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Research Paper

# Influence of lipopolysaccharide on proinflammatory gene expression in human corneal, conjunctival and meibomian gland epithelial cells

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ABSTRACT

*Purpose:* Lipopolysaccharide (LPS), a bacterial endotoxin, is known to stimulate leuokotriene B4 (LTB4) secretion by human corneal (HCECs), conjunctival (HConjECs) and meibomian gland (HMGECs) epithelial cells. We hypothesize that this LTB4 effect represents an overall induction of proinflammatory gene expression in these cells. Our objective was to test this hypothesis.

*Methods:* Immortalized HCECs, HConjECs and HMGECs were cultured in the presence or absence of LPS (15 μg/ml) and ligand binding protein (LBP; 150 ng/ml). Cells were then processed for RNA isolation and the analysis of gene expression by using Illumina BeadChips, background subtraction, cubic spline normalization and GeneSifter software.

*Results:* Our findings show that LPS induces a striking increase in proinflammatory gene expression in HCECs and HConjECs. These cellular reactions are associated with a significant up-regulation of genes associated with inflammatory and immune responses (e.g. IL-1β, IL-8, and tumor necrosis factor), including those related to chemokine and Toll-like receptor signaling pathways, cytokine-cytokine receptor interactions, and chemotaxis. In contrast, with the exception of Toll-like signaling and associated innate immunity pathways, almost no proinflammatory ontologies were upregulated by LPS in HMGECs.

*Conclusions:* Our results support our hypothesis that LPS stimulates proinflammatory gene expression in HCECs and HConjECs. However, our findings also show that LPS does not elicit such proinflammatory responses in HMGECs.

## 1. Introduction

The human ocular surface is inhabited with diversified commensal microorganisms, many of which are bacteria. These organisms account for more than 90% of the eye's surface microbiota, and possess both potent immunoregulatory functions and pathogenic capabilities [1–3]. Indeed, bacteria and their toxins can cause significant, and sometimes irreversible, damage to the ocular surface [4,5]. For example, bacterial keratitis accounts for 41.8%–91.8% of all corneal infections, and is one of the most frequent causes of corneal blindness [6–8]. Further, bacterial conjunctivitis is the major cause of red eye worldwide [9], and bacterial toxins may contribute to the development of obstructed terminal ducts in meibomian gland dysfunction (MGD) [10]. However, the mechanism(s) that underlie the diverse array of these bacterial actions on ocular surface and adnexal epithelial cells have yet to be elucidated.

We hypothesize that one such bacterial mechanism involves a lipopolysaccharide (LPS)-induced proinflammatory gene expression in these epithelial cells. LPS, also known as endotoxin, is a glycolipid of Gram-negative bacteria cell walls that elicits strong inflammatory responses in mammalian cells [11]. LPS is composed of a hydrophilic polysaccharide and a hydrophobic lipid A, which is the toxic component of LPS and activates the host's innate immune system via the Toll-like recepter4/myeloid differentiation factor 2 (TLR4/MD2) receptor complex [12–14]. The lipid transferase LPS-binding protein (LBP) and CD14 catalyze LPS transfer to the TLR4/MD2 complex [14].

In support of our hypothesis, we have previously shown that LPS stimulates leukotriene B4 (LTB4) secretion by human corneal (HCEC), conjunctival (HConjEC) and HMG (HMGEC) epithelial cells, and that this effect is enhanced by co-exposure to LBP [15]. To extend these

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findings, we examined whether LPS and LBP upregulate the expression of numerous inflammatory pathways in these cells.

## 2. Methods

### 2.1. Cell cultures

Immortalized HCEC (from Dr. James Jester, Irvine, CA) [16], HConjEC (from Dr. Ilene Gipson, Boston, MA) [17], and HMGEC [18] were cultured, as previously described [15]. In brief, HCEC and HConjEC were cultured in keratinocyte serum free medium (KSFM) supplemented with bovine pituitary extract (BPE, 25 μg/ml), epidermal growth factor (EGF, 50 ng/ml), penicillin and streptomycin. HMGEC were cultured in KSFM supplemented with 50 μg/ml BPE, 50 ng/ml EGF, penicillin, and streptomycin. Cells were maintained in 75 $cm^2$ flasks and then plated for experiments in 6-well culture dishes (Corning, Lowell, MA). At confluence the cell numbers ranged from 3.8 to 4.9 × $10^5$ cells/well, and varied depending upon the cell type. All cell culture reagents were purchased from Invitrogen Corp. (Carlsbad, CA), except for DMEM/F12, which was obtained from Mediatech, Inc. (Manassas, VA).

After reaching confluence, cells were rinsed with PBS and then cultured in a medium containing DMEM/F12 with 10% FBS, 10 ng/ml EGF, penicillin and streptomycin for 2 days. After this time period, cells (n = 3 wells/cell type/treatment) were incubated in serum-free DMEM/F12 and exposed to vehicle (1% bovine serum albumin [BSA]; Sigma-Aldrich), or LPS (15 μg/ml; E. Coli, strain 0127:B8, lot #050M4094; Sigma-Aldrich, St. Louis, MO) and LBP (150 ng/ml; R&D Systems, Inc. Minneapolis, MN), for 6 h. The LPS and LBP were dissolved in DMEM and the BSA in PBS (Mediatech, Inc., Manassas, VA). The LPS + LBP concentrations used in these studies, as we previously reported [15], were very effective for the stimulation of LTB4 production by ocular surface and adnexal cells.

### 2.2. RNA extraction and gene microarray analysis

Cellular RNA samples were processed for microarray analyses, as previously reported [19].Briefly, total RNA was extracted using RNeasy Mini Kit (Qiagen, Inc., Valencia, CA), according to the manufacturer's instructions. The RNA concentrations and 260/280 nm ratios were determined by using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA). RNA integrity was evaluated by using a RNA Nano 6000 Series II Chip with a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). The RNA samples were further processed by Asuragen (Austin, TX) for quantitation of mRNA levels using microarray expression analysis (HumanHT-12 v.4 Expression BeadChips; Illumina, San Diego, CA), as previously described [20].

Non-log-transformed, background subtracted and cubic spline normalized data were analyzed with commercial software (GeneSifter.net; Geospiza, Seattle, WA). This comprehensive program also generated gene KEGG pathway, ontology and z-score reports. Standardized hybridization intensity values were adjusted by adding a constant, such that the lowest intensity value for a sample equaled 16 [21,22]. BeadChip data were analyzed with Student's t-test (two-tailed, unpaired). All data are accessible for download through the National Center for Biotechnology Information's Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) via series accession number GSE111496.

## 3. Results

### 3.1. LPS + LBP influence on overall gene expression in human ocular surface and adnexal epithelial cells

To determine endotoxin's influence on gene expression in HCECs, HConjECs and HMGECs, we exposed differentiated cells (n = 3 wells/cell type/treatment) to vehicle or LPS + LBP for 6 h and then processed samples for Illumina BeadChip and Geospiza software analyses.

**Table 1**
Influence of LPS + LBP exposure on gene expression in HCECs, HConjECs and HMGECs. Data were evaluated without log transformation. The expression of listed genes was significantly ($p < 0.05$) up regulated in cells exposed to LPS + LBP or vehicle treatment.

| Immortalized Human Epithelial Cell Type | LPS + LBP > Vehicle | Vehicle > LPS + LBP | Total genes |
|---|---|---|---|
| Cornea | 563 | 510 | 1073 |
| Conjunctiva | 786 | 1068 | 1854 |
| Meibomian gland | 613 | 771 | 1384 |

Our results demonstrate that LPS + LBP exert a significant effect on the expression of more than 1000 genes in HCECs, HConjECs and HMGECs (Table 1). The relative direction of this endotoxin impact was similar in all three cell types, with LPS + LBP increasing and decreasing almost the same percentages of genes (i.e. cornea: 52.5% ↑; conjunctiva: 42.4% ↑; meibomian: 44.3% ↑). Some of the most highly up- and down-regulated genes in HCECs, HConjECs and HMGECs following LPS + LBP exposure are shown in Table 2.

There were 9 genes that were upregulated, and 12 genes that were downregulated, in all three cell lines. Examples (with accession numbers) of genes significantly ($p < 0.05$) stimulated by LPS + LBP in all three cell types included those encoding β2-adrenergic receptor (NM_000024), angiopoietin-4 (NM_139314), interleukin-1 (IL-1) receptor-associated kinase 2 (IRAK2; NM_001570), IL-1 receptor antagonist (RA; NM_173843), IL-1α (NM_000575), IL-1β (NM_000576) superoxide dismutase 2 (NM_00102446) and thioredoxin reductase 1 (NM_001093771). Genes significantly ($p < 0.05$) downregulated by LPS + LBP in HCECs, HConjECs and HMGECs included EGF-containing fibulin-like extracellular matrix protein 1 (NM_001039348), G protein-coupled estrogen receptor 1 (NM_001039966), Rab7B (NM_177403), serine protease 23 (NM_007173) and signal transducer and activator of transcription 1 (NM_007315).

### 3.2. Impact of LPS + LBP exposure on pro-inflammatory gene expression in HCECs

Exposure of HCECs to LPS + LBP induced a significant increase in the expression of proinflammatory genes. In fact, of the 20 genes with known functions that were most highly upregulated by LPS + LBP in HCECs, 17 were linked to inflammation (Table 3). This effect was associated with a marked rise in the activity of KEGG pathways mediating a diverse array of inflammatory and immune responses, including cytokine-cytokine receptor interactions and the signaling of Toll-like (TLR), B cell and T cell receptors (Table 4).

Most striking was the influence of LPS + LBP on inflammatory response gene ontologies in HCECs. The endotoxin significantly increased the expression of 52 inflammatory response ontologies with a z-score ≥ 4.0, and another 66 inflammatory response ontologies with z-scores between 2.0 to 4.0. Indeed, 17.7% of the 665 biological process ontologies up-regulated by LPS + LBP were immune-related. Examples of the inflammatory response ontologies stimulated by LPS + LBP in HCECs are shown in Table 5, and include those associated with immune system processes, lymphocyte activation and chemokine production.

### 3.3. Effect of LPS + LBP treatment on pro-inflammatory gene expression in HConjECs

Treatment of HConjECs with LPS + LBP stimulated a significant increase in the activity of numerous pro-inflammatory genes. Of the 25 genes with known functions that were most highly upregulated by LPS + LBP in HConjECs, 15 were linked inflammation (Table 6). This