Defective epithelial barrier function in asthma

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Background: Asthma is a complex disease involving gene and environment interactions. Although atopy is a strong predisposing risk factor for asthma, local tissue susceptibilities are required for disease expression. The bronchial epithelium forms the interface with the external environment and is pivotal in controlling tissue homeostasis through provision of a physical barrier controlled by tight junction (TJ) complexes.

Objectives: To explain the link between environment exposures and airway vulnerability, we hypothesized that epithelial TJs are abnormal in asthma, leading to increased susceptibility to environmental agents.

Methods: Localization of TJs in bronchial biopsies and differentiated epithelial cultures was assessed by electron microscopy or immunostaining. Baseline permeability and the effect of cigarette smoke and growth factor were assessed by measurement of transepithelial electrical resistance and passage of fluorescently labeled dextrans.

Results: By using immunostaining, we found that bronchial biopsies from asthmatic subjects displayed patchy disruption of TJs. In differentiated bronchial epithelial cultures, TJ formation and transepithelial electrical resistance were significantly lower (P < .05) in cultures from asthmatic donors (n = 43) than from normal controls (n = 40) and inversely correlated with macromolecular permeability. Cultures from asthmatic donors were also more sensitive to disruption by cigarette smoke extract. Epidermal growth factor enhanced basal TJ formation in cultures from asthmatic subjects (P < .01) and protected against cigarette smoke–induced barrier disruption (P < .01).

Conclusions: Our results show that the bronchial epithelial barrier in asthma is compromised. This defect may facilitate the passage of allergens and other agents into the airway tissue, leading to immune activation and may thus contribute to the end organ expression of asthma. (J Allergy Clin Immunol 2011;128:549-56.)

Key words: Tight junction, epidermal growth factor, cigarette smoke, asthma, epithelial barrier

Individuals with asthma often have a genetic predisposition, but interactions with environmental factors are critical for disease expression.1 As a barrier to the external environment, the bronchial epithelium is in a key position to translate these gene-environment interactions in asthma. The physical barrier function of epithelia is dependent on cellular integrity and the coordinate expression and interaction of proteins in cell-cell junctional complexes, especially tight junctions (TJs).2,6 TJs are situated at the subapical regions of polarized epithelial cells where they selectively regulate the paracellular passage of molecules and ions7 and restrict the lateral movement of molecules in the cell membrane.8,9 They comprise the integral membrane proteins: claudins, occludin, tricellulins, and junctional adhesion molecules, cytoplasmic cytoskeletal linker proteins zonula occludens (ZO)-1, ZO-2, and ZO-3, and associated signaling molecules and cell cycle regulators that control proliferation and differentiation.2,3,6

Many diseases are exacerbated by, or may develop as a consequence of, loss of epithelial barrier function. Filaggrin, a key protein involved in epidermal differentiation and maintaining skin barrier function, is affected by loss-of-function mutations that are strong predisposing factors for atopic dermatitis (AD).10 More recent studies have also implicated the claudin-1 gene (CLDNI) as a novel susceptibility gene for AD, where it may play a role in barrier dysfunction and T↓2 polarization.11 Other diseases, such as food allergy, appear to be driven by changes in gut epithelial permeability due to environmental stress.12 Although in vitro exposure of airway epithelial cells to the dust mite allergen Dermatophagoides pteronyssinus13 or inhalation exposure of ovalbumin in sensitized mice14,15 both cause epithelial TJ disruption, there are limited studies of TJs in asthma in humans. It has been reported that expression of ZO-1 and E-cadherin is lower in subjects with asthma,16 suggesting a broad defect in adhesion mechanisms. However, the functional consequences of these differences have not been explored. Here, we compared TJ protein expression in bronchial biopsies from healthy and asthmatic subjects and used differentiated epithelial cultures grown from bronchial brushings to evaluate barrier function ex vivo. As smoking is common in asthma and associated with poor symptom control,17
we applied cigarette smoke extract (CSE) to these models to investigate whether defective barrier function might contribute to epithelial susceptibility in asthma and we assessed the ability of epidermal growth factor (EGF) to improve epithelial integrity as proof-of-concept for a novel therapeutic approach. Parts of this work have been presented in abstract form.18-20

METHODS
This article has supplementary methodologic data accessible at this article’s Online Repository at www.jacionline.org.

Clinical characterization of subjects and bronchoscopy
Following ethical approval and written informed consent, subjects were clinically characterized (see Table E1 in this article’s Online Repository at www.jacionline.org) and then underwent fiberoptic bronchoscopy under local anesthesia21 for collection of bronchial biopsies and brushings. All volunteers were nonsmokers and had been free from respiratory tract infections for at least 4 weeks prior to the study.

Immunohistochemistry
Bronchial biopsies were immunostained with mAbs against ZO-1 and occludin (Zymed Laboratories, Inc, San Francisco, Calif) using standard protocols.22 In all cases, analyses were restricted to areas of well-orientated and structurally normal epithelium.

Differentiation of primary bronchial epithelial cell cultures and exposure studies
Bronchial epithelial cells (BECs) were grown from bronchial brushings23 and differentiated at an air-liquid interface (ALI) for 21 days, following a modification of the method of Gray et al.24 Before challenge with freshly prepared CSE, cultures were starved for 24 hours; where indicated, cultures were treated apically with 0.8 nM of EGF prior to and/or after CSE treatment.

Immunocytochemistry
Cultures were trypsinized and cytospins immunostained with antibodies against β-tubulin (Sigma, Poole, United Kingdom), MUC5AC, Ki67, CK5, and CK14 (Abcam, Cambridge, United Kingdom) according to standard protocols. Positively stained cells were quantified using a Leica DMIRB microscope and Qwin software (Leica Camera AG, Solms, Germany).

Whole mount confocal microscopy of ALI cultures and bronchial biopsies
Cultures were fixed in methanol and permeabilized in PBS/0.1% Triton X-100. Whole mount bronchial biopsies were fixed overnight in 4% paraformaldehyde and cleared in dimethyl sulfoxide before permeabilization. Antibodies against ZO-1, occludin, MUC5AC, or β-tubulin were labeled with Alexa Fluor 488, 555, or 647 (Invitrogen, Paisley, United Kingdom). Nuclei were counterstained with Sytox orange or Sytox blue (Invitrogen).

FITC-dextran flux assay
Two milligrams per milliliter of FITC-dextran 4 kDa or 20 kDa (Sigma) was applied to the apical surface of cultures and incubated for 24 hours at 37°C. The dextran passage into the basolateral medium was measured using a Fluoroscan Ascent FL2.5 reader (Thermo Fisher, Loughborough, United Kingdom).

RT-qPCR
Samples were processed into Trizol reagent or RNeasy kit (Qiagen, Crawley, United Kingdom); total RNA was DNase treated (Ambion, Huntingdon, United Kingdom) before cDNA synthesis. Probe and primers for ZO-1, occludin, and the housekeeping genes Ubiquitin C (UBC) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (see Table E2 in this article’s Online Repository at www.jacionline.org) were from PrimerDesign (Southampton, United Kingdom). Data were analyzed using the ΔΔCt method.

Western blotting
Lysates were subjected to SDS-PAGE and Western blotting using anti-ZO-1 or anti-occludin mAbs (Invitrogen, Paisley, United Kingdom), using β-actin as a loading control.

Statistics
For normally distributed data, mean ± standard error of the mean are used and differences between groups analyzed using Student’s t test. Where not normally distributed, data are summarized using the median and interquartile range and were evaluated using nonparametric Wilcoxon rank sum or Mann-Whitney U tests. All the data were analyzed using Prism (GraphPad Software, La Jolla, Calif).

RESULTS
TJs are disrupted in airways of asthmatic subjects
Immunohistochemical analysis of occludin, which plays an indispensable role in the regulation of barrier tightness, and ZO-1, which plays a critical role in the establishment of the beltlike TJs,6,7 was undertaken on bronchial biopsies obtained from normal subjects (n = 7) and asthmatic subjects (n = 19) (Table E1). In well-orientated sections from normal subjects where a clear brush border was evident, occludin and ZO-1 were localized close to the apical surface of the epithelium, forming discrete focal areas of staining between individual columnar cells (Fig 1, A). In biopsies from asthmatic subjects, TJ staining was also evident but this was patchy and irregular with areas of epithelium lacking intercellular staining while other areas appeared normal. In some instances, TJ proteins were missing or reduced in size in areas where a clear brush border was evident; however, in other cases, they were absent from areas of poorly differentiated epithelium. We also observed an increase in cytoplasmic and perinuclear staining in some biopsies. Disruption of

Transwell cultures were fixed and embedded and sections stained with uranyl acetate followed by Reynolds lead stain and viewed on a Hitachi H7000 transmission electron microscope.
TJ staining was observed in biopsies from asthmatic subjects with mild, moderate, and severe asthma, irrespective of treatment or disease severity (Fig 1, A). Semiquantitative scoring of TJs in biopsies containing quantifiable epithelium revealed that those from normal subjects tended to score higher for the presence of TJs (median score 2+) whereas only 3 of 13 interpretable biopsies from asthmatic subjects scored ≥2+. However, TJ proteins form a circumferential band around each epithelial cell to enable formation of an integrated epithelial barrier, we were concerned that quantitation of TJ proteins based on point counting in the biopsies would be compromised in any sections that were not sufficiently well orientated. Therefore, we analyzed TJ protein distribution across the epithelial surface of whole mounts of bronchial biopsies by using confocal microscopy. As suggested by the immunohistochemical analysis, biopsies from normal subjects displayed strong immunofluorescent staining that clearly demarcated each intercellular border, whereas biopsies from asthmatic subjects exhibited a more patchy and irregular pattern of staining (Fig 1, B; see Table E3 in this article’s Online Repository at www.jacionline.org). Although TJ proteins were reduced in asthma, expression levels of ZO-1 and occludin mRNA were comparable in bronchial brushings from normal or asthmatic subjects (see Fig E1 in this article’s Online Repository at www.jacionline.org), suggesting that the effect on TJ proteins involves a posttranscriptional mechanism.

**TJs are disrupted in epithelial cultures from asthmatic subjects differentiated in vitro**

To assess whether the disrupted TJs seen in asthma may be a consequence of inflammatory cell damage or the cytokine milieu, we investigated the formation of TJs in fully differentiated BEC cultures grown from bronchial brushings. Irrespective of the clinical group, cultures formed a fully differentiated pseudostratified secretory epithelium with functionally active cilia by 21 days, which was confirmed by electron microscopy (Fig 2, A). Confocal microscopy showed that differentiated cultures from normal subjects had continuous TJ protein staining, whereas cultures from asthmatic subjects had discontinuous TJs and altered localization of proteins (Fig 2, B and C). Colabeling experiments to identify goblet cells and ciliated cells did not identify differences in TJ protein distribution linked to a specific subtype of cell (Fig 2, B). Analysis of ZO-1 and occludin mRNA levels failed to show a difference in expression levels between the cultures (see Fig E2 in this article’s Online Repository at www.jacionline.org), consistent with our findings in bronchial biopsies. However, Western blots of whole cell lysates.
indicated that cultures from asthmatic subjects had significantly reduced levels of ZO-1 protein and a trend for lower levels of occludin (Fig 2, D), consistent with a posttranscriptional defect, although we could not determine whether this was due to decreased translation or increased degradation. Of interest, the 3 cultures that showed lowest ZO-1 expression also had the lowest transepithelial electrical resistance (TER) readings on day 21.

Consistent with TJ disruption, cultures from asthmatic donors had significantly lower TERs by 21 days of differentiation (Fig 3, A). Extending the differentiation period to 28 days did not improve either TER or TJ staining, suggesting that the barrier defect in the cultures from asthmatic subjects was unlikely to be due to slower differentiation. Post hoc subanalysis of the data based on asthma severity revealed that TER was significantly decreased as a function of disease severity (Fig 3, B). Since previous studies using 16HBE 14o-cells have shown that ZO-1 staining is localized only into a zonula-occludens–like belt in a submerged liquid culture, we also evaluated TERs of ALI cultures that were then placed in a submerged culture. Consistent with the previous report, we found that submersion for 24 hours in minimal medium lacking EGF caused the TER to increase in most cultures tested; however, there remained a significant disease- and severity-related difference between the groups (Fig 3, C and D). Submersion of ALI cultures in the absence of EGF for longer than 24 hours did not change ZO-1 or occludin expression in immunostaining of whole mount ALI cultures (see Fig 4, B).

As TJs control both ion and solute permeability, we investigated the relationship between TER and the passage of 4-kDa and 20-kDa fluoresceinated dextrans across the differentiated epithelial layer in vitro. There was a strong relationship between TER and macromolecular permeability (Fig 3, E), and the permeability of cultures from nonasthmatic and asthmatic subjects to 4-kDa or 20-kDa FITC-dextran was compared (Fig 3, F). Horizontal bars represent median values.

EGF can restore TJs in epithelial cultures from asthmatic subjects without affecting proliferation or goblet cell numbers

EGF is a pleiotropic cytokine that can promote cell proliferation, differentiation, or migration and improve epithelial barrier function. As epithelia are polarized structures, we first established whether the route of administration of EGF affected its ability to improve epithelial barrier function. When applied to the apical surface of differentiated cultures, EGF caused a dose-dependent, significant improvement in TERs of cultures from asthmatic subjects (Fig 4, A; see Fig E4 in this article’s Online Repository at www.jacionline.org), yet it did not affect proliferation (median [interquartile range]: 100% [99%-102%] of control; n = 10; P > .05) or goblet cell numbers (107% [64%-125%] of control; n = 10; P > .05) (see Fig E5 in this article’s Online Repository at www.jacionline.org). In contrast,
Apically applied EGF had no effect on either TER or proliferation of normal cultures (100% [91%-173%] of control; n = 10; P > .05; Fig 4, A; see Fig E5 in this article’s Online Repository at www.jacionline.org), suggesting that the mature epithelial barrier is insensitive to EGF stimulation. The improvement in barrier function in cultures from asthmatic subjects was accompanied by an increase in TJ protein staining (Fig 4, B). Even though apically applied EGF stimulated TJ formation and improved barrier function of fully differentiated epithelial cultures from asthmatic subjects, application of EGF to the basolateral surface of the epithelium failed to improve TER (see Fig E6 in this article’s Online Repository at www.jacionline.org) and there was a 15% (3%-29%) (P < .05, n = 3) increase in the number of CK14+ cells, suggesting squamous differentiation (see Fig E7 in this article’s Online Repository at www.jacionline.org).

**EGF enhances recovery after cigarette smoke–induced injury**

To determine whether fully differentiated cultures from asthmatic donors were more susceptible to environmental insults, we exposed the apical surfaces of cultures to dilutions of freshly prepared CSE. This revealed that BEC cultures derived from asthmatic subjects were much more susceptible to CSE, showing a 50% drop in TER following exposure to 15% CSE whereas the TER of cultures derived from normal controls was unaffected up to 23% CSE (Fig 5, A). Following disruption by exposure to 15% CSE, treatment of cultures from asthmatic subjects with apical EGF accelerated recovery of barrier function as early as 24 hours when compared with treatment of cultures from untreated controls (Fig 5, B). Comparable results were obtained with cultures from normal controls; however in this case, it was necessary to expose the cultures to 30% CSE to achieve a significant drop in baseline TER to allow assessment of recovery (data not shown). In some experiments, cultures were pretreated with apical EGF prior to exposure to CSE as well as during the recovery period (Fig 5, C). In this case, the pretreatment protected the cells against the damaging effect of the CSE and recovery was improved. In all cases, improvement in barrier function was accompanied by restoration of TJs (Fig 5, D), which had been disrupted by CSE exposure (Fig 5, D, panel b).

**DISCUSSION**

We report a disease-related deficiency in epithelial TJs in asthma, both in vivo and in vitro, involving reduced TJ protein localization in junctional complexes and an associated increased permeability of the epithelium to ions and macromolecules. Since the airway epithelium is the first barrier to inhaled insults, such a defect may contribute to the susceptibility of epithelium in asthmatic subjects to environmental stimuli and may help explain bronchial hyperresponsiveness through enhanced penetration of bronchoconstrictor agonists.28 Our observations extend those of de Boer et al, who have previously reported that expression of both ZO-1 and E-cadherin was lower in airway biopsies from asthmatic subjects than from normal subjects.16 Taken together, these studies suggest that a more detailed analysis of TJ and adherens junction proteins and their functionality in bronchial epithelium in asthma is warranted. This should include analysis of claudins in view of the recent finding that reductions in claudin-1 have been observed in AD in association with a defect in bioelectric barrier function.11

Several in vitro studies have demonstrated that proinflammatory cytokines cause disruption of TJs.29 Most notably, IL-13 reduces TJ integrity in BEC cultures29 while TNF-α and IFN-γ disrupt TJs in gastrointestinal epithelial cells, although it appears that their mode of action is different.30 While IL-13 might be implicated as a mediator of TJ disruption in asthma, our in vitro data suggest an IL-13–independent effect, as baseline barrier function was lower in cultures from asthmatic subjects. In support of this, expression of IL-13 mRNA was low in ALI cultures regardless of the severity of the disease (data not shown); however, further experiments would be required to determine whether endogenous IL-13 protein levels are higher in cultures from asthmatic subjects.
and whether this makes a functional contribution to epithelial barrier permeability.

While the asthma group was characterized by increased permeability, there was considerable overlap between the groups, with some cultures from asthmatic donors showing TERs in the normal range. Subgroup analysis revealed that these tended to be from subjects with milder asthma while those in the moderate-severe group had lower TERs irrespective of inhaled corticosteroid use. We also found that as a group, BEC cultures from asthmatic subjects were more permeable to macromolecules than those from normal subjects. However, in the majority of cultures, permeability to 20 kDa dextrans was low, suggesting that for allergens (or their fragments) to penetrate the barrier they must have relatively low molecular weights.

The ability of growth factors such as EGF and keratinocyte growth factor to restore barrier function after injury has been demonstrated in a number of in vitro and in vivo models. We have found that EGF not only restores barrier function after CSE-induced injury but also enhances baseline barrier function of asthma-derived cultures. The effect of EGF when applied apically was dose dependent, but the optimal dose was higher than used in basolateral differentiation medium. Although a recent article suggests that basolaterally applied EGF enhances barrier function through an epidermal growth factor receptor (EGFR)/Rac1/JNK pathway, it remains to be determined whether the same pathway is activated by apically applied EGF either at baseline or when it confers protection against CSE.

On the basis of our results, we propose that growth factor treatment may offer a new therapeutic approach for treating asthma by enhancing the ability of the epithelium to withstand environmental challenges. Our studies have highlighted that the effects of EGF are determined by its route of administration. Apically applied EGF (topical delivery) was found to improve barrier function without affecting proliferation of BEC cultures from asthmatic subjects, whereas basolateral administration of EGF (systemic delivery) increased proliferation without any beneficial effect on barrier function. To explain this, we propose that basolateral application of EGF activates EGFRs on basal cells to trigger proliferation, whereas apical application of EGF activates differentiated columnar cells to promote TJ formation. Since TJ formation restricts the lateral movement of molecules in the cell membrane, the resultant epithelial polarization will confine EGFRs to the basolateral compartment, as observed in vivo. Thus, stimulation via the apical route should become self-limiting as the epithelium becomes fully mature and the receptor and the ligand become spatially separated. This could explain why we observed that apical application of EGF did not have an effect on mature, fully differentiated normal BEC cultures. A similar spatial separation between epithelial growth factors and their receptors by TJ function has been elegantly demonstrated by Vermeer et al. From a therapeutic perspective, topical (inhaled) application of EGF is likely to maximize restoration of barrier function in asthma while minimizing unwanted side effects such as increased proliferation. However, further work would be required to optimize the formulation, dose, and method of delivery of EGF to the airways.

Defects in epithelial barrier function have been observed in Crohn disease and ulcerative colitis. The potential of EGF as

![FIG 5. The sensitivity of BEC cultures to CSE and protection by EGF. To determine the half maximal inhibitory concentrations (IC50), differentiated BEC cultures were exposed to CSE and TERs measured after 5 hours (A). To assess protection by EGF, cultures from asthmatic donors were exposed to 15% CSE and the effect of 0.8 nM EGF on TER recovery was determined 24-hour post-CSE removal (B); in C, cultures were pretreated with EGF (●) or left untreated (○) for 48 hours before being exposed to 15% CSE + EGF and assessed for barrier recovery as in Fig 5, B. In D, TJ integrity was examined by immunostaining for ZO-1: untreated (a), CSE-treated (b), CSE followed by EGF (c), or EGF treated before, during, and after CSE (d).]
a novel treatment for inflammatory bowel disease has been demonstrated in a study involving 12 patients with ulcerative colitis who were given EGF (5 μg) as an enema daily for 14 days. This led to disease remission and improvement in disease activity scores for up to 12 weeks posttreatment, suggesting that restoration of the epithelial barrier normalized tissue responses and downregulated inflammation. Although the study was criticized for a perceived risk that EGF may contribute to hyperproliferation and malignant transformation, our data suggest that the risk would be minimal since the EGF was topically administered. Furthermore, EGF is expressed in the airway submucosal glands and it is well established that it is present normally in many body fluids (including saliva, gastric secretions, tears, breast milk, urine) where it plays a role in maintenance of the epithelial barrier and in healing of damaged mucosa. The restriction of EGF receptors to the basolateral surfaces of noninjured epithelia ensures that the receptor and the ligand are spatially separated to prevent inappropriate activation until epithelial damage allows access of the ligand to induce a rapid repair response that is independent of the influx of inflammatory cells or the activation of local tissue resident cells. A number of studies have identified deficiencies in EGF production in diseases with persistent mucosal damage. These include reduced levels of salivary EGF in diabetic patients linked to the development of oral varus EGF linked to delayed ulcer healing caused by cigarette smoke and diminished levels of salivary and serum EGF in premature infants with necrotizing enterocolitis.

In conclusion, we have identified a lesion in the epithelial barrier in asthma that may explain the susceptibility of airways in asthmatic subjects to environmental agents and bronchoconstrictor agonists and could account for the end-organ expression of asthma. We have also established an in vitro model system that mimics the in vivo phenotype and have used this to study barrier susceptibility and its protection by EGF. We propose that agents that restore epithelial integrity in asthma may offer novel therapeutic approaches for those difficult-to-treat asthmatic subjects who fail to respond to conventional therapy.

We acknowledge Dr Antion Page, Biomedical Imaging Unit, for electron microscopy and Dr Susan Wilson and members of the Histochemistry Research Unit, Southampton General Hospital.

Clinical implications: This work suggests that targeting the barrier defect in asthma may offer a novel therapeutic approach for difficult-to-treat asthmatic patients who fail to respond to conventional therapy.

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METHODS

Unless otherwise stated, all reagents were obtained from Sigma (Poole, United Kingdom). Human wild-type EGF was expressed in Escherichia coli (SinoBio Biotech, Shanghai, China).

Clinical characterization of subjects and bronchoscopy

Following ethical approval by the Southampton and South West Hampshire Local Research Ethics Committees and written informed consent, subjects underwent clinical characterization and fibroptic bronchoscopy under local anesthesia as previously described, allowing collection of bronchial biopsies and brushings. Subjects were characterized according to symptoms, lung function, airway responsiveness to inhaled methacholine (PC_{20}), medication, and skin prick test to common aero-allergens. Asthma severity was assessed according to the Global Initiative for Asthma guidelines. All volunteers were nonsmokers and free from respiratory tract infections for a minimum of 4 weeks prior to inclusion in the study. The clinical details of the subjects involved in each part of the study are shown in Table E1.

Immunohistochemistry

Bronchial biopsies from 7 normal subjects and 19 asthmatic subjects (9 mild; 3 moderate; 7 severe) were processed into glycol methacrylate resin for immunohistochemistry. Semithin 2-μm sections were stained with primary mouse mAbs against ZO-1 (dilution 1/250; clone 1A12; Zymed Laboratories, Inc, San Francisco, Calif) and occludin (dilution 1/250; clone 3F10; Zymed Laboratories, Inc) using standard protocols. Immunostaining was recorded using an Open Zeiss KS 400 Image analysis system (Carl Zeiss, Jena, Germany). In all cases, analyses were restricted to areas of structurally normal and well-oriented epithelium, that is, avoiding tangentially cut epithelium. The extent of staining was assessed by using a semiquantitative score as follows: 3 (strong and regular punctuate staining of TJs along the epithelial surface), 2+ (irregular staining along the epithelial surface, but clear TJs evident), 1+ (patchy staining, only a few TJs evident), and 0 (no staining evident). The analysis was done by one investigator who was blinded to the identities of the specimens.

Primary BEC cultures and ALI cultures

Primary BEC cultures were grown from brushings, as previously described. BECs were cultured in flasks precoated with collagen I (Vitrogen-100, Nutacon, The Netherlands). Bronchial epithelial growth medium (Lonza, Wokingham, United Kingdom) was replenished daily for the first 3 days and thereafter every 2 days. BECs were expanded in culture over 2 to 3 weeks up to passage 2 (p2). To induce differentiation, BECs (p2) were seeded in bronchial epithelial growth medium onto culture inserts (Transwell, 6.5 mm, 0.4 μm pore size; Corning Costar, Bucks, United Kingdom) coated with collagen I. The cells were grown submerged until 90% to 100% confluent, before being taken to the air-liquid interface (ALI). Apical medium was removed (day 0), and basal medium was replaced with 300 μL of ALI medium daily. At the end of treatment, transwells were processed for further analysis.

Statistics

For normally distributed data, mean ± standard error of the mean values are used and differences between groups analyzed using Student’s t test.

CSE exposure and protection by EGF

Smoke from Kentucky research cigarettes #2R1 without filters (Kentucky Tobacco R&D Center, Lexington, Ky) was drawn into 25 mL of BEBM under vacuum over a period of 1 to 2 minutes. The CSE was then passed through a 0.22-μm MilliLex-GS (Millipore, Watford, United Kingdom) filter and used immediately. At day 21, differentiated cultures were starved for 24 hours in minimal medium and cultures were exposed to different concentrations of CSE applied to the apical surface in 100 μL of minimal medium; TER was monitored until CSE-treated groups showed a significant drop to basal levels. CSE was then removed, and cells were washed once with minimal medium and treated apically with minimal medium with or without 0.8 nM EGF for another 24 hours. Alternatively, where indicated, cultures were pretreated for 48 hours with apically applied EGF (0.8 nM) prior to and after CSE treatment.

Ultrastructural analysis by using electron microscopy

Cultures on transwell inserts were fixed for 1 hour in 3% glutaraldehyde, 4% formaldehyde in 0.1 mol/L PIPES buffer (pH 7.2), rinsed, and then postfixed for 1 hour in 1% buffered osmium tetroxide. The cultures were rinsed and cut from the transwell insert before dehydration in an ethanol series. For transmission electron microscopy, membranes were embedded in Spurr resin and following polymerization of the blocks, silver sections were cut on a Leica OMU 3 ultramicrotome. Sections were stained with uranyl acetate followed by Reynolds lead stain and viewed on a Hitachi H7000 transmission electron microscope.

Western blotting

Cell lysates were subjected to SDS-PAGE on a 4% to 15% gradient polyacrylamide gel followed by Western blotting using anti-ZO-1 or anti-occludin mAbs (Invitrogen, Paisley, United Kingdom), with β-actin used as a loading control. The quantification of protein was completed via computer image analysis (Scion Image, Scion Corp, Frederick, Md).

RESULTS

EGF treatment

After differentiation had proceeded for 21 days, cultures were starved for 24 hours in BEBM containing insulin/transferrin/sodium selenite (Sigma, Poole, United Kingdom, 1/100 dilution) and BSA (1.5 μg/mL) (minimal medium) by adding 100 μL onto the apical surface and 300 μL into the basal compartment. Where indicated, cultures were treated with 0.8 nM EGF (5 ng/mL) either apically in 100 μL of minimal medium or basolaterally in 300 μL of medium. The treatments were renewed every day up to 4 days, and TER was monitored daily. At the end of treatment, transwells were processed for further analysis.
Where not normally distributed, data are summarized using the median and interquartile range and were evaluated using nonparametric Wilcoxon rank sum or Mann-Whitney U tests. All the data were analyzed using Prism (GraphPad Software, La Jolla, Calif).

REFERENCES


mRNA Expression of TJ components in bronchial epithelial brushings. mRNA Expression of ZO-1 and occludin in bronchial brushings from normal or asthmatic volunteers was quantified by RT-qPCR. Filled circles, Asthmatic subjects; open circles, healthy, nonasthmatic subjects. BBR, Bronchial brushings; horizontal bars represent median values. There was no significant difference between the groups for mRNA expression of ZO-1 or occludin.
FIG E2. mRNA Expression of TJ components in BEC cultures. mRNA Expression of ZO-1 and occludin in day 21 ALI cultures from normal or asthmatic volunteers was quantified by RT-qPCR. Filled circles, cultures from asthmatic subjects; open circles, cultures from healthy, nonasthmatic subjects. Horizontal bars represent median values. There was no significant difference between the groups for mRNA expression of ZO-1 or occludin.
FIG E3. Correlation between TER and permeability to 4-kDa FITC-dextran. The relationship between TER and permeability to 4-kDa FITC-dextran was measured in transwells whose TER ranged from low to high regardless of subject group. Horizontal bars represent median values.
FIG E4. Dose effect of EGF on epithelial barrier properties. Dose dependency for the effect of apically applied EGF on TERs of cultures from asthmatic subjects (n = 24 wells from 4 subjects); EGF was applied apically for 4 days. There was a dose-dependent increase in TER in response to EGF up to 1.6 nM.
FIG E5. The effect of apically applied EGF on epithelial proliferation and differentiation. The effect of 4 days of exposure to apically applied EGF (0.8 nM) was analyzed on markers of proliferation (Ki67) and differentiation (MUC5AC and β-tubulin); data are expressed as fold change versus control. Apically applied EGF did not significantly affect the proliferation or differentiation markers.
FIG E6. The effect of basolaterally applied EGF on epithelial barrier properties. The effect of basolateral administration of EGF (0.8 nM) on TERs of fully differentiated BEC cultures from asthmatic subjects (n = 8) was determined after 4 days. Horizontal bars represent median values. There was no significant effect of EGF on TER when applied via the basolateral compartment.
The effect of apical or basolaterally applied EGF on cytokeratins expression. The effect of apically or basolaterally applied EGF (0.8 nM) on expression of squamous cell markers (CK5/CK14) was determined by immunohistochemical staining of cytospins prepared from BECs from asthmatic subjects after 4 days of daily treatment. There was a 15% (3% to 29%; \( p < .05; n = 3 \)) increase in numbers of CK14 cells, suggesting squamous differentiation when EGF was applied basolaterally. CK, Cytokeratin.
### TABLE E1. Subject details presented for the whole study group and for each separate study group

<table>
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<th>Study and patient groups</th>
<th>Subject no.</th>
<th>Gender (M/F)</th>
<th>Age (y), mean (range)</th>
<th>FEV(_1) (% predicted), mean (SEM)</th>
<th>PC(_{20}) (SEM) (mg/mL)</th>
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<tr>
<td>Healthy controls</td>
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<td>7/4</td>
<td>35 (20-55)</td>
<td>110.00 (3.44)</td>
<td>&gt;16</td>
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<td>6/5</td>
<td>36 (19-54)</td>
<td>100.54 (4.37)</td>
<td>2.89 (0.70)</td>
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<td>Moderate asthma</td>
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<td>33 (22-50)</td>
<td>99.00 (3.66)</td>
<td>1.89 (0.90)</td>
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<td>Severe asthma</td>
<td>8</td>
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<td>41 (22-71)</td>
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<td>35 (21-64)</td>
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<td>4</td>
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<td>36 (26-54)</td>
<td>111.00 (7.18)</td>
<td>1.61 (0.54)</td>
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<tr>
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<td>3</td>
<td>1/2</td>
<td>33 (22-52)</td>
<td>99.33 (1.45)</td>
<td>0.66 (0.21)</td>
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</tbody>
</table>

F, Female; FEV\(_1\), forced expiratory volume in 1 s; M, male; NA, not available; PC\(_{20}\), the concentration of methacholine that causes a 20% decline in FEV\(_1\); SEM, standard error of the mean.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer and probe</th>
<th>Sequence</th>
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<tr>
<td>ZO-1</td>
<td>Sense primer</td>
<td>AAACAAGCCAGCAGAGACC</td>
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<td>Antisense primer</td>
<td>CGCAGACGATGTTCCATAGTTTC</td>
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<td>Probe</td>
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<td>Sense primer</td>
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<td>Probe</td>
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TABLE E3. Analysis of TJ immunostaining for ZO-1 using whole mount confocal microscopy

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<tr>
<th>Subject group</th>
<th>Staining</th>
<th>Total no.</th>
<th>Intact TJs</th>
<th>Intact + disrupted TJs</th>
<th>Disrupted TJs</th>
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<tr>
<td>Healthy</td>
<td>ZO-1</td>
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<td>0</td>
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<td>Asthma (severe)</td>
<td>ZO-1</td>
<td>1</td>
<td>0</td>
<td>1</td>
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