Expression of Surfactant Protein–A during LPS-Induced Otitis Media with Effusion in Mice

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Abstract

Objective. The objective of this study was to investigate the expression and role of surfactant protein (SP) in the middle ear throughout lipopolysaccharide (LPS)-induced otitis media with effusion (OME).

Study Design. Randomized case-controlled animal model.

Setting. Shandong University, Jinan, China.

Subjects and Methods. SP expression was monitored using reverse transcription polymerase chain reaction (PCR) in normal mice (n = 5). Two groups, control phosphate-buffered saline–injected mice (n = 5) and LPS-injected mice (n = 5), were euthanized 5 days following injection. RNA was extracted for reverse transcription PCR and real-time PCR, and temporal bone samples were used for hematoxylin and eosin staining. LPS was injected into mice, and 5 mice per test were euthanized at 0, 12, 24, 48, 72, and 96 hours following injection. For mRNA expression quantification, reverse transcription PCR and real-time PCR were performed, and proinflammatory cytokine levels were measured by enzyme-linked immunosorbent assay.

Results. SP-A and SP-D expression was detected in normal murine Eustachian tubes. SP-A expression was up-regulated after LPS-induced OME (P = .01). At various time points after LPS injection, concentrations of proinflammatory cytokines (tumor necrosis factor–α [TNF-α], interleukin (IL)–1β, and IL-6) in the middle ear increased significantly (P < .05) and correlated with changes in SP-A expression.

Conclusion. SP-A and SP-D exist in the murine middle ear. The expression of SP-A and TNF-α, IL-1β, and IL-6 was up-regulated in the middle ear of the LPS-induced OME animal model.

Keywords

surfactant protein expression, otitis media with effusion, Eustachian tube mucosa, LPS-induced otitis media

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Otitis media with effusion (OME) is one of the most prevalent infections of early childhood: 60% to 80% of children will have at least 1 episode during their first year of life.1,2 OME may increase a child’s risk for hearing loss and is a major cause of childhood morbidity.3,4 Multiple factors, including bacterial infection, Eustachian tube (ET) dysfunction, and adenoid hypertrophy, affect the onset or development of OME.5,6

In early childhood, before the acquisition of specific immunity, the innate immune response may be critical in OME susceptibility. Surfactant proteins (SP) play important roles in regulating pulmonary surfactant tension and are an important component of the innate immune system. At present, 4 SP have been identified and characterized: SP-A, SP-B, SP-C, and SP-D.7-9 SP-B and SP-C are hydrophobic proteins that associate with lipids to promote the absorption of surfactant phospholipids and to reduce the surface tension. SP-A and SP-D are large, hydrophilic, multimeric proteins belonging to the family of C-type lectins, or collectins, which contribute to the innate immune response. SP-A is the most abundant surfactant-associated protein, with important functions in immune homeostasis in vivo.10-12 As a pattern recognition receptor, SP-A functions in the first line of
defense in the absence of specific antimicrobial antibodies. SP-A osonizes gram-negative bacteria and modifies lipopolysaccharide (LPS) for macrophage binding. SP-A may also regulate proinflammatory cytokines such as interleukin (IL)–1β, IL-6, and tumor necrosis factor–α (TNF-α), which play key roles in fighting infection. LeVine et al found an SP-A deficiency in mice resulted in decreased phagocytic efficacy of macrophages and increased lung inflammation following bacterial infection, providing evidence for the role of SP-A in pathogen clearance.

Currently, there are few studies about the relationship between SP and OME. While expression of SP-A, SP-D, and cellular and subcellular SP-B has been detected in normal human and porcine ETs, the roles of SP during middle ear infection remain unknown. In this study, the OME model was induced with LPS from Klebsiella pneumoniae, which has been proven as effective in inducing OME and biologically similar to the endotoxin extracted from otitis media effusions of OME patients. We provide evidence that SP-A and SP-D are expressed in the middle ear of mice. Also, we demonstrate that induction of SP-A and increased cytokine production are observed in the LPS-induced OME.

Materials and Methods

Subjects

Female BALB/c mice 6 to 8 weeks old with an average mass of 20 g were purchased from the Animal Center of Shandong University. All mice were maintained under pathogen-free conditions. The care, use, and treatment of mice in this study were in strict agreement with guidelines in the Care and Use of Laboratory Animals manual written by the Institute for Laboratory Animal Research, and the protocol was approved by the Institute of Basic Medical Sciences.

Otoscopy examination was performed on all mice prior to treatment to ensure that tympanic membranes were intact and that no middle ear effusion was present. All surgeries were performed under chloral hydrate anesthesia.

Establishment of Experimental OME

Experimental OME was induced by middle ear injection of LPS, as reported previously by Kariya et al. Otoscopic examination was performed for all mice before treatment to ensure that tympanic membranes were normal and that no middle ear effusion was present. When middle ear effusion appears, experimental OME could be diagnosed.

Mice were randomly placed into normal (no injection), control (phosphate-buffered saline [PBS] injection), and experimental (LPS injection) groups for analysis. Following anesthesia, experimental groups received 20 μL LPS (1 mg/mL; Sigma-Aldrich, St Louis, Missouri) via transtympanic injection. PBS (20 μL, 0.01 M) was injected into the middle ears of mice in the control group.

A total of 90 mice were injected with LPS to establish the experimental group. Twenty mice died during the operation. Of the remaining 70 living mice, 57 successfully established the model. Effusion could be observed from the tympanic cavity beginning on day 1 and reached the peak on day 3, while effusion was absent in the middle ear of control group mice.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) and Real-Time PCR

Total RNA was isolated and extracted from ET homogenates of experimental or control mice using the TRIzol Reagent (Invitrogen, Gaithersburg, Maryland) according to the manufacturer’s instructions. The mRNA expression of the SP-A gene was examined using (RT-PCR) with M-MuLV reverse transcriptase in the presence of random hexamer primers. To assess the adequacy of the cDNA and the efficiency of the RT-PCR system, murine GAPDH primers were used as RT-PCR control under the same condition. PCR products were analyzed by gel electrophoresis using 1.5% agarose gel. Stained gels were visualized with the Alphalmager EC system (Alpha Innotech, Santa Clara, California).

Quantitative real-time PCR was performed using SYBR Green reagent in a Mastercycler real-time PCR machine (Realplex 2; Eppendorf, Hamburg, Germany). Reactions were performed twice, and GAPDH values were used to normalize gene expression.

The PCR primer sequences were as follows: SP-A, forward 5′-AGC CTC GAG TGC TGT ATG TGG A-3′ and reverse 5′-TTG CAC TTG ATA CCA GGC ACA AC-3′ (294 bp); SP-B, forward 5′-CCA CCT CCT CAC AAA GAT GAC-3′ and reverse 5′-TTG GGG TTA ATC TGG CTC TGG-3′ (174 bp); SP-C, forward 5′-ATG GAC ATG AGT AGC AAA GAG GT-3′ and reverse 5′-CAC GAT GAG AAG GGC TTT GAG-3′ (119 bp); SP-D, forward 5′-GAA TCA AAG GCG TTT GAG-3′ (285 bp); GAPDH, forward 5′-GGG CAG CCC AGC ACA TCA TCC-3′ and reverse 5′-CCA GCC CCA GCA TCA AAG GTG-3′ (298 bp).

In the first test to investigate SP expression, mucosal tissue mRNA was extracted from normal mice (n = 5) for reverse transcription PCR to analyze both SP-A and SP-D expression. Next, to compare expression between control and experimental groups, PBS-injected mice (n = 5) and LPS-injected mice (n = 5) were euthanized 5 days following injection, and RNA was extracted for RT-PCR and real-time PCR. For mRNA expression quantification, 5 mice were randomly assigned to every time point (t = 0, 12, 24, 48, 72, 96 hours) following LPS injection. A total of 30 mice were euthanized.

Immunohistochemistry

Wild-type BALB/c mice (n = 10) were euthanized by caval dislocation 5 days after middle ear administration of PBS (n = 5) or LPS (n = 5). Temporal bones were removed immediately after killing and processed for histologic examination. Bone specimens were placed in 4% paraformaldehyde for 48 hours and decalcified in 10% ethylenediamine tetra-acetic acid for 7 days at 4°C. After dehydration,
specimens were embedded in paraffin, sectioned at 7-μm thickness, and mounted on glass slides. Sections were deparaffinized, rehydrated, and pretreated with 3% hydrogen peroxide at 37°C for 15 minutes to block endogenous peroxidase. After washing 3 times in PBS for 5 minutes, the sections were blocked with normal goat serum for 30 minutes at 37°C and incubated overnight at 4°C with polyclonal primary antibody to SP-A (Santa Cruz Biotechnology, Santa Cruz, California) diluted in PBS. Slides were incubated in biotinylated goat anti-rabbit antibody for 30 minutes at 37°C, washed in PBS, and then incubated in streptavidin peroxidase for 30 minutes at 37°C. After washing in PBS, diaminobenzine working solution was added for color development. The reaction was stopped with PBS.

**Quantification of TNF-α, IL-1β, and IL-6 in Middle Ear**

The BALB/c mice treated by transtympanic injection of LPS for SP-A mRNA expression analysis using PCR were simultaneously used for quantification of proinflammatory cytokines. Five mice per test were euthanized at 0, 12, 24, 48, 72, or 96 hours, respectively, and 3 tests were performed: TNF-α, IL-1β, and IL-6. The control group was the 0-hour group, in which mice were euthanized immediately after injection. Middle ears were washed transtympanically with 300 μL PBS, and samples were taken from mice at every time point. Concentrations of TNF-α, IL-1β, and IL-6 in collected PBS from the middle ears’ wash were measured by enzyme-linked immunosorbent assay (ELISA) kits (eBioscience, San Diego, California) according to each of the manufacturer’s protocols.

**Statistical Analysis**

SPSS 17.0 (IBM, New York, New York) was used to statistically analyze the data.

**Real-Time PCR.** A t test was used to compare differences of mRNA expression of the SP-A gene in the ET between the control group (PBS) and the LPS-induced OME group 5 days after injection. P < .05 was considered significant.

**TNF-α, IL-1β, IL-6, and mRNA Quantification.** One-way analysis of variance was used to determine statistical significance of changes in mRNA expression and changes in concentrations of each of the proinflammatory cytokines (IL-1β, IL-6, TNF-α) at 0, 12, 24, 48, 72, and 96 hours after LPS injection. For SP-A mRNA expression, the relative expressions of 5 mice at each time point were averaged for 1 test. These average values for each of the 3 tests performed were used in statistical analysis to compare expression across all time points. P < .05 was considered significant, and changes in concentration or expression were analyzed in comparison to the control (0 hours).

**Results**

**Detection of SP in Murine ET**

Extraction of RNA from normal mice without injections (n = 5) was performed for SP detection. From gel electrophoresis analysis of PCR products, SP-A and SP-D mRNA were expressed in the ET mucosa, while SP-B and SP-C were not detected (Figure 1).

**Experimental Otitis Media May Induce the Up-regulation of SP-A Expression**

The SP-A protein was weakly expressed in the control group (PBS group) mucosa of the ET or middle ear (Figure 2). In the experimental group with LPS-induced OME, the protein expression of SP-A was up-regulated (Figure 2B), and mRNA expression (Figure 2C) of the SP-A gene was significantly higher compared with the control (P = .01).

**Expression and Development of SP-A during OME**

After LPS injection, SP-A expression in mRNA increased gradually until its peak at 72 hours after injection. mRNA expression then decreased between 72 and 96 hours. SP-A mRNA expression was significantly higher compared with the control (0 hour) group at every time point (P values of the time points 24, 48, 72, and 96 hours were .01, .006, .004, and .001, respectively). In addition, every time point showed significant differences in expression relative to all other time points, except when comparing the 24-hour and 96-hour groups (P = .214). Gel electrophoresis of RT-PCR products and relative expression of mRNA using real-time PCR are shown in Figure 3A and B, respectively.

**Expression of OME-Related Cytokines in the Middle Ear**

Semiquantitative analysis of ELISA was used to compare concentrations of TNF-α, IL-1β, and IL-6 at each time point (Figure 4). Levels of all 3 cytokines increased significantly compared with 0 hours at each time point after LPS-induced OME (P < .05; TNF-α: P values of the time points 12, 24, 48, 72, and 96 hours were .001, .001, .003, .005, and .012, respectively; IL-1β: P values of the time points 12, 24, 48, 72, and 96 hours are .002, .006, .001, .001, and .009, respectively; IL-6: P values of the time points 12, 24, 48,
72, and 96 hours are .005, .002, .007, .003, and .001, respectively). Within 48 hours after LPS injection, the proinflammatory cytokines peaked and then reduced gradually. At 96 hours, levels of all 3 cytokines were still significantly higher than at time 0 ($P < .05$).

**Discussion**

In this study, we show that SP-A and SP-D are transcriptionally detected in the murine middle ear, and SP-A expression was significantly increased during the pathogenesis of OME. Together with the important role of SP-A in innate immunity regulation, our study provides evidence that SP-A may play a critical role in the body’s reaction to OME. When comparing changes in concentrations of the proinflammatory cytokines with the expression of SP-A over time in this study, SP-A expression, TNF-$\alpha$, and IL-1$\beta$ concentrations all increase for the first 48 hours (Figure 5). SP-A expression peaks at 72 hours, the time point immediately following the peak levels of IL-1 and TNF-$\alpha$. This further suggests a role of SP-A in the pathogenesis and resolution of OME. These 2 cytokines affect vessel repair in endothelial cell walls and up-regulate intercellular adhesion molecule–1 (ICAM-1) expression,$^{18-20}$ and TNF-$\alpha$ plays a role in neutrophil priming, regulation, and apoptosis.$^{21,22}$

**Figure 2.** Expression of surfactant protein (SP)–A in Eustachian tube (ET). Experimental otitis media with effusion mice at 72 hours showed up-regulated expression of SP-A in ET (A, immunohistochemistry; B, reverse transcription polymerase chain reaction (PCR); C, real-time PCR). The arrows showed the positive expression of SP-A. *$P = .01$.

**Figure 3.** Expression of surfactant protein (SP)–A at indicated times after otitis media with effusion (OME). (A) Reverse transcription polymerase chain reaction (PCR) and (B) real-time PCR. Analysis of variance revealed time-dependent increases in SP-A expression after OME.
OME has been shown to cause an increase in expression of ICAM-1, which induces leukocyte adhesion to the vascular wall.\textsuperscript{23-25} Leukocyte and endothelial cell interactions mediate inflammatory responses, and thus, the correlation between SP, present in the ET epithelial lining, and proinflammatory cytokines suggests that SP-A plays a role in neutrophil adhesion during OME. The increasing expression of SP-A for the first 72 hours may help regulate cytokine levels and white blood cell expression in the area of infection, and the lagging decrease in SP-A expression could be a result of TNF-\(\alpha\)-regulated neutrophil apoptosis during the early stages of resolution.

Previous literatures have proven that SP were necessary in ventilating the middle ear and draining secretions to prevent an accumulation and infection of middle ear fluid.\textsuperscript{26-28} At present, several SP have been found to be expressed in the ET and middle ear of normal humans and pigs,\textsuperscript{27,28} while specific SP-A involvement in the surface tension–reducing function of ET surfactant remains unknown. Along with findings by Paananen et al\textsuperscript{15} that SP-A and SP-D genes were expressed in the porcine ET epithelium, our evidence supports the existence of specialized cells in the ET epithelium that express SP-A and SP-D. These proteins may be secreted into the ET lumen during an inflammatory response.

In this study, we analyzed the expression of SP-A and the concentrations of the proinflammatory cytokines such as IL-1\(\beta\), IL-6, and TNF-\(\alpha\). We found that SP-A expression was up-regulated during LPS-induced OME. In addition, concentrations of TNF-\(\alpha\), IL-1\(\beta\), and IL-6 in the middle ear increased significantly in correlation with heightened SP-A expression. As a key component of the innate immune system, SP-A has already been shown to regulate cytokine production and increase the antibacterial and antiviral functions of macrophages.\textsuperscript{29} SP-A has been reported to bind to several receptors including Toll-like receptor 2 and 4 and, consequently, regulate inflammatory responses induced by pathogen-derived products such as peptidoglycan and LPS.\textsuperscript{30-32} SP-A can act either as an opsonin or it can activate macrophage functions resulting in an increased capacity for phagocytosis. Besides stimulation of phagocytosis, SP-A reveals additional immunologically relevant effects: it increases production of superoxide radicals\textsuperscript{33,34} and induces chemotactic migration of macrophages.\textsuperscript{35} These findings, along with our own, confirm that SP-A is an important regulator of the innate immune response. However, the expression and roles of SP-A during the pathogenesis of OME remain unclear. This preliminary study provides evidence that OME induces the expression of SP-A in ET, which may contribute to the resolution of inflammation.
Conclusion

In conclusion, our study provides evidence that SP-A and SP-D are expressed in the mucosa of murine ET and that OME could induce the expression of SP-A, which suggests an important role of SP-A during the immune response to OME. Further study needs to be conducted on the mechanisms of SP-A up-regulation and the relationship between SP-A up-regulation and OME pathogenesis.

Author Contributions

Li Li, design, collection, analysis, and interpretation of the data, drafting the whole manuscript, approval of the manuscript, accountability for all aspects of the work; Xiangrui Guo, collection and analysis of the data, writing and approval of the manuscript, accountability for all aspects of the work; Emily Olszewski, data and statistical analysis interpretation, presentation, writing and revisions; Zhaomin Fan, collection, statistical analysis, drafting the graph and interpretation of the data, final approval, accountability for all aspects of the work; Yuechen Han, collection, data analysis and interpretation, revision, final approval, accountability for all aspects of the work; Yu Ai, collection, data analysis and interpretation, revision, final approval, accountability for all aspects of the work; Lei Xu, collection, analysis and interpretation of the data, writing and revision, final approval, accountability for all aspects of the work; Jiaming Fan, collection, analysis and interpretation of the data, revision, final approval, accountability for all aspects of the work; Yuefang Li, collection, analysis and interpretation of the data, writing and approval of the manuscript, accountability for all aspects of the work; Haibo Wang, design and conduct of the study, collection and interpretation of the data, writing and approval of the manuscript, accountability for all aspects of the work.

Disclosures

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