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Down-Regulation of E-Cadherin in Human Bronchial Epithelial Cells Leads to Epidermal Growth Factor Receptor-Dependent Th2 Cell-Promoting Activity¹

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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

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³ Abbreviations used in this paper: TARC, thymus- and activation-regulated chemokine; ECIS, electric cell-substrate impedance sensing; EGF, epidermal growth factor; EGFR, EGF receptor; TSLP, thymic stromal lymphopoietin; siRNA, small interference RNA; 16HBE, human bronchial epithelial cell line 16HBE 14o–; BEGM, bronchial epithelial growth medium; β_2 m, β_2 -microglobulin; rh, recombinant human.

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bronchial epithelium. Increased permeability of the bronchial epithelium to house dust mite allergen has been associated with enhanced NF- κ B 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 μ g/ml collagen and 10 μ g/ml BSA in EMEM (BioWhittaker) containing 10% FCS (HyClone) supplemented with 100 U/ml penicillin and 100 μ g/ml streptomycin. At 80–90% confluence, cells were passaged or used for experiments. Cells were seeded into 24-well plates or Labteks (Nunc and Life Sciences) in duplicates at a density of 1 \times 10⁵/well, transfected on the subsequent day, and grown for 3 additional days. For preparation of whole-cell lysates or RNA extraction (TRIzol method; Invitrogen Life Technologies), medium was replaced with EMEM containing 0.5% FCS, and cells were rendered quiescent overnight and stimulated with or without EGF (1 ng/ml) for 5–60 min or 6 h. AG1478 (1 μ M; Sigma-Aldrich), a specific inhibitor of EGFR tyrosine kinase activity, was added on each consecutive day after transfection. Specific p38 MAPK inhibitor SB20350 (1 μ M; SmithKline Beecham Pharmaceuticals) and specific MEK/ERK-1/2 inhibitor U0126 (10 μ M; Promega) were added 30 min before stimulation with EGF.

Bronchial epithelial cells were obtained by bronchial brushing in three asthmatic subjects (40–73 years) with mild asthma and atopy for house dust mite, according to standard guidelines (27). Patients were nonsmokers, were not taking corticosteroids, were free of other lung diseases, and had a baseline AMP PC₂₀ (the provocative concentration of AMP causing a 20% fall in forced expiratory volume at 1 s) of <16 mg/ml. The use of any other antiasthma medication was not excluded. The Medical Ethics Committee of the University Hospital of Groningen (Groningen, The Netherlands) approved the study, and signed informed consent was given to participate. Primary cultures of bronchial epithelial cells were established as described previously (28). Cell were cultured on flasks coated with 30 μ g/ml collagen, 30 μ g/ml fibronectin, and 10 μ g/ml BSA in 2.5 ml of serum-free hormonally supplemented bronchial epithelium growth medium (BEGM; Clonetics) containing 100 U/ml penicillin and 100 μ g/ml streptomycin. BEGM has been used before to specifically grow primary epithelial cells (29). We have previously observed that primary epithelial cells from bronchial brushings grown in BEGM all stain positive for pan-cytokeratin (clone AE1/AE3; DAKO) after two passages (data not shown).

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 \times 10⁵/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 \times 10⁵/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 GAAUUGCUCACAUUC. Cells were transfected with 2 μ g of siRNA purified and annealed synthetic oligonucleotides (Eurogentec) in a final concentration of 20 μ M 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 cytopspins, and immunoprecipitation (see “Immunoprecipitation”).

Immunodetection by Western blotting

Total cell lysates were obtained by resuspension of the cells in 1 \times 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 and phosphorylation of p38, ERK, and the 1173 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, 17.3:1) and transferred to a polyvinylidene difluoride membrane (Millipore). Immunodetection of phospho-p38, phospho-Akt, phospho-ERK (New England Biolabs), phospho-EGFR (New England Biolabs), E-cadherin (Santa Cruz Biotechnology), and pan-ERK (Santa Cruz Biotechnology) 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 phospho-ERK, phospho-p38, and phospho-Akt were corrected for the amount of ERK 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- μ m-diameter gold electrode and a larger gold counterelectrode. 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-M Ω resistor to the electrodes at a frequency of 400–40,000 Hz. Before use, the electrodes were coated with collagen (30 μ g/ml) and BSA (10 μ g/ml). For resistance measurements, the electrode array was placed in an incubator, and EMEM, 10% FCS medium (400 μ l/well) was added over the electrodes. After the baseline was established, the wells were inoculated with 400 μ l of cell suspensions (5 \times 10⁴ 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 \times 10⁵) 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 orthovanadate and 1 mM PMSF, and subsequently lysed in 500 μ l 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 μ M pepstatin, 1 μ g/ml aprotinin, Complete, 1 mM PMSF, and 1 mM sodium orthovanadate) for 20 min on ice. Cell lysates were clarified at 10000 \times g for 15 min and precleared with 30 μ l of protein A-Sepharose beads (in a 50% slurry) for 1 h at 4°C. After 1 min of centrifugation at 300 \times g, the cell lysates 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 immunoblotted on a polyvinylidene 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 μ l of iQ SYBR Green Supermix, containing fluorescein to account for well-to-well variation, 0.1 μ M concentration of forward and reverse primer, and 5 μ l of 1/5 diluted cDNA sample were used in a total volume of 25 μ l and added to a 96-well plate. The threshold cycle (C_t) of E-cadherin silenced or EGF-stimulated 16HBE cells was compared with the C_t 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 β_2 -microglobulin (β_2m), with approximately equal amplification efficiency. The ΔC_t was calculated as the difference between the C_t values, determined using the equation $2^{-\Delta C_t}$. The following specific primers pairs were obtained from Biolegio BV: β_2m , 5'-CCAGCAGAGAATGGAAAGTC-3' sense and 5'-GATGCTGCTTACATGTCTCG-3' antisense; TARC, 5'-CACGCAGCTC GAGGGACCAAGTG-3' sense and 5'-TCAAGACCTCTCAAAGGCTTT GCAGG-3' antisense; TSLP, 5'-CAGAGCCCTAACCTTCAACTC-3' sense and 5'-CTTCTTCATTGCCCTGAGTAG-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 \times PCR buffer (Invitrogen Life Technologies), 50 μ M forward and reverse primer, 0.25 μ l of *Taq* polymerase, 2 mM dNTPs, and 75 μ l of MgCl₂ in 25 μ l total volume were added to 2 μ l 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 β_2m 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 β_2m and TARC, respectively. After PCR, 10 μ l of the reaction mixture were run on a 1.5% agarose gel containing 0.2 μ g of ethidium bromide in 1 \times Tris acetate-EDTA. A 100-bp ladder (Pharmacia) was used as DNA marker.

Immunofluorescent stainings

Cytospins 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. Cytospins were washed three times with PBS and incubated for 60 min with the monoclonal anti-TARC 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). Cytospins were washed with PBS for 15 min, and nuclei were stained using 4',6'-diamidino-2-phenylindole 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 for monitoring formation of intercellular junctions (30). Additionally, we measured high-frequency capacitance (40 kHz), which has been shown to be the most sensitive parameter to measure cell attachment. 16HBE cells were seeded on the ECIS electrode, transfected with siRNA on the subsequent day, and monitored by ECIS for 72 h. Resistance of the cells increased in time with the most pronounced effect from 20 to 72 h after transfection (Fig. 1B). Capacitance (which is inversely related to resistance) decreased during the course of the experiment, with the most pronounced effect within the first 20–30 h, indicating that most of the cell attachment occurred in this period (Fig. 1C). Accordingly, it has been described that spreading and attachment occurs in the first phase after seeding of the cells, whereas later 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.

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 non-targeting siRNA (Fig. 2A). When cells were transfected with siRNA for 9 days, E-cadherin levels returned to normal and accordingly, the increase in EGFR phosphorylation was no longer observed (data not shown). To test whether the increase in EGFR phosphorylation was due to increased sensitivity to EGFR, we stimulated cells with recombinant human (rh) EGF (1 ng/ml),

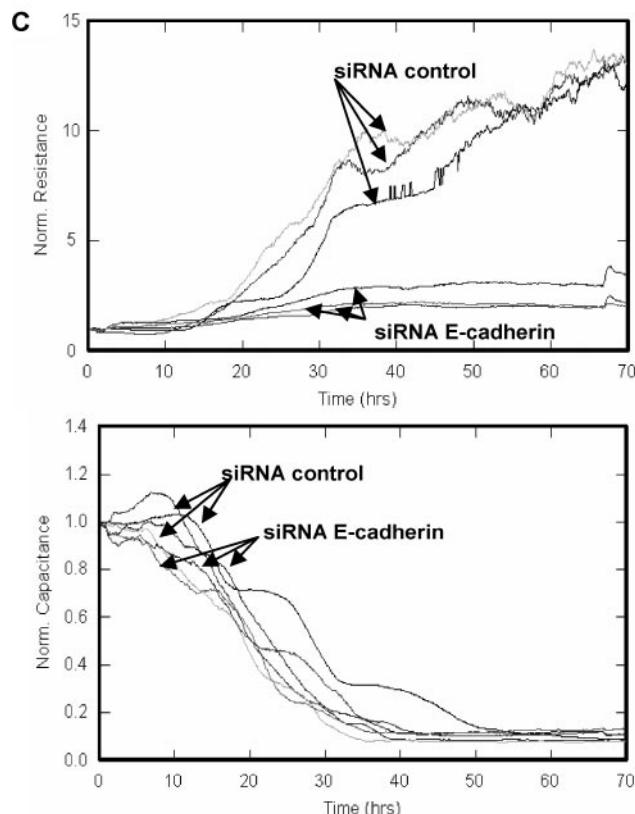
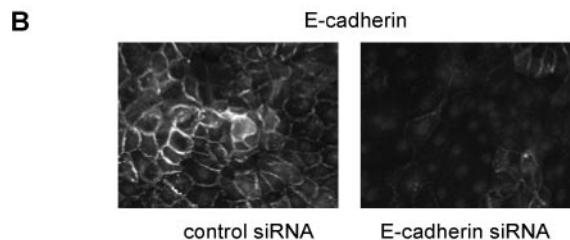
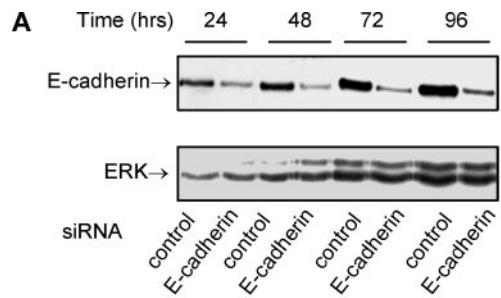


FIGURE 1. siRNA down-regulates E-cadherin expression and E-cadherin-mediated intercellular contact. *A*, 16HBE cells were transfected with nontargeting control siRNA or siRNA specific for E-cadherin. Total cell lysates were prepared on 1, 2, 3, and 4 days after transfection, and E-cadherin was detected by Western blotting as indicated by the arrow. The ERK signal in the lower panel shows equal loading between the presence of the different siRNA oligonucleotides. *B*, 16HBE cells were grown on Labteks and transfected with nontargeting control siRNA or siRNA specific for E-cadherin. After 3 days, E-cadherin was detected by immunofluorescent staining. Representatives are shown. *C*, 16HBE cells were seeded in triplicates into ECIS arrays and transfected with either the nontargeting control siRNA or specific E-cadherin siRNA oligonucleotides on the following day. Resistance was measured immediately after transfection in a time course of 72 h at 400 Hz by ECIS. Results are representative of three independent experiments. Norm., Normalized.

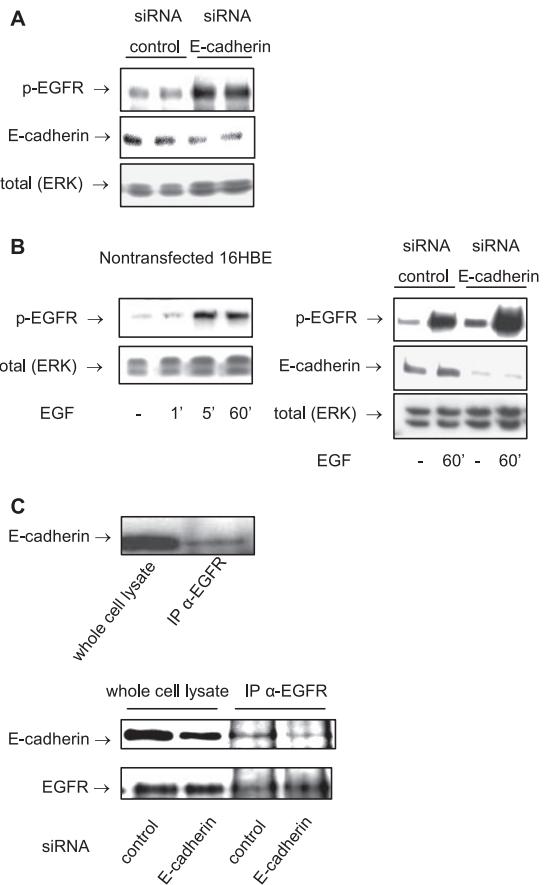


FIGURE 2. siRNA interference of E-cadherin results in increased phosphorylation of EGFR and reduced interaction between EGFR and E-cadherin. *A*, 16HBE cells were transfected with nontargeting control siRNA or siRNA specific for E-cadherin. After 3 days, total cell lysates were prepared and phospho (p-)EGFR, E-cadherin, and ERK were detected by Western blotting as indicated by arrows. A representative of six independent experiments is shown. *B*, Nontransfected 16HBE cells or 16HBE cells transfected with siRNA (3 days) were serum starved overnight and stimulated with or without EGF (1 ng/ml) for 1, 5, or 60 min. Total cell lysates were prepared, and phospho-EGFR, E-cadherin, and ERK were detected by Western blotting as indicated by arrows. Representative blots of three independent experiments are shown. *C*, 16HBE cells were transfected with nontargeting control siRNA or siRNA specific for E-cadherin. After 3 days, whole-cell lysates were prepared and subjected to immunoprecipitation (IP) with anti-EGFR Ab. Immunocomplexes were subjected to SDS, 10% PAGE and stained with anti-E-cadherin as well as anti-EGFR for control. A representative of three independent experiments is shown.

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. 2*B*). 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. 2*C*). 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

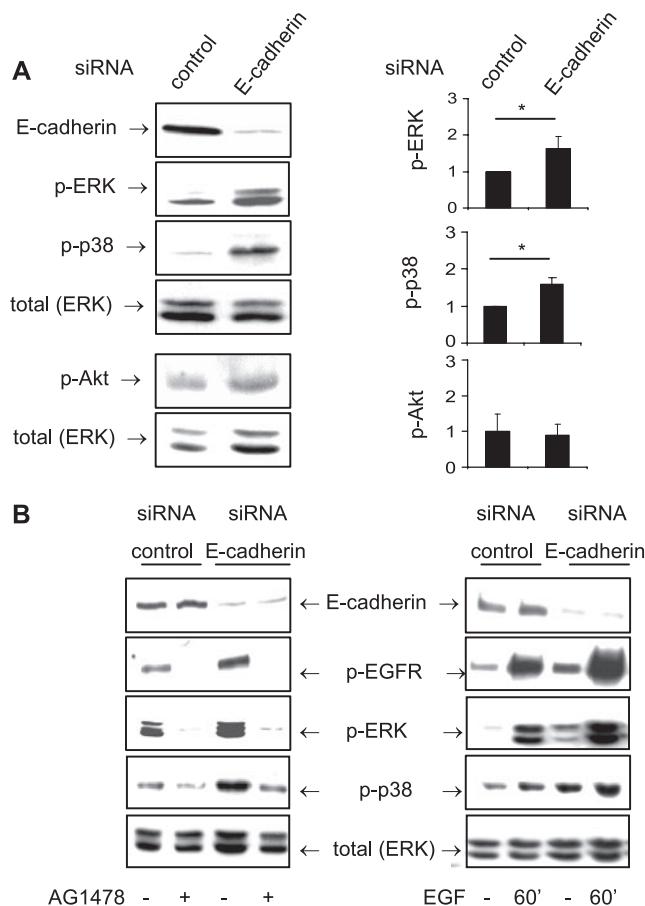


FIGURE 3. siRNA interference of E-cadherin results in EGFR-dependent activation of ERK-1/2 and p38 MAPK signaling. *A*, 16HBE cells were transfected with nontargeting control siRNA or siRNA specific for E-cadherin. After 3 days, total cell lysates were prepared and phosphor (p)-ERK-1/2, phospho-p38, 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. The corresponding diagrams show the mean (\pm SEM) relative phosphorylation levels after normalization for total ERK ($n \geq 3$). *, $p < 0.05$. *B*, 16HBE cells transfected with nontargeting control siRNA or siRNA specific for E-cadherin, grown for 3 days in the presence or absence of tyrophostin AG1478 (1 μ M), 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.

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 tyrophostin (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 E-cadherin down-regulation (Fig. 3B). Furthermore, both basal ERK activation and ERK activation induced by E-cadherin silencing were blocked by AG1478, indicating that ERK-1/2 activation in the 16HBE cells is completely dependent on EGFR. Similarly, the increase in p38 phosphorylation that was induced by E-cadherin silencing was abolished in presence of AG1478, indicating that the increase in p38 phosphorylation induced by E-cadherin silencing (but not basal p38 activity) is dependent on EGFR phosphorylation. Accordingly, we observed that rhEGF induced phos-

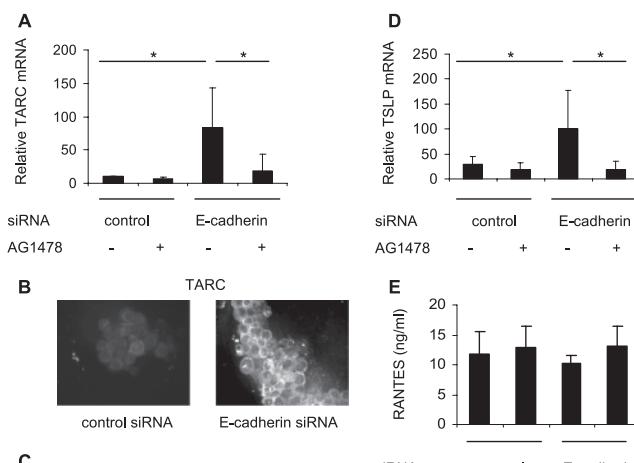


FIGURE 4. siRNA interference of E-cadherin results in EGFR signaling-dependent up-regulation of TARC and TSLP expression, but not RANTES production. *A*, 16HBE cells were transfected with nontargeting control siRNA or siRNA specific for E-cadherin and grown in the presence and absence of tyrophostin AG1478 (1 μ M). 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_2 m. Mean relative TARC mRNA levels \pm 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, cytoplasts 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 μ M), U0126 (10 μ M), and tyrophostin AG1478 (1 μ M) 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_2 m. Mean relative TARC mRNA levels \pm 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 tyrophostin AG1478 (1 μ M). 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_2 m. Mean relative TSLP mRNA levels \pm SEM are shown ($n = 6$). *, $p < 0.05$ 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 tyrophostin AG1478 (1 μ M). After 3 days, RANTES levels were measured in cell-free supernatants by ELISA. Mean relative RANTES levels \pm SEM are shown ($n = 3$). *, $p < 0.05$ between the indicated values.

phorylation of p38 and ERK, which was enhanced when E-cadherin was down-regulated (Fig. 3B). Together, these results suggest that E-cadherin negatively regulates ERK-1/2 and p38 MAPK activation by the inhibition of EGFR phosphorylation. ERK-1/2 and p38 are well-known inducers of multiple transcription factors of which binding sites are present in the TARC promoter, e.g., NF- κ B, AP-1, and GATA (33). Therefore, the ERK-1/2 and p38 pathways may be involved in the regulation of TARC.

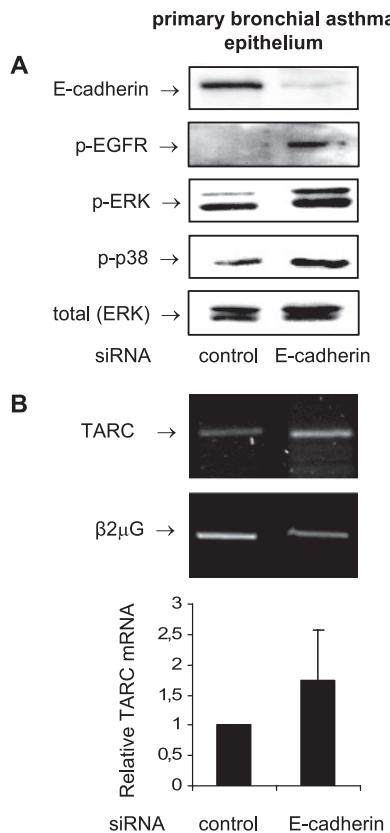


FIGURE 5. siRNA interference of E-cadherin results in EGFR phosphorylation, activation of ERK-1/2 and p38 MAPK, and TARC mRNA expression in bronchial epithelial cell cultures from asthma patients. *A*, Primary epithelial cells derived from bronchial brushings in asthma patients ($n = 3$) were transfected 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. *B*, Primary epithelial cells were transfected with nontargeting control siRNA or siRNA specific for E-cadherin. After 3 days, RNA was isolated, cDNA was synthesized, and TARC expression was determined by RT-PCR. The upper panel shows the TARC signal after 35 cycles and the lower panel shows the $\beta_2\mu G$ signal after 20 cycles (representative of three experiments). Mean relative TARC mRNA levels \pm SEM are shown ($n = 3$).

Down-regulation of E-cadherin results in EGFR-dependent up-regulation of TARC and TSLP

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 a marked up-regulation (8.2 ± 6.0 -fold) 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 immunofluorescent staining we confirmed that this effect could also be observed at protein level (Fig. 4B). To test the involvement of activation of EGFR-dependent signaling in this effect, we used tyrphostin AG1478 throughout the siRNA treatment period. AG1478 reduced TARC mRNA expression, and E-cadherin silencing could no longer up-regulate TARC mRNA expression when AG1478 was present (Fig. 4A). Thus, the up-regulatory effect on TARC expression appears to be dependent on EGFR activity. Next, we tested the effect of rhEGF to confirm that EGFR activation is able to induce TARC expression. Indeed, a significant

increase in TARC mRNA expression was observed upon EGF stimulation (6 h). The EGF-induced TARC expression could be inhibited by selective MEK/ERK-1/2 inhibitor U0126 (10 μ M) and p38 MAPK inhibitor SB203580 (1 μ M), supporting the role of these pathways by in the E-cadherin down-regulation induced expression of TARC (Fig. 4C).

We were interested to test whether the E-cadherin effects are specific for Th2-promoting cytokines and therefore we also studied regulation of TSLP and Th1/Treg cell-attracting chemokine RANTES. Similar to TARC, transfection with E-cadherin-targeting siRNA resulted in an increase (5.32 ± 4.2 -fold) in TSLP mRNA expression over cells transfected with nontargeting siRNA (Fig. 4D). This could again be inhibited by AG1478. In contrast, these effects could not be observed on RANTES protein secretion. High levels of RANTES were secreted by 16HBE cells. When E-cadherin gene expression was silenced, the RANTES secretion levels were not altered (Fig. 4E). Furthermore, AG1478 did not inhibit but instead tended to increase RANTES production. Thus, E-cadherin appears to differentially regulate expression of Th1- and Th2-promoting cytokines by the negative regulation of specific signal transduction pathways.

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- κ B 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 conformation 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- κ B, 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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