RESPIRATORY SYNCYTIAL VIRUS INDUCES LEUKOTRIENE C₄ SYNTHASE EXPRESSION IN BRONCHIAL EPITHELIAL CELLS

LI-HONG SUN,¹ AI-HUAN CHEN,¹ ZI-FENG YANG,¹ JASON J. CHEN,³ WEN-DA GUAN,¹ JIE-LING WU,² SHENG QIN¹ AND NAN-SHAN ZHONG¹

¹State Key Laboratory of Respiratory Disease, The First Affiliated Hospital of Guangzhou Medical University, ²Guangdong Maternal and Children's Hospital, Guangzhou, China, and ³Department of Pathology & Cell Biology, Columbia University, New York, New York, USA

ABSTRACT

Background and objective: Respiratory syncytial virus (RSV) results in acute wheezing in infants and is frequently associated with recurrent wheezing. Although RSV-induced wheezing clinically resembles that of asthma, corticosteroids are not equivalently effective in RSV-associated wheezing. The study sought to determine the mechanisms of RSV-induced wheezing by establishing an in vitro model of RSV-infected human bronchial epithelial cells (16-HBEC).

Methods: Leukotriene C₄ synthase (LTC₄) mRNA expression in 16-HBEC was detected using fluorescence quantitative polymerase chain reaction, and the relative level of LTC₄ mRNA was expressed as quotient cycle threshold (qCT) based on the threshold cycle number value compared with that of β-actin. Cysteinyll leukotrienes (CysLT) in culture supernatant were measured by enzyme-linked immunosorbent assay. RSV-infected 16-HBEC was incubated with gradient concentration of budesonide (BUD) to assess its effects on LTC₄ expression and CysLT secretion.

Results: RSV infection resulted in increased LTC₄ mRNA expression between 48 and 96 h post-infection. High level of CysLT was detected in the supernatant of RSV-infected 16-HBEC. BUD at concentrations of 10⁻¹⁰⁻¹⁰⁻⁷ mol/L did not significantly alter LTC₄ mRNA expression.

Conclusions: RSV infection upregulated LTC₄ expression in HBEC leading to increased CysLT secretion. Such induction was not attenuated by BUD, suggesting that CysLT might contribute to the pathogenesis of RSV-induced wheezing.

Key words: bronchial epithelial cell; corticosteroid; cysteinyll leukotriene; respiratory syncytial virus.

INTRODUCTION

Respiratory syncytial virus (RSV)-related lower respiratory tract infection is a common cause of acute wheezing in infants. Although RSV-induced wheezing clinically resembles asthma, glucocorticoids are not as equivalently effective as in asthma. The activation of a number of host genes during RSV infection results in the induction of pro-inflammatory cytokine and chemokine synthesis such as interleukin (IL)-6, IL-8 and 5-lipoxigenase.² Conceivably, RSV infection may induce inflammatory mediators in airway epithelial cells that might contribute to acute wheezing that presents different responses to glucocorticoids.

Cysteinyll leukotrienes (CysLT) may elicit bronchoconstriction, inflammatory cell infiltration, increased vascular permeability, mucus hypersecretion and airway mucosal oedema. High levels of leukotriene C₄ (LTC₄) have been documented in nasal lavage obtained from infants with bronchiolitis, and the level of LTC₄ was positively correlated with disease severity.³ Furthermore, synthesis and secretion of leukotrienes were not effectively attenuated by glucocorticoids.⁴ It is likely that RSV-induced wheezing is associated with increased CysLT synthesis. In addition, first-line airway defences, airway epithelial
RSV induces LTC,S expression in HBE cells
cells, are able to generate inflammatory mediators and to express LTC,synthase (LTC,S), which may be induced through 5-lipoxygenase signalling pathway leading to increased leukotrienes secretion. To investigate the potential mechanisms of RSV-induced wheezing in infants, we sought to determine if LTC,S expression and leukotrienes secretion in RSV-infected bronchial epithelial cell line, and if they could be suppressed by budesonide (BUD).

METHODS

Cell cultures
Hep-2 cells (human laryngeal carcinoma cell line; Shanghai Institute of Biochemistry and Cell Biology, Shanghai Institute of Biochemistry and Cell Biology, Shanghai, China) and human bronchial epithelial cell (HBEC) line (Southampton University, Southampton, UK) were incubated at 37°C with 5% CO2 in minimum essential medium supplemented with 10% foetal bovine serum (Hangzhou Siqiang Biotechnology Co. Ltd, Hangzhou, China).

RSV preparation and quantification
RSV A2 strain (ATCC VR-1302, from American Type Culture Collection, Rockville, MD, USA) was inoculated to Hep-2 cells. Following sufficient propagation, the viral culture supernatant and cells were stored in a −80°C freezer. This was followed by repetitive freezing and thawing for thrice before centrifugation at 1000 g for 10 min. The supernatant was subsequently stored in 1.5-mL aliquots at −80°C.

A simplified plaque assay11 for RSV quantification was performed using Dulbecco’s modified Eagle’s medium supplemented with 1.5% carboxymethyl cellulose (Sigma, St. Louis, MO, USA) and 2% foetal bovine serum (carboxymethyl cellulose-Dulbecco’s modified Eagle’s medium) as the overlay. Virus sample was serially diluted with Dulbecco’s modified Eagle’s medium and was inoculated to Hep-2 cell monolayer. Five days later, the carboxymethyl cellulose-Dulbecco’s modified Eagle’s medium was aspirated, and the monolayer was fixed with phosphate buffer containing 0.1% crystal violet and 10% formalin for 2 h followed by plaque counting (Fig. S1 in the online supporting information). The plaque forming unit in per millilitre virus sample was calculated by using the formula plaque forming unit = a*b/v (a, the average number of plaques; b, reciprocal value of virus dilution; v, volume of the virus dilution). Stocks of virus contained 2.2 × 10^6 or 1.6 × 10^8 plaque forming unit/mL.

Infection of cells with RSV
Aliquots of 16-HBEC (1.0 × 10^4) were pipetted into a 96-well tissue culture plate and subjected to RSV infection at a serial multiplicity of infection (MOI) of 0.001, 0.01, 0.1, 1 and 10, respectively. The percentage of survival cells was measured by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay12-14 every 24 h between 24 and 120 h post-infection. The plates were also examined under microscope to assess the cytopathic effect. Infection with MOI = 0.1 was found to induce apparent cytopathic effect, while the cell survival rate remains high at various time points (Fig. S2 in the online supporting information); therefore, MOI = 0.1 was employed to determine the kinetics of gene expression of LTC,S and CysLT in HBEC.

16-HBEC was cultured in a six-well tissue culture plate and was infected with RSV at MOI = 0.1 when cell confluence approached to 80%. The virus inoculum was replaced by minimum essential medium containing 2% foetal bovine serum after 2 h incubation, and then the cultures were maintained for up to 120 h. The LTC,S messenger RNA (mRNA) expression in 16-HBEC and CysLT in supernatant were measured every 24 h. The infection of RSV was examined at 72 h post-infection by fluorescence microscopy using fluorescein isocyanate-conjugated RSV monoclonal antibodies (Chemicon International, Inc., Temecula, CA, USA), and RSV fusion gene was targeted by reverse transcriptase-polymerase chain reaction (PCR).

RNA isolation
Trizol reagent (1 mL, Life Technologies, Carlsbad, CA, USA) was added into each well of the six-well tissue culture plate of the 16-HBEC. Total RNA was extracted according to the manufacturer’s instructions.

Fluorescent quantification polymerase chain reaction
RNA (2 μg) was used as template for complimentary DNA synthesis. For fluorescent quantification polymerase chain reaction, the primers for LTC,S were 5′-TACCCAGCCACGTTAGACTG-3′ (forward) and 5′-CGAACAGGTAGACCAGGCCGCG-3′ (reverse), and those for β-actin were 5′-GACTACCTCTAGAGATC-3′ (forward) and 5′-CTCCCTTAATTGCTACGACGAATTT-3′ (reverse). The sequences of the probes were 5′-(FAM)-CTTCCCGTGTCTCTGACCAGC (Eclipse)-3′ for LTC,S and Taqman probe, and 5′-(FAM)- CTGCCATAGCTTACCACACCGG (Eclipse)-3′ for β-actin (Rui Zhen Biological Technology Co. Ltd, Guangzhou, China). PCR was performed using 1 μL complimentary DNA, 25 μL 2×PCR buffer, 0.5 μL (5 U/μL) Taq polymerase (TaKaRa Co. Ltd, Tokyo, Japan), 2 μL of each primer, 0.4 μL of probe (25 mmol/mL), 8 μL of 10 mmol/L deoxyribonucleoside triphosphates (dNTP) Mix and 11.1 μL of deionized water to a total volume of 50 μL. Following denaturation for 5 min at 95°C, PCR amplification was performed by 40 cycles of 94°C for 1 s, 60°C for 20 s and 72°C for 40 s. Quantification of PCR products was achieved by plotting the fluorescence signals versus the cycle numbers at which the signals crossed the baseline.

The threshold cycle number was calculated by using Sequence Detection System software (ID 5 Multicolor Real-Time PCR, Bio-Rad, Inc., Hercules, CA, USA) with an automatic setting of the baseline. Standard curves were derived by plotting the threshold cycle number...
values against the logarithm of the number of copies and that of unknown samples inferred from the regression line. The threshold cycle number value of each sample was adjusted by that of β-actin, and the ratio (qCt) was inversely proportional to both LTC₄S mRNA levels and the gene copies.

**Enzyme-linked immunosorbent assay**

CysLT in the culture supernatant was measured using enzyme-linked immunosorbent assay kits (Cayman, Ann Arbor, MI, USA). Each sample was assayed in duplicate, and the experiment was performed with the same sample twice.

**Infection of cells with inactivated RSV**

For viral inactivation, aliquots of stock RSV virus were exposed to 1800 ml/cm² ultraviolet (UV) radiation for 1 h and referred to as UV-RSV. The 16-HBEC was infected with UV-RSV at MOI = 0.1. The LTC₄S mRNA expression in 16-HBEC and total CysLT in culture supernatant were measured at 72 h after inoculation.

**Study medication**

BUD (AstraZeneca Co. Ltd, Lund, Sweden) was dissolved in ethanol at 10⁻³ mol/L and stored at -20°C. 16-HBEC infected by RSV (MOI = 0.1) were incubated with different concentrations of BUD (10⁻⁶, 10⁻⁴, 10⁻² mol/L) containing 2% foetal bovine serum medium for maintenance culture of 72 h followed by harvesting the cells and supernatant.

**Statistical analysis**

Statistical analysis was conducted using SPSS for Windows version 12.0 (SPSS Inc., Chicago, IL, USA). All variables were presented as mean ± standard deviation. Paired t-test was performed to compare between-group difference, and repeat-measure analysis of variance was employed for among-group comparison when appropriate. Statistical significance was deemed when P < 0.05.

**RESULTS**

**In vitro model of RSV-infected 16-HBEC**

The 16-HBEC did not show evidence of viral cytopathic effect under light microscope after RSV inoculation except for non-specific morphological changes including cell enlargement, increased opacity and clustered dead cells (Fig. 1a). In contrast, HEP-2 cells developed typical cytopathic effect following RSV inoculation (Fig. 1b). The green fluorescence emitted from the cytoplasm of RSV-infected 16-HBEC (Fig. 2a) showed that RSV-specific antigens were localized in the cytoplasm. To demonstrate successful RSV infection in 16-HBEC, the RSV fusion gene fragment (317 bp) was amplified by reverse transcriptase-PCR (Fig. 2b).

**LTC₄S mRNA expression**

RSV infection yielded an increase of LTC₄S mRNA expression in 16-HBEC at 48 h, and the level culmi-

Figure 1 Respiratory syncytial virus (RSV) A2 (multiplicity of infection (MOI) = 0.1) induced cytopathic effect (CPE) in human bronchial epithelial (16-HBEC) cells and HEP-2 cells (arrows). Panel A: Cytopathology of 16-HBEC cells at 72 h following RSV infection. (a) Clusters of cells with CPE (>40); (b) cells with CPE appeared enlarged with increased opacity and clustered cell death (>200). Panel B: Various patterns of CPE were noted in HEP-2 cells following RSV infection (>100). Day 2: helmet-shaped syncitial formation; day 3: irregular map-shaped fusion; day 4: branching-shaped CPE; day 5: mostly fragmented cells and cell death.
RSV induces LTC₄S expression in HBE cells

Figure 2  Respiratory syncytial virus (RSV) A2 infection in human bronchial epithelial (16-HBE) cells. Panel A: Detection of viral antigens by direct immunofluorescence assay (×100): (a) 16-HBE cells were inoculated with RSV at a multiplicity of infection (MOI) of 0.1. After 72 h, the cells were fixed and incubated with anti-RSV rabbit antibody for 30 min. The RSV antigens in cytoplasm appeared in green and the nuclei in red. (b) 16-HBE cells without infection were cultured for 72 h followed by fixation and incubation with anti-RSV antibody. Cells all appeared in red with no RSV antigens in green. (c) HEp-2 cells infected with RSV at MOI = 0.1 for 72 h showed syncytial cell formation (arrows). Panel B: RSV fusion gene was targeted by reverse transcription (PCR). Lane a has the PCR products derived from RSV (317 bp), and Lane b contained a DNA ladder.

Figure 3  Human bronchial epithelial (16-HBE) cells were inoculated with respiratory syncytial virus (RSV) and ultraviolet (UV)-inactivated RSV (multiplicity of infection (MOI) = 0.1). Leukotriene C⁴ synthase (LTC₄S) messenger RNA (mRNA) in cells was assayed by fluorescent quantitation polymerase chain reaction (FQPCR), adjusted by that of β-actin and expressed as quotient cycle threshold (qCt) that was inversely proportional to LTC₄S mRNA gene expression. Cysteinyl leukotrienes (CysLT) were measured by enzyme-linked immunosorbent assay. *P < 0.05; **P < 0.01. (a,c) Effects of RSV on LTC₄S mRNA expression (a) and on CysLT secretion (c) between 24 and 120 h post-infection. Data were expressed as mean ± standard deviation (SD) derived from triplicate. (C) 16-HBE; (D) 16-HBE-RSV (multiplicity of infection = 0.1). (b,d) Effects of UV-RSV on LTC₄S mRNA expression (b) and on CysLT secretion (d) at 72 h after inoculation. Data were expressed as mean ± SD derived from quadruplicate.

In contrast, at 72 h after inoculation with UV-RSV, CysLT secretion did not differ markedly from that in control cells ((10.67 ± 1.97) vs (8.98 ± 1.51), P > 0.05). However, RSV-infected 16-HBEC showed increased secretion of CysLT compared with UV-RSV-inoculated 16-HBEC ((23.54 ± 2.48) vs (10.67 ± 1.97), P < 0.01, Fig. 3d).

Effects of BUD on LTC₄S mRNA expression and CysLT secretion
Because BUD at 10⁻⁴ mol/L led to diffuse cell death under light microscope (Fig. S3 in the online supporting information), the concentration of 10⁻³ mol/L was employed as the upper limit in this study.¹⁶,¹⁷ Noticeably, high concentration of BUD at 10⁻³ mol/L

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Figure 4 Human bronchial epithelial (16-HBE) cells infected by respiratory syncytial virus (RSV) (multiplicity of infection = 0.1) were incubated with budesonide (BUD) at concentrations of 10^{-10}, 10^{-9}, 10^{-8} and 10^{-7} mol/L, respectively, for 72 h. Leukotriene C4 synthase (LTCS) messenger RNA (mRNA) was assayed by fluorescent quantification polymerase chain reaction and adjusted by that of β-actin, expressed as quotient cycle threshold (Cq) that was inversely proportional to LTCS mRNA gene expression. Cysteinyl leukotrienes (CysLT) were measured by enzyme-linked immunosorbent assay. BUD (10^{-10} to 10^{-7} mol/L) did not effectively suppress LTCS mRNA expression or CysLT secretion in RSV-infected 16-HBEC (Fig. 4).

DISCUSSION

RSV-lower respiratory tract infection is a common cause of wheezing in infancy and leads to high rate of hospitalization. Unfortunately, an effective treatment for RSV-induced wheezing is lacking. Despite this anti-asthmatic drugs are frequently prescribed to prevent and treat wheezing pertinent to RSV-lower respiratory tract infection.16,18 A systematic review and meta-analyses involving approximately 1200 children with viral bronchiolitis failed to warrant the use of corticosteroids in RSV-lower respiratory tract infection.20-22 It has been therefore postulated that a few cytokines or inflammatory mediators could be responsible for the development of wheezing.3,23,24

A few studies have documented that secretion of CysLT might be triggered by RSV infection during acute wheezing. Volovitz et al.3 reported an approximately fivefold increase in the concentration of LTC4 in nasopharyngeal secretion obtained from children with RSV-induced bronchiolitis. In another study that determined the levels of CysLT in bronchoalveolar lavage fluid, Kim et al.25 reported significantly higher levels of CysLT in children with RSV-induced bronchiolitis than those in control group. HBEC, acknowledged as airway structural cells, are capable of releasing a spectrum of cytokines and chemokines. Furthermore, HBEC such as human bronchial epithelial (BEAS-2B) cells have been documented to express the 5-lipoxygenase pathway.4 It has been shown that primary HBEC may produce as much as 11 folds of LTC4 than that of 16-HBEC. This may support the hypothesis that the primary cells appear to be a more authentic host of RSV infection due to a significant increase of leukotrienes secretion.6

In an attempt to investigate the impact of RSV infection on 5-lipoxygenase and endothelin-1 expression, Behera et al.4 established an in vitro model of BEAS-2B cells (MOI = 5) yet failed to delineate the cytopathic effect and the optimal condition for observation. In our study, with MOI = 0.1, the cytopathological changes in 16-HBEC were observed at 72 h, while most cells retained their viability. This suggested that at 72 h post-infection with an MOI of 0.1 could be deemed as the optimal condition for observation of LTCS mRNA expression and CysLT secretion in HBEC.

A limited number of publications investigate CysLT synthesis in bronchial epithelial cells with RSV infection. Behera et al.4 showed that RSV infection upregulated 5-lipoxygenase expression in BEAS-2B cells and led to more intensive CysLT synthesis and secretion. Our study demonstrated that RSV infection significantly upregulates LTCS mRNA expression and CysLT synthesis in epithelial cells. However, LTCS expression and CysLT synthesis was only upregulated at 72 h or more following RSV infection. Villenave et al.23 and Yoon et al.25 reported that IL-6 and IL-8 was significantly upregulated at 24 h or less in HBEC with RSV infection. Furthermore, leukotrienes reportedly upregulated IL-6 expression2 possibly due to a positive feedback from the inflammatory responses. If there was a biphasic induction of LTCS and other cytokines such as IL-6, then the early-phase increase in IL-6 and IL-8 may be responsible for late-phase augmented release of CysLT. Additionally, 16-HBEC inoculation with UV-RSV did not show any increased LTCS mRNA expression and CysLT secretion, suggesting that those increases in RSV infected 16-HBEC

References


were likely correlated with the activity of RSV, and the release of inflammatory mediators in airway epithelial cells might contribute to the pathogenesis of RSV-induced acute wheezing.

The impact of corticosteroids on synthesis and release of leukotrienes in asthmatic patients has been inconclusive. Tanaka et al.28 documented considerably reduced urinary Leukotriene E4 (LTE4) in asthmatic patients who received inhaled fluticasone propionate (800 μg once daily) for 2 weeks. In other studies,23,29 however, neither urinary LTE4 nor sputum leukotriene E4 were altered by high-dose inhaled corticosteroids (>1000 μg daily). Our results suggested that inhaled corticosteroid might not effectively suppress the upregulation of LTC4 mRNA expression and CysLT secretion in RSV-infected 16-HBE cells. This may be helpful to interpret the different effects of corticosteroids between asthmatic patients and children with RSV-induced bronchiolitis. The concentration of BUD (10⁻⁸ mol/L) used in this study was higher than that employed in literatures (10⁻⁸ to 10⁻⁷ mol/L).30,31 The effect of BUD on cell apoptosis has been of interest, as suggested by inhibition of apoptosis in HBEC induced by microbial infection via Mitogen-activated protein kinase (MAPK) pathway32 but corticosteroids was able to induce apoptosis of airway epithelial cells.30 Although it is reported that phospholipase in human lung mast cells is insensitive to corticosteroid and dexamethasone could not block leukotriene pathway enzymes including LTC4,34 desensitization of glucocorticoids receptors may partly explain the no response to steroid.27,28 The precise mechanism why BUD is ineffective in inhibiting LTC4 mRNA expression has yet to be elucidated. It is unknown if BUD could effectively suppress the expression of LTC4 and CysLT in primary HBEC with higher levels. The lack of measurement of LTC4 at protein levels may therefore be a limitation of the study.

Our study revealed that the increase of CysLT synthesis in bronchial epithelial cells correlated with RSV infection and that BUD was unable to attenuate CysLT synthesis. Further studies of corticosteroids on LTC4 mRNA expression and CysLT secretion in children with acute RSV bronchiolitis will be undertaken to provide additional clinical evidence.

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Supporting Information
Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Figure S1 Plaques of RSV A2 in HEP-2 cells. Serial 10-fold dilutions of RSV A2 strain were inoculated to HEP-2 cell monolayer in duplicate. The monolayer was stained at day 5. Control wells have HEP-2 cells without RSV.

Figure S2 Cell viability of RSV-infected 16-HBE cells by MTT assay with different MOI (0.001, 0.01, 0.1, 1 and 10) and at different times (24, 48, 72, 96 and 120 h post-infection). Cell survival rate was calculated by comparing the optical density of RSV-infected cells with that of uninfected controls at the same time point. Data were expressed as mean ± SD derived from six wells.

Figure S3 Cytopathology of 16-HBE cells at 72 h following incubation with 10−4 mol/L BUD (×100). Diffused cell death was observed (arrows).