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Modulation of human bronchial epithelial cells by pneumococcal choline binding protein A

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ABSTRACT

Choline binding protein A (CbpA) is an important adhesin and a determinant of virulence for *Streptococcus pneumoniae*. Binding to epithelial cells of host mucosal surfaces by pneumococcal CbpA is essential for pneumococcus to initiate colonization and to trigger subsequent invasive pneumococcal infections. In this study, we examined the immunopathologic mechanisms for the activation of human bronchial epithelial cells by CbpA in pneumococcal infections. Adhesion molecules, cytokines, and chemokines were assessed by flow cytometry, quantitative real-time polymerase chain reaction, and enzyme-linked immunosorbent assay, respectively. Intracellular signaling molecules were investigated by enzyme-linked immunosorbent assay or transcription factor assay. CbpA could upregulate cell surface expression of adhesion molecule intercellular adhesion molecule–1 on human bronchial epithelial cells. CbpA could also induce the release of inflammatory cytokine interleukin-6, and chemokines CCL2, CXCL1, and CXCL8 from human bronchial epithelia cells. CbpA-mediated induction of these mediators was differentially regulated by extracellular signal-regulated kinase, c-Jun N-terminal protein kinase, p38-mitogen–activated protein kinase, and nuclear factor–κB pathways. CbpA was also found to participate in the induction of IL-6, CCL2, CXCL1, and CXCL8 in the airways of mice upon intranasal challenge with *S. pneumoniae*. Our study therefore suggests that pneumcoccal CbpA plays an immunopathophysiologic role by activating human bronchial epithelial cells in pneumococcal infections.

1. Introduction

Streptococcus pneumoniae are a major cause of infectious diseases, including otitis media, sinusitis, pneumonia, sepsis, and meningitis that are associated with significant morbidity and mortality worldwide, and pneumococcal infectious diseases are still major threats to human health worldwide [1,2]. Current strategies to treat these infections with antibiotics are complicated by the rapid emergence of antibiotic-resistant pneumococcal strains [3]. Although vaccination with pneumococcal conjugate vaccine could result in a decline of pneumococcal diseases caused by vaccine-type pneumococcus, it could lead to nonvaccine serotype *S. pneumoniae* replacement [4]. Therefore, a better understanding of the pathogenesis of pneumococcal diseases, especially the interaction between *S. pneumoniae* and host cells, is central to developing new ways to control pneumococcal infectious diseases.

Pneumococcal capsular polysaccharide has long been recognized as an essential virulence factor for pneumococcal infections [5]. In recent years, it has been widely accepted that pneumococcal surface-exposed proteins are pivotal for the cell wall physiology, and play an important role in the pathogenesis of pneumococcal infections [6]. The surface of pneumococcus is decorated with a family of choline-binding proteins [7]. Choline binding protein A (CbpA) is an important adhesin and a determinant of virulence for pneumococcus. It can bind host proteins, including factor H, secretory IgA (sIgA) via the secretory component and C3 secreted by epithelial cells [8,9]. Moreover, the interaction between polymeric immunoglobulin receptor and CbpA mediates translocation of pneumococcus across nasopharyngeal epithelial cells into the bloodstream [10]. In a murine model, it was found that CbpA contributed to prolonged pneumococcal nasopharyngeal colonization and to the transition to the lower respiratory tract [11]. Taken together, CbpA plays a critical role in bridging pneumococcus and host cells.

The human respiratory mucosal surfaces with bronchial epithelial cells constitute the first barrier that prevents invading pneumococcus to transmigrate into the lungs [12,13]. Human bronchial epithelial cells are a potent source for expressing and releasing inflammatory cytokines, chemokine,s and adhesion molecules, which play a critical role in initiating, amplifying, maintaining or

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modulating airway inflammation [14]. However, the potential of pneumococcal surface virulence protein CbpA to modulate the function of human bronchial epithelial cells has not been investigated. To further elucidate the immunopathologic mechanisms for the activation of human bronchial epithelial cells in pneumococcal infections, we investigated the intracellular signaling mechanisms regulating the expression of cytokine, chemokine and adhesion molecule in well-differentiated primary bronchial epithelial cells grown on air–liquid interface activated by pneumococcal CbpA.

2. Subjects and methods

2.1. Reagents

Human recombinant tumor necrosis factor–α (TNF-α) was obtained from PeproTech (Rocky Hill, NJ). Ultra-purified lipopolysaccharide (LPS) from *Escherichia coli* K12 strain without any contamination by lipoprotein was purchased from InvivoGen Corp. (CA). IκB-α phosphorylation inhibitor BAY11-7082, extracellular signal–regulated kinase (ERK) inhibitor PD98059, c-Jun N-terminal protein kinase (JNK) inhibitor SP600125, p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580, phosphatidylinositol 3– kinase (PI3K) inhibitor LY294002, and Janus Kinase JAK inhibitor AG-490 were purchased from Calbiochem Corp. (CA). SB203580 was dissolved in water, whereas PD98059, LY294002, SP600125, AG-490 and BAY11-7082 were dissolved in dimethyl sulfoxide (DMSO). In all studies, the concentration of DMSO was 0.1% (vol/vol).

2.2. Mice

Female 5– to 6-week-old BALB/c mice were obtained from and raised at Chongqing Medical University at Chongqing. All animal experiments were done in accordance with the Institutional Animal Care and Use Committee's guidelines at the Chongqing Medical University at Chongqing.

2.3. Pneumococcal culture supernatants and recombinant CbpA

CbpA-deficient (CbpA−) mutant TIGR4 pneumococcus was established as described previously [15]. Briefly, the upstream and downstream CbpA flanking regions were amplified from genomic DNA preparations in strain TIGR4 by polymerase chain reaction (PCR) using specific primer pairs, whereas the Janus cassette was amplified by PCR from chromosomal DNA of *S pneumoniae strain* CP1296. The PCR products of the Janus cassette and the CbpA-flanking sequences were digested by appropriate restriction enzymes and ligated. The ligation mixtures were then used to transform parent TIGR4. The transformants were selected for resistance to kanamycin (150 μg/ml) on blood agar plates. The correct insertion of Janus cassette in the *cbpA* locus was confirmed by PCR amplification, DNA sequencing, and Southern hybridization. The mutant pneumococcus were cultured in C+Y medium in 5% CO_2 at 37°C to exponential phase. After centrifugation (3000 *g* for 30 minutes), culture supernatants were collected, filtered (0.2 μm pore), and concentrated (10-fold) (Vivaspin). Protein concentrations in the concentrated culture supernatants (CCS) were determined (Bio-Rad). Western blot confirmed the presence of CbpA in TIGR4 CCS and its expected absence in the CCS derived from mutant strain TIGR4 (data not shown).

Recombinant CbpA (amino acids 26–488; molecular weight of approximately 80 kDa) was cloned in *Escherichia coli* by PCR amplification from genomic DNA of strain TIGR4 as described by us previously [16]. The coding sequence for mature CbpA includes regions exposed on the surface of pneumococcus. The coding sequence for CbpA used for protein expression was cloned into plasmid pET-32a(+) vector (Novagen, Darmstadt, Germany) with *E coli* DH5α as the bacterial host. For recombinant CbpA expression, each recombinant PET32a(+) plasmid vector was transcloned into the *E coli* expression strain BL21(DE3). Recombinant CbpA was initiated by induction with isopropyl-β-d-thiogalactoside (IPTG), and recombinant CbpA protein was purified from the soluble fraction of the recombinant *E coli* lysates by use of metal affinity chromatography columns and buffers (Novagen) according to the manufactures' instructions. The purified CbpA contained a plasmid-encoded thioredoxin (TRX) and a poly-histidine(His) tag. For future studies for biologic functions of recombinant CbpA, we removed the His tag and TRX fusion protein from the TRX-His-CbpA protein. Briefly, after dialysis against PBS, recombinant enterokinase was added at a 1:200 (unit/weight) ratio of protease to fusion CbpA protein. By confirming successful and complete protein cleavage by 10% sodium docecyl sulfate&polyacryamide gel electrophoresis (SDS–PAGE) analysis, EKapture agarose was added to remove the residual recombinant enterokinase and the column of Ni^{+}-NTA agarose resin was used to isolate and remove the TRX-His from the CbpA recombinant protein. Finally, we got the target CbpA protein which was then lyophilized and stored at −20°C until use. Anti-CbpA antibody was generated by immunization of mice with recombinant CbpA.

2.4. Endotoxin-free solutions

Cell culture medium was purchased from Gibco Invitrogen Corporation (CA) free of detectable LPS (<0.1EU/ml). All other solutions were prepared using pyrogen-free water and sterile polypropylene plasticware. No solution contained detectable LPS, as determined by the Limulus amoebocyte lyase assay (sensitivity limit 12 pg/ml; Biowhittaker, Inc., MD).

2.5. Culture of human primary bronchial epithelial cells grown on air–liquid interface

Primary bronchial epithelial cells (PBEC) were obtained from ScienCell Research Laboratories and were grown in serum-free bronchial epithelial cell medium containing 0.5 ng/ml human recombinant epidermal growth factor, 52 mg/ml bovine pituitary extract, 0.1 ng/ml retinoic acid, 0.5 mg/ml hydrocortisone, 5 mg/ml insulin, 10mg/ml transferrin, 0.5 mg/ml epinephrine, 6.5 ng/ml triiodothyronine, 50 mg/ml gentamicin, and 50 pg/ml amphotericin-B at 37°C in a humidified 5% CO_2 atmosphere. Media were changed every 48 hours until PBEC were 90% confluent. An air–liquid interface model was created as described by us previously [31]. Briefly, PBEC between passages 1 and 3 were plated onto 12-mm collagen-coated polyester transwell inserts (pore size 0.4 mm; Corning, Inc., Corning, Inc., NY) in differentiation media containing 50% Dulbecco's modified Eagle's medium in bronchial epithelial cell medium with supplements described above. Within 7 days of submerged culture conditions, these epithelial cells attained 100% confluence. PBEC were then exposed to an apical air interface by removing the apical medium and exposing cells only to medium on their basal surface. They were allowed to grow for an additional 14 days in air–liquid interface to reach full differentiation. Under these conditions, PBEC formed a well-differentiated mucociliary phenotype and they were characterized using immunofluorescent assays with antibodies cytokeratin-18, -19, and vimentin.

2.6. Protein array analysis of chemokines and cytokines in culture supernatant

The expression profile of 79 different cytokines in basolateral supernatants of PBEC was assessed semi-quantitatively using antibody-based RayBioTM human cytokine array V (RayBiotech Inc., CA).

2.7. Quantitative real-time polymerase chain reaction

Total RNA was extracted through use of RNeasy (Qiagen, Chatsworth, CA) from 1×10^6 cells 8 hours after stimulation. Extracted RNA was reverse transcribed into first-strand cDNA from 1 μg of