.

**IL-8 does not transcriptionally up-regulate MUC5AC promoter activity**

Mediators can regulate MUC5AC gene expression at both the transcriptional and posttranscriptional levels (reviewed in Ref. 10). To determine whether the observed IL-8-mediated increase in MUC5AC mRNA abundance resulted from a transcriptional mechanism, the effect of IL-8 on MUC5AC promoter activity was evaluated. A549 cells were transiently transfected with a plasmid containing a luciferase reporter gene under control of the MUC5AC promoter, which is up-regulated by LPS (13) and IL-1β (16) and repressed by dexamethasone (40). Luciferase activity was evaluated 24 h after exposure of the transfected cells to IL-8. Concentrations of IL-8 at levels that increased MUC5AC mRNA abundance did not alter luciferase activity (Fig. 6). These results indicate that IL-8 does not transcriptionally up-regulate expression of the MUC5AC mucin gene.

**IL-8 increases the stability of the MUC5AC transcript**

To determine whether the IL-8-mediated increase on MUC5AC mRNA abundance resulted from posttranscriptional regulation, we examined the effect of IL-8 on mucin mRNA stability. To that end, transcription was inhibited by actinomycin D, a RNA polymerase II inhibitor, following incubation of cells in the presence or absence of IL-8 for 20 h, and the rate of MUC5AC mRNA decay was determined. In the absence of IL-8, the half-life (defined as the time at which 50% of mRNA remained) of MUC5AC mRNA in A549 cells was ~4 h (Fig. 7A), in good agreement with previously reported data (17, 18). After IL-8 exposure, the approximate half-life of MUC5AC mRNA in A549 cells was increased to 14 h in agreement to that observed with TNF (17) and NE (18). Similarly, in H292 cells, IL-8 increased the half-life of MUC5AC mRNA ~3-fold (from 4.25 h in unstimulated cells to 13.5 h in stimulated cells) (Fig. 7B). This 3-fold increase in mRNA half-life can account for most of the 3- to 4-fold increase in MUC5AC steady-state mRNA levels observed after exposure of these cell lines to IL-8, suggesting that the primary mechanism of IL-8 regulation of MUC5AC gene expression is posttranscriptional.
FIGURE 8. The 3′-UTR sequence of MUC5AC mRNA and location of the 45-mer riboprobes used for REMSA. The sequence shown was determined by analyses of the MUC5AC 3′-UTR in genomic DNA isolated from primary human foreskin fibroblasts and NCI-H292 cells. It is identical in sequence and size to that reported in Ref. 51, except for a G→C transversion, which is capitalized, bolded, and underlined. The translation termination codon in riboprobe 1 is boxed and bolded. The sequences in the 10 slightly overlapping 45-mer RNA riboprobes that covered the 3′-UTR of the MUC5AC gene in toto are indicated by dashed lines.

FIGURE 9. REMSA analyses of putative RBP binding sites in the 3′-UTR of the MUC5AC transcript following exposure of lung cells to vehicle or IL-8. Protein extracts (PE) (Cy, cytoplasmic and N, nuclear) were isolated from A549 cells exposed to control vehicle or IL-8 (25 nM) for 20 h, incubated with biotin-labeled riboprobes, electrophoresed, and analyzed as described in Materials and Methods. A, REMSA analyses with biotin-labeled riboprobes 3, 6, 9, and 10. Control lanes with Cy and N extracts are also shown. B, Quantification of nuclear complexes identified by * in A (n = 3). Statistically significant differences are indicated by asterisks: **, p < 0.01; and ***, p < 0.001.

IL-8 alters the binding of RBP to cis-sequences in the 3′-UTR of MUC5AC

Regulation of gene expression at the posttranscriptional level is typically mediated by binding of RBP to cis-sequences in the 3′-UTR of target mRNA (36, 49). To determine whether RBP regulated MUC5AC expression, we first determined the 3′-UTR sequence of MUC5AC, because there is variation in sequence and size of the three published sequences (42, 50, 51). To resolve these discrepancies, we conducted sequence analyses of genomic DNA isolated both from primary human foreskin fibroblasts and from NCI-H292 cells. Sequences from the primary cells and cancer cell line were identical and yielded a 3′-UTR of 438 nt (Fig. 8). This was identical in sequence and size to the 3′-UTR of the MUC5AC sequence reported in Ref. 51, except for a G→C transversion, as indicated in Fig. 8.

On the basis of these findings, 10 slightly overlapping 45-mer RNA riboprobes that covered the 3′-UTR of MUC5AC in toto (Fig. 8) were synthesized and biotinylated at the 5′-end for detection by streptavidin. Riboprobes were incubated with nuclear or cytoplasmic extracts isolated from control or IL-8-exposed A549 cells, and RBP complexes were identified by REMSA. Data indicated that specific riboprobes form nuclear and/or cytoplasmic protein complexes that presented with different electrophoretic mobilities and that IL-8 altered the profile of specific complexes resulting in induction/increase of some complexes or disappearance/decrease of others. The most consistently observed and marked effects were with nuclear RBP bound to riboprobe-3, -9, or -10 (Fig. 9), as well as cytoplasmic complexes bound to riboprobe-10. Riboprobe-6 did not form RBP complexes with either cytoplasmic or nuclear extracts. Taken together, these results demonstrated that cellular factors, predictably RBP and/or small RNA, bind to specific sequences in the 3′-UTR of the MUC5AC transcript in a lung epithelial cell line and that IL-8 alters RBP levels in these cells.

Discussion

Studies from several laboratories over the past decade have demonstrated that specific inflammatory mediators and byproducts can increase the expression of individual mucin genes in vitro in lung epithelial cells. The present study indicates that the chemokine IL-8, which is a predominant proinflammatory mediator activated in response to pulmonary inflammation (20), should be added to this list. Most of these mediators regulate secretory mucin genes at the transcriptional level (reviewed in Ref. 10). However, our study shows that IL-8 mediates MUC5AC gene regulation at the posttranscriptional, rather than the transcriptional, level and that the posttranscriptional effects are translated into increased MUC5AC mucin protein. In contrast, two other inflammatory mediators—TNF-α and NE—regulate MUC5AC gene expression at both the posttranscriptional (17, 18) and the transcriptional level (15, 16). Interestingly, IL-8 also increased MUC5B mRNA levels in two cancer cells lines (Fig. 3) and increased the stability of the MUC5B transcript (data not shown). However, the IL-8-induced effect on MUC5B expression was not translated into increased MUC5B mRNA or protein in primary-differentiated HBE cells, suggesting that IL-8 mediates MUC5B gene expression differently in cancer cell lines than in a more physiological model. Whether IL-8 regulates MUC5B expression at the transcriptional level in lung epithelial cells and/or cell lines has not been investigated.

The finding that some inflammatory mediators regulate mucin genes at the posttranscriptional, as well as the transcriptional, level is perhaps not surprising given that inflammatory mediators are prevalent in inflamed lungs for several days. Specific mediators initiate transcription, which is typically rapid and transient, whereas other mediators appear to increase transcript stability to sustain mucin expression. Some genes that are activated in human airways during inflammation, as well as genes implicated in airway remodeling in lung diseases (52), are regulated at the posttranscriptional level (53, 54). Posttranscriptional regulation of mucin genes may have evolved to allow sustained expression of mucins as part of the innate immune response. An increased understanding of mechanisms whereby mediators posttranscriptionally regulate mucin gene expression may lead to new pharmacological agents to decrease overproduction of MUC5AC mucin and thus mitigate the
contribution of mucus overproduction/obstruction to morbidity and mortality in lung diseases.

Regulation of gene expression at the posttranscriptional level is classically mediated by the binding of RBP to cis-sequences in the 3′-UTR of mRNA (36, 37). Our data show that complexes, which presumably include RBP, bind to specific sequences in the 3′-UTR of the MUC5AC transcript and that the levels of specific bound complexes are altered following exposure of lung cells to IL-8. Studies are ongoing to identify RBP that bind to specific cis-sequences in the 3′-UTR of the MUC5AC gene.

That the observed IL-8-induced increase in MUC5AC mRNA abundance in two respiratory tract-derived cancer cell lines is also observed in primary-differentiated HBE cells indicates that the effect of IL-8 on MUC5AC mucin gene expression is physiologically relevant. The fold increase is lower in HBE cells than in cancer cell lines (1.8–vs 3–4 fold), which may reflect the lower population of mucin-expressing cells in the differentiated HBE cell model system of ciliated, basal, and goblet/mucus cells (47). A previous report concluded that IL-8 did not mediate mucin gene expression in primary-differentiated HBE cells, because minimal increases in MUC5AC or MUC5B mRNA were detected by RT-PCR after 16 or 24 h exposure to low IL-8 concentrations (10 and 50 ng/ml) (19). In the current study, primary-differentiated HBE cells were exposed to increasing concentrations of IL-8, and mucin gene expression was evaluated by quantitative real-time PCR. MUC5AC mRNA expression was not altered at the lowest IL-8 concentration evaluated (10 ng/ml = 1.25 nM) (data not shown), in agreement with the results of Chen et al. (19). However, a significant increase in MUC5AC mRNA abundance was observed at higher IL-8 concentrations (6.25, 25, and 75 nM) (Fig. 4). In contrast, MUC5B mRNA expression was significantly increased in HBE cells only at the highest IL-8 concentration (75 nM) used in this study. Because IL-8 concentrations in the airway secretions of patients with obstructive airway diseases are in the 1–21 nM range (21–27), MUC5C mRNA transcripts levels may be stabilized in preference to MUC5B transcripts in inflamed airways. However, local levels of IL-8 may be markedly increased during exacerbations in asthmatic patients and could account for the high levels of MUC5B mucin in sputum from a patient with status asthmaticus (55).

IL-8, which is activated in response to inflammation (20), is secreted in the lungs by macrophages as well as lung epithelial cells (28). IL-8 recruits neutrophils to the site of inflammation (29) and accounts for most of the neutrophil-dominated chronic inflammation in CF airways (30). Epithelial cells in the lower and upper respiratory tracts of CF patients have an augmented production of IL-8 (23, 32–34), and IL-8 may account for the proinflammatory phenotype in CF lungs (reviewed in Ref. 35). Importantly, human NE also increases the abundance of MUC5AC mRNA in AS49 cells and in primary-differentiated HBE cells and increases the stability of the MUC5AC transcript in AS49 cells (18). That in vivo IL-8 recruits neutrophils that can release NE to the lung raises the possibility of a positive feedback cascade that could be operative in inflamed airways of patients with CF and/or COPD, where IL-8 levels and neutropenia abound.

This study suggests that IL-8, in addition to being a potent neutrophil chemoattractant, also increases the abundance of mRNA of mucin genes. This newly identified function may contribute to sustaining overproduction of mucins in patients with chronic obstructive airway diseases. Therapeutic strategies to decrease IL-8 may prove to be an effective approach in the management of inflammation and mucin overproduction in CF, acute asthma, and chronic bronchitis.

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Disclosures

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References