An Antimicrobial Peptide Modulates Epithelial Responses to Bacterial Products

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INTRODUCTION

The respiratory epithelium and mucociliary clearance system (MCS) of the middle ear, nose, and paranasal sinuses play a key role in the primary defense system of the upper respiratory tract. Changes in the respiratory epithelium caused by infection and inflammation can lead to reduced mucociliary clearance due to loss of ciliated cells, squamous metaplasia, and secretory cell hyperplasia. Mucus hypersecretion is an important characteristic of otitis media with effusion (OME), chronic sinusitis, chronic rhinitis, and chronic bronchitis. A variety of stimuli can affect the function of epithelial cells, including inflammatory mediators released in response to smoke, and microbial components such as lipopolysaccharide (LPS) from gram-negative bacteria and lipoteichoic acid (LTA) from gram-positive bacteria. It has previously been demonstrated that LPS and LTA increase chemokine, cytokine, and mucus production in middle ear epithelial cells.

Staphylococcus aureus, Streptococcus pneumoniae, non-typeable Haemophilus influenzae, Moraxella catarrhalis, and Pseudomonas aeruginosa are involved in the pathogenesis of chronic sinusitis. Inflammation caused by these organisms is partly mediated by their cell membrane components LPS and LTA. LPS has been frequently detected in airway secretions during upper respiratory tract infection. In addition to its proinflammatory activity, it has been reported to induce metaplastic changes in the respiratory epithelial cells. LTA is the major glycolipid found in most gram-positive bacteria and has most of the biochemical and physiologic properties of LPS. LPS and LTA have been shown to affect the mucociliary clearance function of the respiratory epithelium.

Antimicrobial peptides play an important role in innate host defense, and are thought to be particularly important at mucosal surfaces that form the initial barrier between the host and the external environment. Defensins and cathelicidins are the principal families of human antimicrobial peptides. They are mainly produced by leukocytes and epithelial cells, and display antimicrobial activity against a broad spectrum of bacteria, fungi, and enveloped viruses. The only member of the cathelicidin family identified in humans is hCAP-18 (human cationic antibacterial protein of 18kDa); LL-37 is its carboxy-terminal antibacterial peptide, which comprises 37 amino acid residues. Expression of hCAP-18/LL-37 is shown in neutrophils and throughout epithelia in many organs, including surface epithelia of conducting airways and submucosal glands. LL-37 is considered to play an important role in the first line of defense against local and systemic infection and in systemic invasion of pathogens at sites of inflammation and wounds. LL-37 has been shown to have a broad-spectrum antimicrobial activity and is able to neutralize LPS.

We previously reported the development of the novel LL-37 derived antimicrobial peptide P60.4-Ac.
The culture was terminated after 2 weeks at air-liquid interface.

The culture was processed for further analysis and only the outgrowth was analyzed.

**Fixed, Embedding in Paraffin, and Histologic Analysis**

The microporous membranes were fixed in 2% paraformaldehyde at room temperature for 20 minutes by placing the paraformaldehyde under and on top of the membrane. Subsequently the membranes were dehydrated through a graded series of ethanol, starting at 50% ethanol for 3 minutes each step. Absolute ethanol was replaced by UltraClear (Klinipath, Duiven, The Netherlands) for 5 minutes. UltraClear was removed and replaced by liquid paraffin and incubated for 2 hours in a 58°C incubator. Next the inserts were removed from their well and the liquid paraffin was poured out of the insert. The insert was placed upside down and stored for 24 hours at room temperature. A preheated scalpel was used to remove the membrane from the inserts and the membrane was cut in two pieces. Both parts of the membrane were embedded in paraffin. After embedding in paraffin, 4-μm sections were cut and stained with hematoxylin-eosin (HE).

**Experimental Design**

A total of five experiments were performed with sphenoid sinus samples of five patients. LPS from *Salmonella typhi*, LTA from *Staphylococcus aureus* (Sigma, St. Louis, MO) and P60.4-Ac were added basally in the medium of the air-liquid interface cultures. Basal medium including LPS, LTA, and P60.4-Ac was refreshed every 2 days. P60.4-Ac was synthesized as previously described.15 Every experiment performed consisted of a negative control, LPS (1 μg/mL), LTA (1 μg/mL) or P60.4-Ac, all in duplicate. To investigate the effect of P60.4-Ac on the induced effect by LPS or LTA, two different concentrations of peptide (100 ng/mL and 1,000 ng/mL) were incubated with or without LPS and LTA.

**Image Analysis**

The thickness of the mucosal layer was quantified by digital image analysis (Qwin, Leica Microsystems, Wetzler, Germany). Adjacent digital images (400× magnification) covering the full diameter of the culture filter were taken. On each image, six measurements of the epithelial layer thickness and number of cell layers were conducted. This way, in total, approximately 80 locations per filter were analyzed. A second investigator (JJG) also scored the images.

**Statistical Analysis**

The number of cell layers and the thickness of the mucosal layer were analyzed for statistical differences using the Student’s *t* test for paired samples. Differences at a *P* value of <.05 were considered statistically significant.

**RESULTS**

**LPS and LTA Increase Thickness of the Mucosal Layer**

Morphologic changes in cultured human sphenoid epithelial cells after incubation with LPS or LTA were described in detail previously.6 In short, sphenoid mucosa cultured for 2 weeks differentiated from pseudostatified epithelium to squamous and ciliated epithelium with few goblet cells (Fig. 1A). In the presence of LPS and LTA (1 μg/mL), an increased thickness of the mucosal layer (LPS: *P* = .028; LTA: *P* = .007), and an increase of the number of cell layers (LPS: *P* = .005; LTA: *P* = .009) was observed.
These results indicate that these bacterial products induce cell proliferation and differentiation.

**P60.4-Ac Neutralizes LPS- and LTA-Induced Effects on Air-Liquid Interface Cultured Sphenoid Cells**

To study the neutralizing effect of P60.4-Ac on LPS and LTA, two concentrations of P60.4-Ac with and without LPS or LTA were added to the basal medium of air-liquid interface cultures of sphenoid mucosa. 100 ng/mL P60.4-Ac significantly \( (P = .017) \) inhibited the ability of LPS (1 μg/mL) to increase the thickness in the epithelial layer (Figs. 1D and 2). There was no significant effect on the thickness of the mucosal layer and no effect in the number of cell layers when 1 μg/mL P60.4-Ac was combined with LPS (Figs. 1E, 2, and 3). Both 100 ng/mL and 1,000 ng/mL P60.4-Ac significantly inhibited the ability of LTA to increase the epithelial thickness (100 ng/mL peptide: \( P = .008 \); 1,000 ng/mL peptide: \( P = .009 \)) (Figs. 1F and G, and Fig. 2). When P60.4-Ac at 1,000 ng/mL was added alone to the cultures (Fig. 1H), the thickness of the epithelial layer was higher, but this effect was not statistically significant \( (P = .107) \).

The analysis of number of cell layers showed similarly significant results compared to the results of the thickness of the mucosal layer. When 100 ng/mL P60.4-Ac was added to LPS \( (P = .004) \) or when 100 ng/mL and 1,000 ng/mL P60.4-Ac was added to LTA, a marked suppression of the number of epithelial layers was noted (100 ng/mL peptide: \( P = .018 \); 1 μg/mL peptide: \( P = .014 \) (Fig. 3). P60.4-Ac alone at 1,000 ng/mL had a non-significant \( (P = .08) \) effect on epithelial cell layers.

**DISCUSSION**

The upper respiratory tract is lined with a ciliated pseudostratified epithelium containing cilia-bearing and secretory cells. The explants used in our culture experiments displayed similar morphologic characteristics. The outgrowth of the explants contained more flattened cells, and ciliated cells were less abundantly present than in the original biopsy specimen. The cultured sphenoid sinus epithelial cells had developed a more squamous phenotype with few goblet cells. High concentrations of LPS and LTA induced thickening of the mucosal layer. These results indicate that LPS and LTA are able to induce metaplasia and hyperplasia of respiratory sinus epithelial cells in culture.

P60.4-Ac, a synthetic antimicrobial peptide based on the structure of the cathelicidin LL-37, has neutralizing effects on the pro-inflammatory activity of LPS and LTA.\(^{15}\)

The aim of this study was to investigate whether P60.4-Ac could neutralize the LPS- and LTA-induced effects on cultured sphenoid mucosa cells. In air-liquid interface

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**Fig. 1.** Light microscopic photographs (400× magnification) of sphenoid mucosa cultured at air liquid interface. (A) Negative control (culture medium); (B) with 1 μg/mL lipopolysaccharide (LPS); (C) with 1 μg/mL lipoteichoic acid (LTA); (D) with 1 μg/mL LPS and 100 ng/mL peptide 60.4-Ac; (E) with 1 μg/mL LPS and 1 μg/mL peptide; (F) with 1 μg/mL LTA and 100 ng/mL peptide; (G) with 1 μg/mL LTA and 1 μg/mL peptide; (H) with 1 μg/mL peptide. Differences in thickness and number of the cell layers in the epithelial layer were noted between stimulated and control-treated cultures.

**Fig. 2.** Effect of lipopolysaccharide (LPS) (1 μg/mL) and lipoteichoic acid (LTA) (1 μg/mL) in the presence or absence of P60.4-Ac on the thickness of the epithelial layer in air-exposed cultured sphenoid mucosa. After a 2-week culture period, thickness of the cultured epithelial layer was analyzed by image analysis. Data were analyzed for statistical differences by Student t-test for paired samples. *< .05 versus control.
cultures of sphenoid epithelial cells, the peptide P60.4-Ac blocked the ability of LPS and LTA to change the morphology of the epithelial layer. These results indicate that P60.4-Ac can neutralize the LPS- and LTA-induced effect on cultured sphenoid epithelial cells.

A variety of stimuli can affect the function of epithelial cells, including microbial components such as LPS and LTA. These microbial components activate epithelial cells through interaction with toll-like receptors (TLRs). TLR4 and TLR2 are expressed on epithelial cells and are involved in cellular responses to LPS and LTA, respectively. Cationic antimicrobial peptides such as LL-37 are known to bind and neutralize LPS and LTA. There appears to be a direct relation between the antimicrobial and LPS-neutralizing properties of LL-37. The hydrophobic and amphipatic features of LL-37 are involved in its ability to neutralize LPS, which involves a direct interaction between LL-37 and LPS, as well as the ability of LL-37 to dissociate LPS aggregates. Although not studied in detail, LTA neutralization by LL-37 may also involve hydrophobic and amphipatic features as well as direct interactions.

Activation of the epithelial cells by LPS or LTA leads to a host innate immune response involving the recruitment of neutrophils and mucin production, but may also be involved in the repair of epithelial cell damage. Repair of epithelial damage includes epithelial proliferation, and both processes have been shown to be mediated in part by activation of the epidermal growth factor receptor (EGFR). It has recently been shown that the mechanism of epithelial cell activation by LPS that results in proliferation and wound repair involves both TLR4, Duox-1, and EGFR signaling pathways. The mechanism by which LTA causes epithelial proliferation has not yet been elucidated, but may involve similar signaling pathways.

LPS and LTA have been shown to cause a dose-dependent increase in thickness of the epithelial layer as well as an increase in mucus-producing cells in cultured epithelial cells, suggesting that bacterial products may induce mucus cell metaplasia, proliferation, and hypersecretion. Results of animal studies also showed the effects of bacterial products on airway mucosa. Addition of endotoxin to the middle ear cavity resulted in proliferation and the formation of secretory epithelium. A single and repeated intratracheal instillation of LPS in mouse lungs induces mucus cell metaplasia and hyperplasia, whereas long-term intratracheal instillation of LPS in hamsters also resulted in mucus cell hyperplasia and persistent lung pathology.

A variety of stimuli, including LL-37, cause epithelial activation via EGFR. LL-37 causes epithelial cell proliferation and wound closure, which is mediated in part by transactivation of the EGFR via metalloprotease-mediated cleavage of membrane-anchored EGFR ligands and activation of mitogen-activated protein kinases (MAPK). Based on the structural and functional homology between LL-37 and P60.4-Ac, we hypothesize that this latter peptide employs similar signaling pathways as LL-37.

For treatment of upper and lower airway infections, antibiotics are still the most frequently used therapy. However, bacterial resistance to antibiotics has increased during the last decades. As a result there is an increasing need to discover, develop, and introduce new drugs. Besides their direct antimicrobial function, antimicrobial peptides can play an important role in the protection of epithelial surfaces through other activities, including their ability to neutralize the proinflammatory activity of the bacterial products LPS and LTA. Antimicrobial peptides have multiple roles as mediators of inflammation, with impact on epithelial and inflammatory cells influencing a range of processes, including immunity, cytokine release, and chemotaxis.

Antimicrobial peptides are interesting candidates as templates for development of novel antibiotics because of their broad-spectrum antimicrobial activity and the observation that bacteria may not easily develop resistance against them. In addition, other activities of antimicrobial peptides distinct from their antimicrobial activity may contribute to their efficacy in vivo.

CONCLUSION

In conclusion, we demonstrate that important morphologic changes induced by LPS and LTA may lead to a disturbance of the mucociliary clearance system in vivo are inhibited by P60.4-Ac. Whether these in vitro findings can be translated to the in vivo situation and can be of
clinical benefit in future management of OME and sinusitis remains to be investigated.

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**BIBLIOGRAPHY**


