Down-Regulation of E-Cadherin in Human Bronchial Epithelial Cells Leads to Epidermal Growth Factor Receptor-Dependent Th2 Cell-Promoting Activity

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Airway epithelial cells are well-known producers of thymus- and activation-regulated chemokine (TARC), a Th2 cell-attracting chemokine that may play an important role in the development of allergic airway inflammation. However, the mechanism responsible for up-regulation of TARC in allergy is still unknown. In the asthmatic airways, loss of expression of the cell-cell contact molecule E-cadherin and reduced epithelial barrier function has been observed, which may be the result of an inadequate repair response. Because E-cadherin also suppressed multiple signaling pathways, we studied whether disruption of E-cadherin-mediated cell contact may contribute to increased proallergic activity of epithelial cells, e.g., production of the chemokine TARC. We down-regulated E-cadherin in bronchial epithelial cells by small interference RNA and studied effects on electrical resistance, signaling pathways, and TARC expression (by electric cell-substrate impedance sensing, immunodetection, immunofluorescent staining, and real-time PCR). Small interference RNA silencing of E-cadherin resulted in loss of E-cadherin-mediated junctions, enhanced phosphorylation of epidermal growth factor receptor (EGFR), and the downstream targets MEK/ERK-1/2 and p38 MAPK, finally resulting in up-regulation of TARC as well as thymic stromal lymphopoietin expression. The use of specific inhibitors revealed that the effect on TARC is mediated by EGFR-dependent activation of the MAPK pathways. In contrast to TARC, expression of the Th1/Treg cell-attracting chemokine RANTES was unaffected by E-cadherin down-regulation. In summary, we show that loss of E-cadherin-mediated epithelial cell-cell contact by damaging stimuli, e.g., allergens, may result in reduced suppression of EGFR-dependent signaling pathways and subsequent induction of Th2 cell-attracting molecule TARC. Thus, disruption of intercellular epithelial contacts may specifically promote Th2 cell recruitment in allergic asthma. The Journal of Immunology, 2007, 178: 7678–7685.

Allergic immune disorders like atopic asthma are characterized by Th2-mediated inflammation. In sensitized individuals, allergen exposure induces an increase in Th2 cell infiltration and Th2 cytokine expression. Cells of the innate immune system, especially airway epithelial cells, may play an important role in driving the immune response in respiratory allergy. The airway epithelium is the first line of defense to inhaled aeroallergens but may also affect the outcome of the immune response by the production of various proinflammatory mediators, including chemokines (1–8). Chemokine receptors are expressed in different patterns on T cell subsets and may therefore be responsible for the selective recruitment of Th2 cells to the asthmatic airways (9). One of the chemokine receptors that is preferentially expressed on Th2 cells is C-C chemokine receptor CCR4 (9). Increased numbers of CCR4+ T cells have been observed in the airways upon allergen inhalation (4). Thymus- and activation-regulated chemokine (TARC)3 is one of the ligands for CCR4, and there is now emerging evidence that TARC is crucial for T cell recruitment to the asthmatic airways (2, 4, 6, 10–12). In asthma, a correlation has been observed between the expression of TARC and thymic stromal lymphopoietin (TSLP; Ref. 13), a cytokine that has been shown to induce TARC expression in dendritic cells and to activate these cells to trigger Th2 cell polarization (7). Airway epithelial cells have been shown to express both TARC and TSLP (7, 13). However, the mechanisms responsible for the exaggerated release of these proallergic factors by the airway epithelium in asthma have remained largely unclear.

The airway epithelial barrier is often disrupted in asthma patients, with evidence for shedding of ciliated cells. An inadequate repair response and inability to restore cell-cell contacts after damaging stimuli, e.g., aeroallergens, may be responsible for the damaged and activated phenotype of the bronchial epithelium. This is suggested by the increased expression of repair mediators, e.g., epidermal growth factor receptor (EGFR) and TGF-β at sites of ciliated cell detachment (14–18). Loss of barrier function may enhance access of inhaled allergens to underlying APCs. In addition, it may lead to increased proinflammatory activity of the
bronchial epithelium. Increased permeability of the bronchial epithelium to house dust mite allergen has been associated with enhanced NF-κB activity as well as increased expression of proinflammatory cytokines (19, 20, 21), which implies that reduction of epithelial cell-cell contacts may have immunomodulatory consequences.

Epithelial barrier function is provided by the formation of intercellular junctions. An important molecule in the maintenance of epithelial integrity is E-cadherin, an adhesion molecule that mediates intercellular contact through homophilic interactions (22). E-cadherin is predominantly located in adherens junctions and linked to the actin cytoskeleton via β-catenin (23). Damage to the epithelium may result in loss of E-cadherin membrane expression and intercellular contacts. It has recently been demonstrated that E-cadherin membrane expression is reduced in bronchial biopsies from asthma patients at the sites of epithelial lesions (24). This may have important consequences, given that E-cadherin is known to negatively regulate multiple signaling pathways. For instance, E-cadherin has been described to suppress MEK/ERK-1/2 signaling in squamous carcinoma cells by a yet unidentified mechanism (25). Furthermore, loss of E-cadherin expression has been demonstrated to increase activity of receptor tyrosine kinases, e.g., EGFR, although the functional implications for this activation have not been defined (26). In view of these observations, we questioned whether down-regulation of E-cadherin and disruption of the epithelial barrier may have consequences for the proallergic activity of airway epithelial cells and expression of proinflammatory chemokines (e.g., TARC). We silenced E-cadherin by small interference RNA (siRNA). We demonstrate that the down-regulation of E-cadherin expression is associated with increased EGFR downstream signaling and a subsequent increase in expression of Th2-attracting chemokine TARC.

Materials and Methods

Cell culture

The human bronchial epithelial cell line 16HBE 14o− (16HBE) was provided by Dr. D. C. Gruenert (University of California, San Francisco, CA). 16HBE cells were cultured on dishes coated with 30 μg/ml collagen and 10 μg/ml BSA in EMEM (BioWhittaker) containing 10% FCS (HyClone) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. At 80–90% confluence, cells were passaged or used for experiments. Cells were not taking corticosteroids, were free of other lung diseases, and had not been treated with antiasthma medication and were not taking corticosteroids, were free of other lung diseases, and had not been treated with antiasthma medication and were not taking corticosteroids, were free of other lung diseases, and had not been treated with antiasthma medication. When 90% confluent, cells were passaged with the use of trypsin and further cultured for use at passage 2. A culture period of at least 2–3 wk was required to obtain sufficient cells for experimentation, rendering it unlikely that any possible antiasthma medication would have interfered with our results. Cells were seeded into 24-well plates at a density of 1 × 10^4/well, transfected on the subsequent day and grown for 3 additional days. Medium was then replaced with basal medium (Clonetics), and the cells were rendered quiescent overnight.

Transfection with E-cadherin and nontargeting siRNA

E-cadherin gene expression was down-regulated in the bronchial epithelial cells by the use of siRNA. Epithelial cells were seeded in 24-well plates or Labteks at 1 × 10^5/well and transfected with siRNA with a specific target sequence for human E-cadherin or with nontargeting control oligonucleotides (Eurogentec). The target sequence for E-cadherin was GCA GAUUGUCUCACAAUUUC. Cells were transfected with 2 μg of siRNA purified and annealed synthetic oligonucleotides (Eurogentec) in a final concentration of 20 μM by the high-efficiency transfection method using Oligofectamine (Invitrogen Life Technologies). Three days after transfection, cells were subjected to preparation of total cell lysates, RNA isolation, preparation of cytosins, and immunoprecipitation (see “Immunoprecipitation”).

Immunodetection by Western blotting

Total cell lysates were obtained by resuspension of the cells in 1 × sample buffer (containing 2% SDS, 10% glycerol, 2% 2-ME, 60 mM Tris-HCl (pH 6.8) and bromophenol blue) and boiling for 5 min. Expression of E-cadherin, phosphorylation of p38, ERK, and the N73 tyrosine residue of EGFR were analyzed by Western blotting. We used total ERK as a loading control. Total levels of ERK were not affected by siRNA silencing of E-cadherin (data not shown). Samples were loaded on an SDS, 10% PAGE gel (Acrylamide-bisacrylamide, 173:1) and transferred to a polyvinylidene difluoride membrane (Millipore). Immunodetection of p38, p44/42, phosphorylated p38 and ERK, and phosphorylated EGFR was performed by standard procedures, and the detection was performed by enhanced chemiluminescence according to the manufacturer’s guidelines (ECL; Amersham). Quantification of phosphorylation levels was performed by densitometry of the films using ImageMaster (Pharmacia). Densitometry values of phosphorylated ERK, p38, and p90/p85 and p90/p85-Akt were corrected for the amount of total protein present in the samples.

Resistance measurements

The resistance of cells in culture was measured using the electric cell-substrate impedance sensing (ECIS) technique as described previously (30). Electrode arrays, relay bank, lock-in amplifier, and software for the ECIS measurements were obtained from Applied Biophysics. Each electrode array consists of eight wells, which are 1 cm in height and 0.5 cm² in area; each well contains a 250-μm-diameter gold electrode and a larger gold current electrode. The large and small electrodes are connected via the relay bank to a phase-sensitive lock-in amplifier, and AC current is applied through a 1-MΩ resistor to the electrodes at a frequency of 400–40,000 Hz. Before use, the electrodes were coated with collagen (30 μg/ml) and BSA (10 μg/ml). For resistance measurements, the electrode array was placed into an incubator, and EMEM, 10% FCS medium (400 μl/well) was added over the electrodes. After the baseline was established, the wells were inoculated with 400 μl of cell suspensions (5 × 10^4 cells/well) in triplicate. The cells were then allowed to attach and spread overnight and were transfected with siRNA as described previously; resistance was monitored by ECIS.

Immunoprecipitation

16HBE cells (2 × 10^6) were seeded in triplicate in coated 12-well plates, transfected with siRNA on the subsequent day, grown for 3 additional days, washed with ice-cold PBS containing 1 mM sodium orthovandate and 1 mM PMSF, and subsequently lysed in 500 μl of lysis buffer (50 mM Tris (pH 7.4), 10% glycerol, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 1 mM DTT, 1 μg peptatin, 1 μg aprotinin, Complete, 1 mM PMSF, and 1 mM sodium orthovandate) for 20 min on ice. Cell lysates were clarified at 10000 × g for 15 min and precleared with 30 μl of protein A-Sepharose beads (in a 50% slurry) for 1 h at 4°C. After 1 min of centrifugation at 300 × g, the supernatants were subjected to immunoprecipitation with a polyclonal Ab specific for EGFR (Santa Cruz Biotechnology) and incubated rotating at 4°C for 1 h. Protein A-Sepharose beads were added to each sample and incubated rotating for 3 h to bind to the primary
Abs. Next, immune complexes were washed three times with lysis buffer. The precipitates were subjected to SDS, 10% PAGE and immunobilized on a polyvinyliodene difluoride membrane; immunocomplexes were detected using ECL, which was performed according to the manufacturer’s guidelines. The E-cadherin Ab was purchased from Santa Cruz Biotechnology.

**Real-time RT-PCR**

cDNA was synthesized as described previously (31). Expression of TARC and TSLP mRNA was analyzed by quantitative real-time PCR using the Bio-Rad MyiQ Single-Color detection system (Bio-Rad Laboratories, Life Science Group) according to the manufacturer’s guidelines. In short, 15 μl of iQ SYBR Green Supermix, containing fluorescein to account for well-to-well variation, 0.1 μM concentration of forward and reverse primer, and 5 μl of 1/5 diluted cDNA sample were used in a total volume of 25 μl and added to a 96-well plate. The threshold cycle (Ct) of E-cadherin silenced or EGF-stimulated 16HBE cells was compared with the Ct generated by the reference sample (the 16HBE cells transfected with nontargeting siRNA or unstimulated 16HBE cells). Expression of the TARC gene was normalized to expression of the housekeeping gene β2-microglobulin (β2m), with approximately equal amplification efficiency. The ΔCt was calculated as the difference between the Ct values, determined using the equation 2−ΔCt. The following specific primers pairs were obtained from Biologio BV: β2m, 5′-CCACAGAGAGAACTGAG-3′ sense and 5′-GATGCTGCTTACATGTCTCG-3′ antisense; TARC, 5′-CACGCAGCGATGCCTGTACG-3′ sense and 5′-CTTCCATGTTGACATTGCGAG-3′ antisense; TSLP, 5′-CAGAGCCCTAAATCCTCAA-3′ sense and 5′-CTTCCCTATTGCTGTTAGT-3′ antisense. PCR conditions were: 94°C for 10 min, 40 cycles of 94°C, 30 s; 59°C, 30 s; 72°C, 30 s, and 5 min at 72°C. Due to limited cell numbers and the lower quality of cDNA, we used conventional PCR for the amplification of cDNA samples derived from the primary epithelial cell cultures. In short, 10× PCR buffer (Invitrogen Life Technologies), 50 μM forward and reverse primer, 0.25 μl of Taq polymerase, 2 mM dNTPs, and 75 μl of MgCl2 in 25 μl total volume were added to 2 μl of cDNA. The same primer pairs as real-time PCR were used. PCR conditions were: 94°C for 5 min, 20 cycles of 94°C, 30 s; 55°C, 30 s; 72°C, 30 s for detection of β2m and 35 cycles of 94°C, 30 s; 58°C, 30 s; 72°C, 30 s for detection of TARC. With these primers, the amplified products were 268 and 214 bp for β2m and TARC, respectively. After PCR, 10 μl of the reaction mixture were run on a 1.5% agarose gel containing 0.2 μg of ethidium bromide in 1× Tris acetate-EDTA. A 100-bp ladder (Pharmacia) was used as DNA marker.

**Immunofluorescent stainings**

Cytosplins were fixed in PBS-buffered paraformaldehyde (4%) for 60 min, permeabilized in PBS containing 0.2% Triton X-100 for 10 min, and blocked with 10% goat serum in PBS for 60 min. Cytosplins were washed three times with PBS and incubated for 60 min with the monoclonal anti-TARAh Ab (1/100; R&D Systems) or polyclonal anti-E-cadherin Ab (1/100; Santa Cruz Biotechnology), which were detected by incubation with Alexa green 488-labeled anti-goat IgG conjugate (1/400) or Alexa green 488-labeled anti-rabbit IgG conjugate (1/400; Molecular Probes). Cytosplins were washed with PBS for 15 min, and nuclei were stained using 4′,6′-diamindino-2-phenyldindole in Vectashield (Vector Laboratories). Fluorescence was analyzed by fluorescence microscopy (Zeiss).

**Measurement of RANTES protein levels**

RANTES levels were detected in cell-free supernatants, using an ELISA kit according to the manufacturer’s guidelines (R&D Systems).

**Statistical analysis**

We used the Wilcoxon signed rank test for paired observations to test for significance in the analyses concerning chemokine expression.

**Results**

**Down-regulation of E-cadherin by siRNA**

We hypothesized that down-regulation of E-cadherin-mediated cell-cell contact may have important consequences for the activation status of bronchial epithelial cells, leading to reduced suppression of specific signaling pathways and a subsequent increase in Th2-attracting chemokine expression. We tested our hypothesis by down-regulation of E-cadherin using siRNA oligonucleotides with an E-cadherin-specific target sequence. We used the bronchial epithelial cell line 16HBE, which highly expresses E-cadherin. Transfection of the 16HBE cells with E-cadherin-specific siRNA resulted in a marked down-regulation of total E-cadherin expression when compared with transfection with nontargeting control siRNA, an effect already observed at day 1, remaining for at least 4 days and being most pronounced at day 3 (Fig. 1A). In addition, we performed immunofluorescent staining and observed reduced membrane localization of E-cadherin protein after transfection with E-cadherin-specific siRNA (Fig. 1B). We observed no obvious changes in cell confluence or morphology.

**Changes in epithelial resistance**

To functionally test the effect of E-cadherin down-regulation, we monitored real-time changes in epithelial resistance using ECIS. With this technique, the attachment of adhesive cells on gold-film electrodes is measured by the increasing resistance as a result of blocking the current path by insulating plasma membranes. Once a confluent monolayer is formed, tight intercellular contacts restrict the current flow between neighboring cells, leading to a further increase in resistance. We measured the resistance at a low frequency (400 Hz), which has been demonstrated to be relatively insensitive to changes caused by cell attachment and is the most sensitive parameter to strictly the current flow between neighboring cells, leading to a further increase in resistance. We measured the resistance at a low frequency (400 Hz), which has been demonstrated to be relatively insensitive to changes caused by cell attachment and is the most sensitive parameter to

**Increased EGFR phosphorylation after E-cadherin silencing**

Next, we were interested to examine the consequences of down-regulation of E-cadherin-mediated cell-cell contact on epithelial activity. E-cadherin has been described to reduce ligand binding to EGFR and mobility of EGFR in the plasma membrane through physical interaction between E-cadherin and EGFR. We observed basal activation and phosphorylation of EGFR in the 16HBE cells, possibly due to autocrine release of EGFR ligands (e.g., EGF, TGF-α, human bronchial EGF, which are expressed by 16HBE cells; Ref. 32). When the cells were transfected with siRNA E-cadherin for 3 days, a marked increase in basal EGFR phosphorylation occurred when compared with cells transfected with nontargeting siRNA (Fig. 1C). This indicates that siRNA silencing of E-cadherin indeed leads to functional down-regulation of E-cadherin and reduced formation of intercellular junctions. Down-regulation of E-cadherin did not affect the high-frequency capacitance measurements, indicating that the observed changes in resistance reflect establishment of tight intercellular contacts (30). Cells transfected with siRNA targeting E-cadherin showed a far less pronounced increase in resistance during the time course of the experiment when compared with cells transfected with nontargeting siRNA (Fig. 1C). This indicates that siRNA silencing of E-cadherin indeed leads to functional down-regulation of E-cadherin and reduced formation of intercellular junctions. Down-regulation of E-cadherin did not affect the high-frequency capacitance measurements, indicating that the observed changes in resistance are specifically due to reduced intercellular contacts and not caused by alterations in the spreading and attachment phase.
which induced a clear increase in EGFR phosphorylation after 5 and 60 min. When E-cadherin was down-regulated, EGF (1 ng/ml) further enhanced EGFR phosphorylation (Fig. 2B). These results suggest that E-cadherin negatively regulates phosphorylation and activation of the EGFR. We confirmed the interaction between E-cadherin and EGFR by coimmunoprecipitation of E-cadherin with anti-EGFR Ab (Fig. 2C). As expected, less E-cadherin was precipitated when E-cadherin was down-regulated by siRNA.

**Downstream effects of EGFR activation by E-cadherin silencing**

To further investigate the consequences of increased EGFR phosphorylation by E-cadherin down-regulation, we studied effects on downstream signaling pathways that can be induced by EGFR activation, e.g., the MEK/ERK-1/2 and p38 MAPK as well as the PI3-K/Akt pathways. We observed that in 16HBE cells transfected with E-cadherin siRNA, the basal activation p38 and ERK, but not...
Akt, was increased 3 days after transfection when compared with treatment with control siRNA (Fig. 3A), in conjunction with the effects on EGFR phosphorylation. To confirm the involvement of EGFR activation, we used the EGFR tyrosine phosphorylation inhibitor tyrphostin (AG1478) during the siRNA transfection period. As expected, the presence of AG1478 blocked basal activation of EGFR and abolished the EGFR phosphorylation that was induced by the indicated values. B, 16HBE cells were transfected with nontargeting control siRNA or siRNA specific for E-cadherin and grown in the presence and absence of tyrphostin AG1478 (1 μM), U0126 (10 μM), and tyrphostin AG1478 (1 μM) were added 30 min before EGF stimulation. TARC expression was determined by real-time PCR and related to the expression of the housekeeping gene β-m. Mean relative TARC mRNA levels ± SEM are shown (n = 3), *, p < 0.05 between the indicated values. C, 16HBE cells were grown to confluence, serum starved overnight, and stimulated with or without EGF (1 ng/ml) for 6 h. SB203580 (1 μM), U0126 (10 μM), and tyrphostin AG1478 (1 μM) were added 30 min before EGF stimulation. TARC expression was determined by real-time PCR and related to the expression of the housekeeping gene β-m. Mean relative TARC mRNA levels ± SEM are shown (n = 3), *, p < 0.05 between the indicated values. D, 16HBE cells were transfected with nontargeting control siRNA or siRNA specific for E-cadherin and grown in the presence and absence of tyrphostin AG1478 (1 μM). After 3 days, RNA was isolated, cDNA was synthesized, and TARC expression was determined by real-time PCR and related to the expression of the housekeeping gene β-m. Mean relative TARC mRNA levels ± SEM are shown (n = 3), *, p < 0.05 between the indicated values. E, 16HBE cells were transfected with nontargeting control siRNA or siRNA specific for E-cadherin and grown in the presence and absence of tyrphostin AG1478 (1 μM). After 3 days, RANTES levels were measured in cell-free supernatants by ELISA. Mean relative RANTES levels ± SEM are shown (n = 3), *, p < 0.05 between the indicated values.

**FIGURE 3.** siRNA interference of E-cadherin results in EGFR-dependent activation of ERK-1/2 and p38 MAPK signaling. A, 16HBE cells were transfected with nontargeting control siRNA or siRNA specific for E-cadherin. After 3 days, total cell lysates were prepared and phospho (p-)ERK-1/2, phospho-p38, phospho-Akt, E-cadherin, and ERK were detected by Western blotting as indicated by arrows. A representative of at least three independent experiments is shown. B, 16HBE cells transfected with nontargeting control siRNA or siRNA specific for E-cadherin, grown for 3 days in the presence or absence of tyrphostin AG1478 (1 μM), serum starved overnight, and stimulated with or without EGF (1 ng/ml) for 60 min. Total cell lysates were prepared and phospho-ERK-1/2, phospho-p38, phospho-Akt, E-cadherin, and ERK were detected by Western blotting as indicated by arrows. A representative of at least three independent experiments is shown.

**FIGURE 4.** siRNA interference of E-cadherin results in EGFR signaling-dependent up-regulation of TARC and TSLP expression, but not RANTES production. A, 16HBE cells were transfected with nontargeting control siRNA or siRNA specific for E-cadherin and grown in the presence and absence of tyrphostin AG1478 (1 μM). After 3 days, RNA was isolated, cDNA was synthesized, and TSLP expression was determined by real-time PCR and related to the expression of the housekeeping gene β-m. Mean relative TARC mRNA levels ± SEM are shown (n = 6), *, p < 0.05 between the indicated values. B, 16HBE cells were transfected with nontargeting control siRNA or siRNA specific for E-cadherin. After 3 days, cytospins were prepared, and TARC was detected by immunofluorescent staining. Representatives of three independent experiments are shown. C, 16HBE cells were grown to confluence, serum starved overnight, and stimulated with or without EGF (1 ng/ml) for 6 h. SB203580 (1 μM), U0126 (10 μM), and tyrphostin AG1478 (1 μM) were added 30 min before EGF stimulation. TARC expression was determined by real-time PCR and related to the expression of the housekeeping gene β-m. Mean relative TARC mRNA levels ± SEM are shown (n = 3), *, p < 0.05 between the indicated values. D, 16HBE cells were transfected with nontargeting control siRNA or siRNA specific for E-cadherin and grown in the presence and absence of tyrphostin AG1478 (1 μM). After 3 days, RNA was isolated, cDNA was synthesized, and TARC expression was determined by real-time PCR and related to the expression of the housekeeping gene β-m. Mean relative TARC mRNA levels ± SEM are shown (n = 3), *, p < 0.05 between the indicated values. E, 16HBE cells were transfected with nontargeting control siRNA or siRNA specific for E-cadherin and grown in the presence and absence of tyrphostin AG1478 (1 μM). After 3 days, RANTES levels were measured in cell-free supernatants by ELISA. Mean relative RANTES levels ± SEM are shown (n = 3), *, p < 0.05 between the indicated values.
activation is able to induce TARC expression. Indeed, a significant
effect on TARC expression appears to be dependent on EGFR ac-

When AG1478 was present (Fig. 4). Thus, the up-regulatory ef-
fication could no longer up-regulate TARC mRNA expression

To examine the effect of activation of signaling transduction by E-cadherin silencing on expression of Th2-attracting chemokine TARC, we studied mRNA and protein expression of TARC. Transfection with E-cadherin siRNA resulted in increased expression of TARC mRNA expression over cells transfected with nontargeting siRNA (Fig. 4A). TARC protein levels in supernatant were often below the detection level, but by Western blotting as indicated by arrows. Representative blots are shown. B. Primary epithelial cells were transfected with nontargeting control siRNA or siRNA specific for E-cadherin. After 3 days, total cell lysates were prepared and E-cadherin, phospho-EGFR, phospho-ERK-1/2, phospho-p38, and ERK were detected by Western blotting as indicated by arrows. Representative blots are shown. A. Primary epithelial cells derived from bronchial brushings in asthma patients

Effects in primary bronchial asthma epithelium

To test the relevance of our finding for human asthmatic airway epithelium, we also used primary epithelial cultures obtained from asthma patients and transfected them with E-cadherin siRNA. In accordance to our findings in the 16HBE cell line, silencing of E-cadherin in these cells resulted in increased EGFR phosphorylation and activation of the ERK-1/2 and p38 pathways as well (Fig. 5A). In addition, transfection with E-cadherin-targeting siRNA resulted in an increase in TARC mRNA expression over cells transfected with nontargeting siRNA (Fig. 5B).

Discussion

Respiratory allergy is characterized by Th2-mediated airway inflammation. TARC is a Th2-attracting chemokine that may play a dominant role in the attraction of Th2 cells to the sites of allergic inflammation, i.e., the asthmatic airways. Airway epithelial cells are known to express TARC, yet the mechanisms responsible for the increased expression of this chemokine in allergen-induced asthma have not been identified (4, 6). In the asthmatic airways, the epithelium displays a damaged phenotype, with loss of ciliated cells, reduced membrane expression of the cell-cell contact molecule E-cadherin and increased expression of proinflammatory mediators and repair markers, including EGFR (14). In addition to mediating cell-cell contact, the homophilic adhesion molecule E-cadherin is known to negatively regulate multiple signaling pathways. Reduced control of relevant signaling molecules may have important consequences for the transcriptional regulation of specific chemokines. For instance, the production of TARC has been described to involve MAPK-regulated transcription factors, e.g., NF-κB and AP-1 (33).

In the current study, we demonstrate for the first time that siRNA silencing of E-cadherin results in increased expression of TARC, which is likely mediated by the activation of EGFR-dependent signaling. We observed that down-regulation of E-cadherin leads to increased phosphorylation of EGFR. EGFR, a receptor tyrosine kinase with intrinsic tyrosine kinase activity, can induce phosphorylation of a variety of intracellular substrates, of which the MAPK pathway is the most well-known component. Accordingly, we observed increased activation of the EGFR downstream MEK/ERK-1/2 and p38 MAPK pathways. These findings suggest that E-cadherin expression inhibits EGFR activation and
limits its downstream signaling. It has previously been demonstrated that E-cadherin and EGFR colocalize, which impairs mobility of EGFR in the plasma membrane and reduces ligand binding efficiency to EGFR (26). These effects appeared dependent on the adhesive extracellular region and not on the β-catenin-binding site of E-cadherin. The complex formation between E-cadherin and EGFR may restrict switching of the EGFR to an active configuration and result in density-dependent inhibition of cellular activity. Our results are in line with these findings, showing increased ligand-dependent phosphorylation of EGFR when E-cadherin-mediated cell-cell contact and interaction between EGFR and E-cadherin are reduced. Consequently, we observed an increase in EGFR-dependent expression of Th2 cell-attracting chemokine TARC as a result of enhanced phosphorylation of the ERK and p38 MAPK pathways. In addition to the MAPK-regulated transcription factors AP-1 and NF-κB, the promoter of TARC contains putative binding sites for GATA, STAT, and AML-1 (33). However, none of these is known to be regulated by E-cadherin. Instead, E-cadherin can regulate gene transcription through negative control of the wingless type (Wnt)/β-catenin pathway. No binding site in the TARC promoter has been described for the β-catenin-associated transcription factors T cell factor and lymphoid enhancer factor-1, and currently it is unknown whether activation of the Wnt/β-catenin pathway by E-cadherin down-regulation could contribute to the up-regulatory effect on TARC.

In contrast to TARC, production of the Th1/Treg cell-attracting chemokine RANTES was not enhanced by E-cadherin silencing, whereas EGFR inhibitor AG1478 rather up-regulated than inhibited RANTES. This suggests a selective effect of EGFR activation on Th2-promoting cytokines. Indeed, the transcriptional activation of Th1/Treg and Th2 cell-attracting chemokines is differentially regulated. In contrast to the effects on TARC expression, inhibition of EGFR has been described to increase RANTES expression in keratinocytes, which was likely mediated by inhibition of MEK/ERK-1/2 activation (34). Furthermore, p38 MAPK inhibitor SB202190 failed to inhibit RANTES and MIP-1 expression in EBV-immortalized B cells, whereas it markedly reduced expression of TARC and macrophage-derived chemokines another CCR4 ligand (33). These effects may be related to differences in the potential transcription elements of the promoter region. In line with these findings, we have observed differential effects of allergen exposure on TARC and RANTES levels in sputum of asthma patients. Although TARC release was significantly increased upon allergen inhalation, RANTES levels were unaltered and even tended to decrease (I. H. Heijink, A. J. M. van Oosterhout, D. S. Postma, E. Vellenga, unpublished observation). Many aeroallergens, including house dust mite, cockroaches, fungi and pollen contain protease activity (35–39), which can disrupt E-cadherin-mediated epithelial cell-cell contacts (40–42). Accordingly, we have observed that house dust mite reduces membrane expression of E-cadherin in bronchial epithelial cells (I. H. Heijink, A. J. M. van Oosterhout, D. S. Postma, E. Vellenga, unpublished observation). In this way, epithelial damage and down-regulation of E-cadherin by protease-containing aeroallergens might selectively induce expression of Th2 cell-promoting cytokines by a mechanism involving EGFR phosphorylation and activation of specific signal transduction pathways. Interestingly, down-regulation of E-cadherin also resulted in an increase in the expression of TSLP, a cytokine that is thought to play an important role in the direction of allergic responses and that has been associated with increased TARC expression in the asthmatic airways (13). We were able to confirm our findings of E-cadherin down-regulation on EGFR-dependent MAPK activation and TARC expression in bronchial epithelial cells derived from asthma patients. Furthermore, we have recently demonstrated that stimulation with dust mite extract results in the up-regulation of TARC mRNA expression in primary epithelial cultures from asthma patients and that dust mite-induced TARC expression in bronchial epithelial cells is mediated in an EGFR and MAPK-dependent manner (43). These findings illustrate the functional downstream relevance of E-cadherin down-regulation in human bronchial epithelium.

Together, our findings may have important implications for pathological disorders associated with reduced E-cadherin expression. We demonstrate that loss of E-cadherin-mediated contact between airway epithelial cells results in reduced suppression of signaling pathways involved in the expression of TARC, as well as up-regulation of TSLP expression. This effect is likely mediated by the increased phosphorylation of EGFR as a result of reduced interaction between E-cadherin and EGFR. In this way, disruption of E-cadherin-mediated junctions between epithelial cells may result in specific Th2 cell recruitment and promote Th2-type allergic inflammation.

Disclosures

The authors have no financial conflict of interest.

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