CCL20/CCR6 Feedback Exaggerates Epidermal Growth Factor Receptor-Dependent MUC5AC Mucin Production in Human Airway Epithelial (NCI-H292) Cells

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Mucous hypersecretion is an important feature of obstructive airway diseases such as asthma, chronic obstructive pulmonary disease, and cystic fibrosis. Multiple stimuli induce mucin production via activation of an epidermal growth factor receptor (EGFR) cascade, but the mechanisms that exaggerate mucin production in obstructive airway diseases remain unknown. In this study, we show that binding of CCL20, a G protein-coupled receptor (GPCR) ligand that is upregulated in the airways of subjects with obstructive airway diseases, to its unique GPCR CCR6 induces MUC5AC mucin production in human airway epithelial (NCI-H292) cells via metalloprotease TNF-α-converting enzyme (TACE)-dependent EGFR activation. We also show that EGFR activation by its potent ligand TGF-α induces reactivation of EGFR via binding of endogenously produced CCL20 to its receptor CCR6 in NCI-H292 cells but not in normal human bronchial epithelial (NHBE) cells, exaggerating mucin production in the NCI-H292 cells. In NCI-H292 cells, TGF-α stimulation induced two phases of EGFR phosphorylation (EGFR-P). The second EGFR-P was TACE-dependent and was responsible for most of the total mucin induced by TGF-α. Binding of endogenously produced CCL20 to CCR6 increased the second EGFR-P and subsequent mucin production induced by TGF-α. In NHBE cells, TGF-α-induced EGFR activation did not lead to significant CCL20 production or to EGFR rephosphorylation, and less mucin was produced. We conclude that NCI-H292 cells but not NHBE cells produce CCL20 in response to EGFR activation, which leads to a second phase of EGFR-P and subsequent exaggerated mucin production. These findings have potentially important therapeutic implications in obstructive airway diseases.

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Abbreviations used in this article: COPD, chronic obstructive pulmonary disease; EGFR, epidermal growth factor receptor; EGFR-P, EGFR phosphorylation; GPCR, G protein-coupled receptor; NHBE, normal human bronchial epithelial; siRNA, small interfering RNA; TACE, TNF-α-converting enzyme; TAPI-1, TNF-α protease inhibitor-1.

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tion by its ligand TGF-α stimulates a positive feedback pathway involving CCL20/CCR6 in NCI-H292 cells but not in normal human bronchial epithelial (NHBE) cells, exaggerating mucin production in the NCI-H292 cells.

Materials and Methods

Reagents

Recombinant CCL20 and TGF-α were purchased from R&D Systems (Minneapolis, MN). AG1478, EGFR-neutralizing Ab (Ab-3), TNF-α protease inhibitor-1 (TAPI-1), pertussis toxin, cycloheximide, and PD98059 were purchased from Calbiochem (La Jolla, CA).

Cell culture

Human pulmonary mucoepidermoid carcinoma (NCI-H292) cells were purchased from American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 medium containing 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), and 25 mM HEPES at 37°C in a humidified 5% CO2 water-jacketed incubator. NCI-H292 cells have been shown to express EGFR (30), EGFR proligands (31), MUC5AC mucin (12), and CCL20 (27). Because cell lines such as NCI-H292 show variability in their responses to stimuli and inhibitors at different passages, all experiments were performed with cells from passages 80 to 90.

Proliferating NHBE cells were purchased from Lonza (Walkersville, MD) and grown in immersed culture in bronchial epithelial cell growth medium (Lonza) according to the recommendations of the supplier. NHBE cells were grown in immersed culture instead of air–liquid interface to maintain culture conditions similar to those for the NCI-H292 cells and because NHBE cells in immersed culture have been shown to express MUC5AC mucin (32, 33) and CCL20 (17). Experiments with NHBE cells were performed with passages 2 to 4 to limit variable responses. Confluent cultures were serum-starved (NCI-H292) or incubated with epidermal growth factor-free bronchial epithelial cell growth medium (NHBE) for 2 h before the addition of various concentrations of CCL20 (0.1, 0.3, 1, 3, and 10 ng/ml) or TGF-α (5 ng/ml). Chemical inhibitors or neutralizing Abs were added 30 min before stimulation with CCL20 or 30 min before or at various times after stimulation with TGF-α. Cell lysates and supernatants were harvested at various times after stimulation for measurement of EGFR-P (up to 6 h), CCL20 (up to 24 h), and MUC5AC mucin (24 h).

Small interfering RNA preparation and transfection of cells

TACE small interfering RNA (siRNA) knockdown and confirmation of specific TACE silencing were performed as described previously (8). TACE expression was knocked down ~90% by this method (8). For CCR6 siRNA knockdown, subconfluent (50–60%) NCI-H292 cells were transfected with CCR6 siRNAs (SMARTpool L-005453; Dharmacon, Lafayette, CO) or nontargeting control siRNA (Non-targeting pool D-001810; Dharmacon) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. CCR6 expression was knocked down ~80% 72 h after transfection by this method (measured by immunoblot, see Fig. 2B).

Measurement of EGFR-P, CCL20, and MUC5AC mucin

EGFR-P in cell lysates and CCL20 in cell culture supernatants were measured by sandwich ELISA kits according to the manufacturer’s instructions (Duoset I.C. R&D Systems). EGFR-P results were expressed as picograms of EGFR-P per microgram of total protein. Protein concentrations in cell lysates were measured using a bicinchoninic acid-based protein assay kit (Pierce, Rockford, IL). CCL20 results were expressed as picograms of CCL20 per milliliter of supernatant.

MUC5AC protein in cell lysates and in cell culture supernatants was measured by ELISA as described previously (12). Total MUC5AC protein was normalized to total protein in cell lysate and expressed as micrograms of mucin per microgram of total protein. In experiments using the monoclonal EGFR- or CCL20-neutralizing Abs, the standard MUC5AC ELISA protocol was modified: a rabbit polyclonal Ab against MUC5AC mucin (H-160; Santa Cruz Biotechnology, Santa Cruz, CA) and a goat anti-rabbit IgG (secondary Ab)–HRP conjugate were used to measure MUC5AC mucin protein. In experiments using EGFR- or CCL20-neutralizing Abs, the Abs did not change total cell protein values significantly (data not shown).

Immunoblotting

NCI-H292 and NHBE cell lysates were prepared, and immunoblotting was performed as described previously (34). In brief, equal amounts of protein were separated by SDS–7.5% PAGE, transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA), and blotted with mAbs against either human CCR6 (R&D Systems) or human EGFR (R&D Systems). Bound Ab was visualized with ECL (Amersham, Piscataway, NJ). Immunoblots were scanned, and band intensities were quantified with National Institutes of Health Image 1.63 software (developed at the National Institutes of Health and available for free download at rsbweb.nih.gov).

Statistical analysis

All data are expressed as means ± SD. One-way ANOVA was used to determine statistically significant differences between groups (p < 0.05 for the null hypothesis).

Results

CCL20 induces mucin production via CCR6- and TACE-dependent EGFR activation

Because mucins are produced via EGFR-P (12), and because binding of CCL20 to its receptor CCR6 has been shown to induce EGFR-P in colon carcinoma cells (24), we examined whether CCL20 could induce mucin production in human airway epithelial cells via EGFR-P. In NCI-H292 cells, exogenous CCL20 induced EGFR-P time-dependently (maximal at 30 min; Fig. 1A, left panel) and induced mucin production dose-dependently (Fig. 1B, left panel). However, in NHBE cells, exogenous CCL20 did not induce EGFR-P (Fig. 1A, right panel) or mucin production (Fig. 1B, right panel) significantly. In NCI-H292 cells, pretreatment with the EGFR-selective inhibitor AG1478, with an EGFR-neutralizing Ab, or with TAPI-1, a relatively selective metalloprotease TACE inhibitor (35), prevented CCL20-induced EGFR-P at 30 min (Fig. 1C) and prevented mucin production (Fig. 1D) completely. These results indicate that CCL20 stimulates mucin production in NCI-H292 cells but not in NHBE cells, and they suggest that TACE-dependent EGFR proligand cleavage and subsequent ligand-dependent EGFR activation are responsible for CCL20-induced mucin production in the NCI-H292 cells. The CCL20 receptor CCR6 was expressed in NCI-H292 cells and to a lesser extent in NHBE cells (Fig. 2A). To examine whether CCR6 is involved in CCL20-induced EGFR-P and mucin production in NCI-H292 cells, we knocked down CCR6 expression ~80% in these cells by transfection of CCR6 siRNA (Fig. 2B). The efficiency of TACE siRNA knockdown in NCI-H292 cells (90%) has been documented previously (8). CCR6 siRNA or TACE siRNA knockdown prevented CCL20-induced EGFR-P (Fig. 2C) and mucin production (Fig. 2D) completely, whereas a nontargeting siRNA control had no significant effect on either response. These results suggest that CCR6 and TACE are responsible for CCL20-induced EGFR-P and subsequent mucin production in NCI-H292 cells.

Stimulation of EGFR by its ligand TGF-α results in a second phase of EGFR activation, which increases total mucin production in NCI-H292 cells but not in NHBE cells

We hypothesized that various stimuli that cause EGFR activation could result in CCL20 production and secretion, leading to CCL20/CCR6-dependent EGFR reactivation and to additional mucin production. Because EGFR ligands bind to EGFR and activate the receptor directly, here we chose the potent EGFR ligand TGF-α as the stimulus. First we compared the effects of stimulation with TGF-α on EGFR-P in NCI-H292 cells and NHBE cells: In the control state (incubation with medium alone) and after stimulation with TGF-α, EGFR-P was greater in the NCI-H292 cells compared with that in the NHBE cells (Fig. 3A), which corresponded with the markedly higher levels of EGFR in the NCI-H292 cells versus those in the NHBE cells in the control state (Fig. 3B). After

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stimulation with TGF-α, EGFR-P peaked at ~0.2 h in both cell types and then declined steadily, reaching a nadir ~2 h after stimulation (Fig. 3A). In NCI-H292 cells, EGFR-P then increased a second time, peaking at ~4 h and then declining to control levels by 6 h (Fig. 3A, open bars). However, in NHBE cells, there was no second phase of EGFR-P (Fig. 3A, hatched bars).

Next we examined the time-related effects of EGFR-P on TGF-α-induced mucin production in the two cell types: Pretreatment with the EGFR inhibitor AG1478 prevented EGFR-P (data not shown) and mucin production (Fig. 4) induced by TGF-α in both cell types completely. When AG1478 was added to NCI-H292 cells 3 h after TGF-α stimulation (i.e., after the initial EGFR-P and before the second EGFR-P, see Fig. 3A), mucin production was still inhibited significantly (Fig. 4, left panel). However, in NHBE cells, the addition of AG1478 at 3 h had no significant effect on total TGF-α-induced mucin production (Fig. 4, right panel), confirming that no further EGFR-P after 3 h is involved in mucin production in the NHBE cells. When AG1478 was added 6 h after TGF-α stimulation (i.e., after the second EGFR-P in NCI-H292 cells, see Fig. 3A), mucin production was not inhibited significantly in either cell type (Fig. 4). Together, these results indicate that TGF-α induces a second phase of EGFR-P in NCI-H292 cells but not in NHBE cells, and they suggest that this second EGFR-P increases total mucin production in the NCI-H292 cells.

**TACE-dependent EGFR reactivation increases TGF-α–induced mucin production**

Because TGF-α–induced EGFR activation has been shown to lead to reactivation of EGFR in some permanent epithelial cell lines metalloprotease-dependently (36, 37), we examined whether metalloprotease-dependent EGFR proligand cleavage contributes to the second EGFR-P and subsequent mucin production in NCI-H292 cells: Addition of an EGFR-neutralizing Ab to NCI-H292 cells 3 h after TGF-α stimulation prevented the second peak of EGFR-P at 4 h completely (Fig. 5A) and inhibited total mucin production markedly (Fig. 5B), implicating ligand binding to EGFR in these responses. However, addition of the EGFR-neutralizing Ab 6 h after TGF-α had no significant effect on mucin production (Fig. 5B), implicating ligand binding to EGFR between 3 and 6 h in the increased mucin response. In cells that...
were not stimulated with TGF-α, the EGFR Ab had no significant effect on EGFR-P at 4 h (Fig. 5A) or on mucin production (Fig. 5B).

The source of EGFR ligands causing EGFR reactivation could either be endogenous (i.e., produced by the epithelial cells) or exogenous (e.g., due to the TGF-α added to the culture medium). Because metalloprotease TACE-dependent cleavage of EGFR proligands leads to EGFR-P and to mucin production in airway epithelial cells (8), we examined whether TACE is involved in the second EGFR-P and in downstream mucin production: Pre-treatment with TAPI-1, a relatively selective TACE inhibitor (35), suppressed the second EGFR-P (Fig. 5A) and mucin production (Fig. 5B) induced by TGF-α markedly. In cells that were not stimulated with TGF-α, TAPI-1 had no significant effect on EGFR-P at 4 h (Fig. 5A) or on mucin production (Fig. 5B). A cysteine protease inhibitor (leupeptin, 50 μM) or a serine protease inhibitor (aprotinin, 10 μg/ml) had no significant effect on these responses (data not shown). When TAPI-1 was added 3 h after TGF-α stimulation, mucin production was still inhibited significantly (Fig. 5B). Furthermore, when added 6 h after TGF-α stimulation (i.e., after the second EGFR-P, see Fig. 3A), TAPI-1 had no significant effect on mucin production (Fig. 5B), suggesting that the TAPI-induced suppression of mucin production was due to inhibition of the second EGFR-P. TACE siRNA knockdown inhibited the second peak of EGFR-P at 4 h (Fig. 5C) and suppressed mucin production (Fig. 5D) induced by TGF-α markedly, whereas a nontargeting siRNA control had no significant effect on EGFR-P (Fig. 5C) or on mucin production (Fig. 5D), confirming a role for TACE in these responses. Together, these results suggest that metalloprotease TACE contributes to production of the second EGFR-P and subsequent mucin production in NCI-H292 cells.

FIGURE 3. TGF-α induces two phases of EGFR-P in NCI-H292 cells and a single phase of EGFR-P in NHBE cells. A, EGFR-P was measured in NCI-H292 cells (open bars) and in NHBE cells (hatched bars) incubated with TGF-α (5 ng/ml) starting at time 0 h (control) for the times indicated. An arrow denotes the peak of the second phase of EGFR-P in NCI-H292 cells at 4 h. Values are means ± SD; n = 5. *p < 0.05 compared with control. †p < 0.05 compared with control and EGFR-P at 2 h (NCI-H292). B, EGFR was measured by immunoblot in NCI-H292 cells (H292; left) and in NHBE cells (right). A blot representative of three independent experiments is shown. Band intensities were quantified (n = 3), and the intensity of the NCI-H292 band was set arbitrarily to 1. Values are means ± SD; n = 3. *p < 0.05 compared with NCI-H292.

FIGURE 4. The second EGFR-P increases mucin production in NCI-H292 cells. MUC5AC mucin in cell lysates (solid bars) and in cell supernatants (open bars, NCI-H292; hatched bars, NHBE) was measured in NCI-H292 cells (left panel) and in NHBE cells (right panel) incubated for 24 h with medium alone (−TGF-α; control) or with added TGF-α (+TGF-α). The EGFR-selective inhibitor AG1478 (10 μM) was not added (−) or was added 0, 3, or 6 h after TGF-α stimulation. Values are means ± SD; n = 5. *p < 0.05 compared with control. †p < 0.05 compared with +TGF-α alone.

FIGURE 5. TACE promotes the second EGFR-P and subsequent mucin production. A, EGFR-P was measured in NCI-H292 cells incubated for 4 h with medium alone (−TGF-α; control) or with added TGF-α (+TGF-α). Inhibitors were not added (−) or an EGFR-neutralizing Ab (5 μg/ml) was added 3 h after TGF-α stimulation (i.e., 1 h before the peak of the second EGFR-P) or the TACE inhibitor TAPI-1 (30 μM) was added 30 min before TGF-α stimulation. Values are means ± SD; n = 5. *p < 0.05 compared with control, †p < 0.05 compared with +TGF-α alone. B, MUC5AC mucin in cell lysates (solid bars) and in cell supernatants (open bars) was measured in NCI-H292 cells incubated for 24 h with medium alone (−TGF-α; control) or with added TGF-α (+TGF-α). Inhibitors were not added (−) or an EGFR-neutralizing Ab was added 3 or 6 h after TGF-α stimulation or TAPI-1 was added 0, 3, or 6 h after TGF-α stimulation. Values are means ± SD; n = 5. *p < 0.05 compared with control, †p < 0.05 compared with +TGF-α alone. C and D, EGFR-P (C) and MUC5AC mucin (D) in cell lysates (solid bars) and in cell supernatants (open bars) were measured in NCI-H292 cells treated with Lipofectamine 2000 alone (−) or in NCI-H292 cells transfected with TACE siRNA (100 nM) or nontargeting siRNA (100 nM; NON) and incubated for 72 h before addition of medium alone (−TGF-α; control) or TGF-α (+TGF-α) for 4 h (EGFR-P) or for 24 h (mucin). Values are means ± SD; n = 3. *p < 0.05 compared with control, †p < 0.05 compared with +TGF-α alone.
Binding of endogenously produced CCL20 to CCR6 increases TGF-α-induced mucin production via EGFR reactivation

Because binding of exogenous CCL20 to its receptor CCR6 induced mucin production in NCI-H292 cells via TACE-dependent EGFR activation (Figs. 1, 2), we reasoned that binding of endogenously produced CCL20 to CCR6 could lead to EGFR reactivation-dependent mucin production. Because the CCL20 receptor CCR6 has been reported to couple to Gi proteins (38), we examined the effects of the Gi protein inhibitor pertussis toxin on the second EGFR-P and subsequent mucin production: Pretreatment with pertussis toxin suppressed the second peak of EGFR-P at 4 h (Fig. 6A) and mucin production (Fig. 6B) induced by TGF-α in NCI-H292 cells markedly, suggesting that Gi protein signaling is required for the responses. In addition, pretreatment with the protein synthesis inhibitor cycloheximide suppressed the second EGFR-P markedly (Fig. 6A), implicating new protein synthesis in EGFR reactivation. These results suggest that an initial EGFR-P induced by TGF-α stimulates new protein synthesis that leads to GPCR-dependent EGFR reactivation and subsequent mucin production.

To examine whether new CCL20 synthesis and secretion induced by TGF-α increases mucin production via EGFR rephosphorylation, first we measured CCL20 released into the medium at various times after stimulation with TGF-α. In NCI-H292 cells, CCL20 levels were increased relative to control at 3 h, peaked at 4 h, and were not further elevated at 24 h after TGF-α stimulation (Fig. 7). The increase in CCL20 levels between 2 and 4 h after TGF-α stimulation paralleled the increase in EGFR-P during the second phase of EGFR-P (see Fig. 3A). However, in NHBE cells, CCL20 levels did not increase significantly after stimulation with TGF-α (Fig. 7). In NCI-H292 cells, preincubation with AG1478 prevented TGF-α–induced CCL20 production completely (data not shown), implicating the initial EGFR-P induced by TGF-α in CCL20 synthesis and release in the NCI-H292 cells.

Next, we examined whether binding of endogenously produced CCL20 to CCR6 stimulates TGF-α–induced mucin production via EGFR rephosphorylation. In NCI-H292 cells, pretreatment with a CCL20-neutralizing Ab suppressed the second EGFR-P (Fig. 8A, left panel) and inhibited total mucin production induced by TGF-α dose-dependently (Fig. 8B, left panel). In cells that were not stimulated with TGF-α, the CCL20-neutralizing Ab had no significant effect on EGFR-P at 4 h (Fig. 8A, left panel) or on mucin production (Fig. 8B, left panel). CCR6 siRNA knocked down suppressed the second EGFR-P significantly (Fig. 8A, right panel) and suppressed mucin production (Fig. 8B, right panel) induced by TGF-α, whereas a nontargeting siRNA control had no significant effect (Fig. 8A and 8B, right panels), confirming that CCR6...
ERK1,2 does not lead to mucin production downstream of the first but not the second phase of EGFR activation

Because EGFR ligand-induced mucin production has been shown to occur via activation of ERK1,2 (39), we examined the role of ERK1,2 activation over time in TGF-α-induced mucin production in NCI-H292 cells. Pretreatment with the MEK inhibitor PD98059, which prevents ERK1,2 activation, had no significant effect on the first EGFR-P (data not shown) but suppressed the second EGFR-P markedly (Fig. 9A) and prevented mucin production induced by TGF-α completely (Fig. 9B). However, when the MEK inhibitor was added to NCI-H292 cells 3 h after TGF-α stimulation (i.e., after the initial EGFR-P and before the second EGFR-P, see Fig. 3A), the second EGFR-P (Fig. 9A) and mucin production (Fig. 9B) were not inhibited significantly, indicating that ERK1,2 activation after 3 h is not involved in these responses. In cells that were not stimulated with TGF-α, the MEK inhibitor had no significant effect on EGFR-P at 4 h (Fig. 9A) or on mucin production (Fig. 9B). However, pretreatment with the MEK inhibitor prevented TGF-α-induced CCL20 production at 4 h completely (Fig. 9C), implicating ERK1,2 activation downstream of the initial EGFR-P in TGF-α-induced CCL20 production. Together, these results suggest that ERK1,2 activation leads to mucin production downstream of the initial EGFR-P and via promotion of a CCL20/CCR6-dependent second EGFR-P but that ERK1,2 does not lead to mucin production downstream of the second EGFR-P. The present findings are shown as a schematic (Fig. 10).

Discussion

In this study, we show that binding of CCL20 to its unique GPCR CCR6 induces MUC5AC mucin production in human airway epithelial (NCI-H292) cells. CCR6 siRNA knockdown prevented mucin production in response to exogenous CCL20, confirming that the mucin response to CCL20 involved an interaction with its receptor, CCR6. The CCL20/CCR6 interaction resulted in EGFR activation and mucin production, both of which were prevented by the EGFR-selective inhibitor AG1478 and by pretreatment with an EGFR-neutralizing Ab, which blocks EGFR interaction with EGFR ligands, implicating ligand-dependent EGFR activation in CCL20-induced mucin production.

Metalloproteases have been shown to cleave EGFR proligands and to release soluble ligands, making them available for binding to EGFR (40). Among the metalloproteases known to cleave EGFR proligands on the airway epithelial surface (9, 41) is TACE (8). A metalloprotease inhibitor with relative selectivity for TACE [TAPI-1 (35)] and TACE siRNA knockdown prevented EGFR activation and mucin production induced by CCL20. Notably, mice that lack the CCL20 receptor CCR6 have been reported to produce less mucin than that produced by wild-type mice in response to infection with respiratory syncytial virus (42), suggesting a role for CCL20/CCR6 in mucin production in vivo; the signaling leading to mucin production was not explored in that study. The current findings implicate a CCL20 → CCR6 → TACE → EGFR proligand cleavage → EGFR-P signaling cascade in the mucin response to exogenous CCL20.

Having shown that the interaction of the GPCR ligand CCL20 with its receptor CCR6 stimulates an EGFR cascade resulting in mucin production, we investigated further the potential pathophysiologic implications of this novel pathway for mucin production. Because the expression of EGFR (3, 43, 44) and of CCL20 (14–16) in airways is increased in obstructive airway diseases, we hypothesized that activation of EGFR could stimulate endogenous production and secretion of CCL20. To test this hypothesis, we examined the effect of EGFR activation on CCL20 production and on subsequent mucin production. As a stimulus, we chose the potent EGFR ligand TGF-α because of the established role of TGF-α in mucin production (8, 12, 28, 29) and because TGF-α binds to and activates EGFR directly, inducing signals downstream from EGFR selectively. For these studies, we chose NCI-H292 cells, a widely used and available model system for mucin production (reviewed in Ref. 26), because various stimuli including EGFR ligands (e.g., TGF-α) induce mucin production in these cells EGFR-dependently (12) and because NCI-H292 cells express CCL20 inducibly (27).

In this study, we show that TGF-α–induced EGFR activation results in ERK1,2-dependent CCL20 production and secretion. Consistent with previous studies (12, 30), we found that stimulation of NCI-H292 cells with TGF-α induces mucin production. In addition, we found that TGF-α–induced EGFR activation results in TACE-dependent reactivation of EGFR. This led to further mucin production downstream of the second phase of EGFR-P, resulting in an exaggerated mucin response. Pretreatment with a CCL20-neutralizing Ab, which prevents CCL20-dependent activation of its receptor CCR6, and CCR6 siRNA knockdown suppressed the second EGFR-P and total mucin production induced by TGF-α, implicating CCL20/CCR6 in TACE-dependent EGFR reactivation and subsequent mucin production. The current findings show that EGFR activation stimulates a positive feedback mechanism involving CCL20/CCR6-dependent
activation of a TACE surface cascade, resulting in EGFR reactivation and further mucin production (shown as a schematic in Fig. 10).

Because EGFR (43), EGFR ligands (43), and CCL20 (14, 16) are expressed sparsely in normal airways, we hypothesized that a CCL20/CCR6 positive feedback mechanism may not be present in NHBE cells. We found that, similar to previous reports showing CCR6 expression in normal colon epithelium (45) and in normal lung (16), NHBE cells express the CCL20 receptor CCR6 in the control state, albeit at lower levels than those in NCI-H292 cells. However, addition of the CCR6 ligand CCL20 did not increase EGFR-P and mucin production in NHBE cells significantly, perhaps due to the lower levels of CCR6 or of EGFR in these cells versus those in NCI-H292 cells. In NHBE cells, direct stimulation of EGFR with TGF-α resulted in mucin production via a single phase of EGFR activation and did not lead to a significant increase in CCL20 production.

A lack of CCL20 production and secretion in NHBE cells compared with that in asthmatic bronchial epithelial cells stimulated with the allergen Der p 1 has been reported (15), suggesting that increased CCL20 production in response to proinflammatory stimuli may be an intrinsic feature of epithelial cells in chronic inflammatory airway diseases. Because the production of chemokines such as CCL20 is regulated largely via gene transcription (46), we speculate that the greater airway epithelial production of CCL20 in obstructive airway diseases (14–16) could be due to increased CCL20 gene transcription by NFs that transactivate the CCL20 promoter such as NF-κB (45, 47), Sp1 (48), and epithelium-specific Ets NFs (48). The basis of increased airway epithelial CCL20 production in obstructive airway diseases and the potential involvement of EGFR signaling in this increase need further exploration. Subsequent studies should be performed in primary airway cells from subjects with disease (e.g., COPD, asthma) to examine the role of CCL20/CCR6 in exaggerated mucin production in these cells. The current findings show that EGFR activation does not induce appreciable CCL20 production in NHBE cells, and they show that feedback reactivation of EGFR in these cells does not occur. Thus, in contrast to NCI-H292 cells, mucin production is not further increased by a CCL20/CCR6 feedback mechanism in NHBE cells.

The binding of CCL20 to its unique GPCR CCR6, which is expressed on immature dendritic cells (23) and on memory T cells (49), is well known to induce chemotaxis of these cells (49, 50). In studies comparing CCR6-deficient mice and wild-type mice, CCL20/CCR6 has been shown to increase Th2-type airway inflammation in response to Ag (51) and to increase Th1-type airway inflammation in response to cigarette smoke (19). These results suggest that an interaction between CCL20 produced by the airway epithelium or by immune cells recruited to the airways (52) and CCR6 modulates airway inflammation in response to inhaled stimuli. To our knowledge, CCR6 protein expression in airway epithelium has not been examined in obstructive airway diseases such as COPD, asthma, and cystic fibrosis. In colon epithelium, CCR6 has been reported to be expressed constitutively (45) and, like its cognate ligand CCL20 (53–55), to be upregulated in inflammatory bowel diseases (54, 55). This suggests that epithelial CCL20/CCR6 signaling could contribute to the pathophysiology of inflammatory bowel diseases and raises the possibility that similar upregulation of epithelial CCR6 in inflammatory airway diseases could occur. While information on CCR6 expression in airways is currently lacking, increased expression of the CCR6 ligand CCL20 has been clearly demonstrated in COPD (16) and cystic fibrosis airways (14) and in primary bronchial epithelial cells from asthmatics (15), suggesting that increased epithelial CCL20/CCR6 signaling could occur in subjects with obstructive airway diseases.

Repeated metalloprotease-dependent EGFR activation after stimulation with EGFR ligands (i.e., positive feedback) has been observed in some permanent epithelial cell lines previously (36, 37). Since then, many GPCRs including the Gi protein-coupled CCL20 receptor CCR6 (24, 38) have been shown to activate EGFR metalloprotease-dependently (13). In the current study, we found that in NCI-H292 cells stimulated with TGF-α, CCL20/CCR6 blockade inhibited most of the mucin production downstream of EGFR reactivation but only suppressed the second EGFR-P partially, implicating EGFR feedback due to CCL20/CCR6 in exaggerated mucin production (data not shown). Notably, we found that inhibition of ERK1,2 downstream of the second but not the first EGFR-P suppressed cell proliferation but had no effect on mucin production induced by TGF-α, suggesting that different GPCRs (e.g., CCR1,2, CCR6) induce different patterns of cell activation (e.g., with or without a second phase of ERK1,2 activation), leading to divergent EGFR responses (e.g., cell proliferation, mucin production). We speculate that multiple epithelial EGFR responses (e.g., production of mucins, chemokines, and angiogenic factors; cell proliferation, cell invasiveness) could be exaggerated by GPCR feedback, thereby contributing to the pathophysiology of various airway diseases (e.g., asthma, COPD, cystic fibrosis, lung adenocarcinoma) that are characterized by increased levels of EGFR signaling. For example, the cyclooxygenase-2 product and GPCR ligand PGE2 has recently been shown to increase cell invasiveness via EGFR feedback in biliary carcinoma cells (56). Thus, the identification of G protein-coupled ligand–receptor interactions (e.g., CCL20/CCR6) that exaggerate specific EGFR responses (e.g., mucin) via feedback could lead to targeted therapy for deleterious epithelial cell responses. Testing this idea will be an important subject of future studies.

**FIGURE 10.** Schematic of the positive feedback loop between the EGFR and CCL20/CCR6 signaling pathways in NCI-H292 cells. Multiple inhaled stimuli activate EGFR via production of a soluble form of the EGFR ligand TGF-α (8, 22) or other EGFR ligands (not shown). TGF-α (diamond, left side) binds EGFR directly, inducing a first phase of EGFR-P (pY, left side) that results in the activation of ERK1,2 and in subsequent production and secretion of MUC5AC mucin (small box) and CCL20. Secreted CCL20 binds to its unique G protein–coupled receptor CCR6, resulting in the metalloprotease TACE (scissors)-dependent cleavage of EGFR proligands, inducing a second phase of EGFR-P (pY, right side). The second EGFR-P due to CCR6 signaling increases mucin production in NCI-H292 cells ERK1,2-independently (large box) but is absent in normal cells.

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In summary, we show that binding of CCL20 to its unique G protein-coupled receptor CCR6 induces mucin production in permanent human airway epithelial (NCI-H292) cells via metalloprotease TACE-dependent activation of an EGFR cascade. We also show that EGFR activation induced directly by the potent EGFR ligand TGF-α stimulates the production and secretion of CCL20 in NCI-H292 cells but not in NHBE (normal) cells, resulting in TACE-dependent EGFR reactivation via a novel CCL20/CCR6 positive feedback loop that leads to additional mucin production in NCI-H292 cells. These findings are important because the expression of EGFR and of CCL20 in airways is increased in airway diseases characterized by mucous plugging such as asthma (15, 43), COPD (16, 44), and cystic fibrosis (3, 14). Our findings suggest that components of this positive feedback loop such as CCL20/CCR6 and TACE could be novel therapeutic targets for exaggerated mucin production.

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Disclosures

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References


