TNF-α–mediated bronchial barrier disruption and regulation by src-family kinase activation

Michelle A. Hardyman, PhD,[a] Emily Wilkinson, BSc,[a] Emma Martin, BMedSci,[a] Nivenka P. Jayasekera, MD,[a,b] Cornelia Blume, PhD,[a] Emily J. Swindle, PhD,[a] Neil Gozzard, PhD,[c] Stephen T. Holgate, MD, DSc,[a,b] Peter H. Howarth, MD, DM,[a,b] Donna E. Davies, PhD,[a,b] and Jane E. Collins, PhD[a,b] *Southampton and Slough, United Kingdom*

Background: Because TNF-α is increased in severe asthma, we hypothesized that TNF-α contributes to barrier dysfunction and cell activation in bronchial epithelial cells. We further hypothesized that src-family kinase inhibition would improve barrier function in healthy cells in the presence of TNF-α and directly in cultures of severe asthmatic cells where the barrier is disrupted.

Objectives: We assessed the effect of TNF-α, with or without src-family kinase inhibitor SU6656, on barrier properties and cytokine release in differentiated human bronchial epithelial cultures. Further, we tested the effect of SU6656 on differentiated primary cultures from severe asthma.

Methods: Barrier properties of differentiated human bronchial epithelial air-liquid interface cultures from healthy subjects and subjects with severe asthma were assessed with transepithelial electrical resistance and fluorescent dextran passage. Proteins were detected by immunostaining or Western blot analysis and cytokines by immunoassay. Mechanisms were investigated with src kinase and other inhibitors.

Results: TNF-α lowered transepithelial electrical resistance and increased fluorescent dextran permeability, caused loss of occludin and claudins from tight junctions with redistribution of p120 catenin and E-cadherin from adherens junctions, and also increased endogenous TNF-α, IL-6, IL-1β, IL-8, thymic stromal lymphoprotein, and pro–matrix metalloprotease 9 release. SU6656 reduced TNF-α–mediated paracellular permeability changes, restored occludin, p120, and E-cadherin and lowered autocrine TNF-α release. Importantly, SU6656 improved the barrier properties of severe asthmatic air-liquid interface cultures. Redistribution of E-cadherin and p120 was observed in bronchial biopsies from severe asthmatic airways.

Conclusions: Inhibiting TNF-α or src kinases may be a therapeutic option to normalize barrier integrity and cytokine release in airway diseases associated with barrier dysfunction. (J Allergy Clin Immunol 2013;132:665-75.)

Key words: *Airway, bronchial, barrier, epithelial, TNF-α, cytokines, proMMP-9, src kinase, SU6656*

The pulmonary airway epithelium resides at a critical external interface, exposed to harmful aerosols and pathogens. The proximal bronchial epithelium comprises columnar-ciliated cells and mucus-secreting goblet cells supported by basal cells, to generate a selective permeability barrier to control fluid loss, entry of pathogens, and inappropriate immune reactions in the subepithelial lung mucosa.[1] In surface epithelial cells, tight junctions (TJs) encircle the subapical regions of lateral cell membranes to regulate permeability via the paracellular pathway and to restrict lateral movement in the cell membrane, while intercellularly connecting to the actin cytoskeleton.[2] The physical barrier function of TJs relies on the expression and interaction of protein complexes, including integral membrane proteins, claudins, occludin, tricellulins, Marvel D and junctional adhesion molecules, cytoskeletal linker proteins, zonula occluden (ZO)-1, ZO-2, and ZO-3, cingulin and 7H6, and associated signaling and cell cycle regulators that control junction assembly, proliferation, and differentiation.[3-7] The TJ barrier appears to discriminate on the basis of size and charge[2] encompassing a leak pathway for large solutes and a pore pathway (approximately 4 Å) of variable pore density for small uncharged molecules and ions. Further selectivity of paracellular ion permeability is contributed by the approximate 27-member claudin family of variably coexpressed proteins.[8,9] Occludin has been shown to be important in cytokine-mediated barrier responses, and perturbations in occludin result in independent modification of ion and macromolecular permeability.[10,11]

Below the TJ is the actin-linked zonula adherens junction (AJ),[12,13] whereas keratin-linked desmosomes provide additional cohesion.[14] The AJ, comprising E-cadherin and catenins, is implicated in assembly and maintenance of TJs.[15] E-cadherin and ZO-1 associate with catenins in the initial stages of *de novo* junction assembly before formation of mature tight and AJs.[16] Perturbation experiments have shown that E-cadherin is important for TJ assembly in Madin-Darby canine kidney epithelial (MDCK) cells[15] and transformed human bronchial epithelial (16HBE) cells[17]; therefore, its regulation must be considered in the context of barrier control in the airway.[18,19]

From [a]the Academic Unit of Clinical and Experimental Sciences and [b]Southampton NIHR Respiratory Biomedical Research Unit, Sir Henry Wellcome Laboratories, University of Southampton Faculty of Medicine, University Hospital Southampton; and [c]UCB Celltech, Slough.

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Corresponding author: Jane E. Collins, PhD, Clinical and Experimental Sciences Unit, Sir Henry Wellcome Laboratories, Level E, South Block, Mailpoint 813, University Hospital Southampton, Southampton SO16 6YD, United Kingdom. E-mail: J.E.Collins@soton.ac.uk.

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Abbreviations used
AJ: Adherens junctions
ALI: Air-liquid interface
ECL: Electrochemiluminescence
EVOM: Epithelial volt ohm meter
FITC: Fluorescein isothiocyanate
HBEC: Human bronchial epithelial cell
HRP: Horseradish peroxidase
LDH: Lactate dehydrogenase
mAb: Monoclonal antibody
MDCK: Madin-Darby canine kidney epithelial
MMP: Matrix metalloprotease
MSD: Meso Scale Discovery
SFK: Src family kinase
TER: Transepithelial electrical resistance
TIMP: Tissue inhibitor of metalloproteinase
TJ: Tight junction
TSLP: Thymic stromal lymphoprotein
ZO: Zonula occluden

Epithelia in inflamed asthmatic airways exhibited disruption of TJs and increased permeability, especially in severe disease.[1,20] TNF-α is increased in airways of severe, corticosteroid refractory patients with asthma and implicated in airway pathology,[21,22] whereas in healthy subjects inhalation of TNF-α triggers airway constriction, hyperresponsiveness, and sputum neutrophilia.[23] TNF-α promotes epithelial barrier dysfunction in other tissues,[2] and previous studies reported that TNF-α in conjunction with IFN-γ caused TJ disruption in cultured bronchial epithelia.[24,25] However, information is scarce on the specific effects of TNF-α on claudins, barrier changes, and epithelial-specific cytokine release. Modeling airway inflammation with bronchial epithelial air-interface cultures challenged with TNF-α, we hypothesized that TNF-α disrupts TJs of human airway epithelium and promotes inflammatory cytokine release. We also investigated mechanisms that control these responses and found that Src family kinases and modifications to E-cadherin and p120 catenin were involved. We confirmed that p120 is disrupted in severe asthma and that SU6656 improves barrier function in air-liquid interface (ALI) cultures from donors with severe asthma. Our data suggest that barrier disruption may be amenable to pharmacologic intervention in severe asthmatic disease. Parts of this work were published in abstract form.[26]

METHODS

For additional information on the assessments, analyses, and measurements used, please see the Methods section in this article's Online Repository at www.jacionline.org.

Clinical characterization of subjects and bronchoscopy

Following ethical approval and informed consent, subjects underwent fiber optic bronchoscopy under local anesthesia for collection of bronchial biopsies and brushings.[27] All volunteers were nonsmokers and were free from respiratory tract infections for at least 4 weeks before the study. Further clinical information is included in Table E1 (in the Online Repository available at www.jacionline.org).

Culture and differentiation of primary bronchial epithelial cells

Human bronchial epithelial cell (HBEC) cultures were grown from bronchial brushings[28] and differentiated for 21 days at an ALI.[20] Differentiated cultures were treated basolaterally with TNF-α (10 ng/mL) or other agents as detailed in the Results section.

Measurement of TER and assessment of epithelial barrier integrity

The transepithelial electrical resistance (TER) was measured with an epithelial volt ohm meter (EVOM) with STX2 electrode (World Precision Instruments, Aston, United Kingdom). Fluorescein isothiocyanate (FITC)-dextran 4 kDa (Sigma-Aldrich, Poole, United Kingdom) was applied to cells apically and incubated for 3 hours at 37°C. Basolateral dextran passage was analyzed with a Fluoroskan Ascent FL2.5 reader (Thermo Fisher, London, United Kingdom).

Exogenous cytokines, small molecule inhibitors, and neutralizing antibodies

TNF-α (R&D Systems Europe Ltd, Abingdon, United Kingdom) was reconstituted in PBS-0.1% BSA; SU6656[29] (Sigma-Aldrich), matrix metalloprotease 9 (MMP-9) inhibitor 1 (Merck, London, United Kingdom), and GM6001 (Millipore, Nottingham, United Kingdom) were reconstituted in dimethyl sulfoxide and diluted for use; vehicle controls were routinely included. Cells were preincubated with inhibitors for 2 hours before adding TNF-α.

Immunocytochemistry

Junctional proteins were detected in ALI cultures by immunofluorescent staining with the use of mouse monoclonal antibodies (mAbs) against p120 catenin (BD Biosciences, Oxford, United Kingdom), claudin-3, claudin-4, occludin, and E-cadherin and rabbit anti–claudin-8 (all Invitrogen, Paisley, United Kingdom) with the nuclear counterstain, 7-amino-actinomycin D (Sigma-Aldrich). Staining was assessed with a confocal Leica SP5 microscope with the use of identical settings between conditions.

Immunohistochemistry

Bronchial biopsies were acetone-fixed and embedded in glycolmethacrylate resin. Sections (2 μm) were immunostained with mAbs against E-cadherin (Invitrogen), p120 catenin (BD Biosciences), and neutrophil elastase (clone NP57; Dako, Cambridgeshire, United Kingdom) with the use of standard protocols.[30] In all cases, analyses were restricted to areas of well-orientated and structurally intact epithelium.

Western blot analysis

Cell lysates were subjected to SDS-PAGE and Western blot analysis with the use of anti-p120 catenin (Y228 and Y280), anti–total p120 catenin (BD Biosciences), and anti–β-actin with horseradish peroxidase–electrochemiluminescence (HRP-ECL) detection (Amersham Biosciences, London, United Kingdom).

RT-qPCR for TNF-α

Samples were processed with RNeasy kit (Qiagen, Manchester, United Kingdom) for total RNA before cDNA synthesis. Primers for TNF-α with ubiquitin C and glyceraldehyde 3-phosphate dehydrogenase housekeeping genes (PrimerDesign, Southampton, United Kingdom) were used to quantify expression with the use of a TaqMan 7900HT machine (Applied Biosystems, Foster City, Calif) and analyzed with the ΔΔCt method.

MSD multiplex cytokine array and ELISA assays

Conditioned media were assayed for IL-1β, IL-6, IL-8, TNF-α, IL-10, and MMP-9 with the use of an electrochemiluminescence immunoassay according to the manufacturer's protocol (Meso Scale Discovery [MSD], Rockville, Md). Conditioned media were assayed for thymic stromal lymphoprotein

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