Lipopolysaccharide-Induced Expression of Intercellular Adhesion Molecule-1 and Chemokines in Cultured Human Corneal Fibroblasts

Naoki Kumagai,1 Ken Fukuda,2 Youichiro Fujitsu,1 Ying Lu,1 Nobubiko Chikamoto,1 and Teruo Nisaida1

PURPOSE. Invasion of bacteria into the corneal stroma induces the infiltration of leukocytes and subsequent corneal ulceration. The role of corneal fibroblasts in the detection of bacterial invasion into the stroma was investigated by examining the in vitro expression of the receptor complex for lipopolysaccharide (LPS), a common component of Gram-negative bacteria, as well as the possible effects of LPS on both the expression of adhesion molecules and the release of chemokines in cultured human corneal fibroblasts.

METHODS. Expression of the LPS receptor complex, intercellular adhesion molecule (ICAM)-1, and the chemokines interleukin (IL)-8 and monocyte chemotactic protein (MCP)-1 was examined by reverse transcription–polymerase chain reaction, enzyme-linked immunosorbent assay, or immunofluorescence analysis.

RESULTS. Corneal fibroblasts were found to contain transcripts encoding toll-like receptor-4, CD14, and MD-2, all of which are components of the LPS receptor complex. The expression of ICAM-1 at the surface of corneal fibroblasts and the amount of ICAM-1 mRNA in the cells were both increased by LPS. Similarly, LPS increased both the release of IL-8 and MCP-1 by corneal fibroblasts as well as the amounts of the corresponding mRNAs in these cells. These various effects of LPS were potentiated by the presence of a low concentration of human serum.

CONCLUSIONS. Corneal fibroblasts may play an important role in the defense system of the cornea by recognizing the presence of LPS and subsequently expressing adhesion molecules and chemokines that promote leukocyte infiltration. (Invest Ophthalmol Vis Sci. 2005;46:114–120) DOI:10.1167/iovs.04-0922

Corneal fibroblasts are the principal cellular component of the corneal stroma. These cells contribute to the maintenance of the normal structure of the stroma through the synthesis and degradation of stromal collagen and by secreting various bioactive substances. We have shown that corneal fibroblasts, but not corneal epithelial cells, probably play an important role in the local accumulation and activation of leukocytes in the cornea by producing various chemokines1–4 and expressing adhesion molecules5 in response to stimulation with inflammatory cytokines. We have also shown that corneal fibroblasts possess the ability to engulf bacteria and other foreign bodies.6 Corneal fibroblasts may thus function as sentinel cells in the defense of the cornea against various external agents.

Bacterial corneal ulcer is a major cause of loss of vision in both developing and developed countries. This condition results from the destruction of collagen fibrils in the corneal stroma by collagenolytic enzymes produced as a consequence of bacterial infection.7 Pathologic examination has revealed the presence of many infiltrated leukocytes, including neutrophils and macrophages, in or surrounding corneal ulcers.7 Both activated fibroblasts and infiltrated leukocytes may contribute to the degradation of stromal collagen.8,9 We have demonstrated an interaction between bacteria and resident corneal fibroblasts, in that elastase produced by Pseudomonas aeruginosa both degrades collagen directly and activates collagen-degrading matrix metalloproteinases produced by corneal fibroblasts.9 However, the mechanism of leukocyte infiltration into the infected cornea has remained unknown. Lipopolysaccharide (LPS), a component of the cell membrane of Gram-negative bacteria, is a potent secretagogue for a variety of cytokines produced by inflammatory cells.10 LPS in tear fluid enters the cornea by diffusion at sites of injury.11 Injection of LPS into the corneal stroma induces an acute inflammatory reaction that is characterized by infiltration of neutrophils and other mononuclear cells and subsequent corneal ulceration. This reaction is suppressed by pretreatment with corticosteroids.12,13 These observations suggest the possibility that corneal fibroblasts detect the presence of LPS and trigger the local infiltration of leukocytes through expression of chemokines and adhesion molecules. However, the role of corneal fibroblasts in the LPS-induced inflammatory reaction has not been determined.

LPS is a cation-shaped amphiphatic molecule with a large hydrophobic component and a small hydrophilic domain.14 The lipophilic portion of LPS, known as lipid A, is highly conserved structurally and is necessary for endotoxic activity.15 In an aqueous environment, LPS forms polymeric aggregates, with the hydrophilic polysaccharide component facing outward and the lipid A region facing inward.14 Polymeric LPS binds to leukocytes poorly and fails to provoke responses at low concentrations. LPS-binding protein (LBP), an acute-phase serum protein produced mostly by the liver, promotes the biological effects of LPS by rendering it monomeric and thereby exposing lipid A, the active component.16 LBP then transfers LPS monomers to the binding site of CD14 on the cell surface.17 The addition of LBP to serum-free cell cultures thus results in enhancement of LPS-induced cellular responses by a factor of 100 to 1000.18 LPS receptors are composed of toll-like receptor (TLR)-4 and CD14.19 TLR-4 possesses both extracellular and intracellular domains and mediates intracellular sig-
naling in response to LPS stimulation. CD14 does not contain an intracellular domain and does not mediate LPS signaling directly. It functions in mammalian cells to recognize a diverse array of bacterial constituents, including LPS, and facilitates LPS signaling through TLR-4. The protein MD-2 has also recently been shown to be essential for the correct intracellular distribution of, and LPS recognition by, TLR-4.

In the current study, we investigated the role of corneal fibroblasts in LPS-induced inflammation associated with bacterial corneal ulceration. The results showed that cultured human corneal fibroblasts expressed TLR-4, CD14, and MD-2, and that stimulation with LPS derived from various bacterial species induced the production by these cells of the chemokines interleukin (IL)-8 (or CXCL8) and monocyte chemotactic protein (MCP)-1 (or CCL2) and of intercellular adhesion molecule (ICAM)-1. Our observations suggest that the recognition of LPS by corneal fibroblasts and the consequent activation of these cells contribute to bacterial corneal ulceration.

METHODS

Materials

Eagle’s minimum essential medium (MEM), fetal bovine serum, and phosphate-buffered saline (PBS) were obtained from Invitrogen/Gibco (Grand Island, NY). All media and cytokines used for cell culture were endotoxin minimized. LPS derived from Escherichia coli, Salmonella minnesota, and P. aeruginosa was obtained from Sigma-Aldrich (St. Louis, MO). Tissue culture dishes were from Greiner Bio-One (Kens- muenster, Austria), and eight-well chamber slides were from Nalg Nunc (Naperville, IL). Human recombinant tumor necrosis factor (TNF)-α was from GenzymeTechne (Minneapolis, MN). A mouse monoclonal antibody (mAb) to ICAM-1 was from PharMingen (San Diego, CA), normal mouse immunoglobulin G (IgG) was from Santa Cruz Biotechnology (Santa Cruz, CA), horseradish peroxidase–conjugated goat antibodies to mouse IgG (1:500 dilution). The cells were washed with PBS and then observed by fluorescence microscope (Axioskop 50; Carl Zeiss, Oberkochen, Germany).

Isolation and Culture of Human Corneal Fibroblasts

Four human corneas were obtained from Mid-America Transplant Service (St. Louis, MO), Northwest Lions Eye Bank (Seattle, WA), and The Eye Bank of Wisconsin (Madison, WI). The donors were white males and females ranging in age from 4 to 65 years. After the center of each donor cornea was punched out for corneal transplantation surgery, the remaining rim of the tissue was used for the present experiments. The human material was used in strict accordance with the basic principles of the Declaration of Helsinki. Corneal fibroblasts were prepared and cultured as described previously. Each donor cornea was digested separately with collagenase to provide a suspension of corneal fibroblasts. The cells from each cornea were cultured independently in MEM supplemented with 10% fetal bovine serum in 60-mm dishes until they had achieved ~90% confluence. They were used for the present studies after four to six passages. The purity of the cell cultures was judged on the basis of both the distinctive morphology of corneal fibroblasts and their reactivity with antibodies to vimentin in immunofluorescence analysis. No contamination with corneal epithelial cells was detected.

Whole-Cell ELISA for ICAM-1

A whole-cell ELISA for ICAM-1 was performed as described, with minor modifications. Corneal fibroblasts (1 × 10^6 cells per well) were grown in 96-well, flat-bottomed microtiter plates for 72 hours. The culture medium was then changed to serum-free MEM, and the cells were incubated for an additional 24 hours. After replacement of the medium with MEM supplemented with various concentrations of LPS or TNF-α (positive control), in the absence or presence of 0.5% human serum (AB type), the cells were incubated for up to 48 hours, washed twice with PBS, and fixed for 15 minutes at room temperature with PBS containing 1% paraformaldehyde. The cells were then washed with PBS containing 0.1% bovine serum albumin (BSA), incubated for 1 hour at 37°C with an mAb to ICAM-1 (1:10,000 dilution) in PBS containing 1% BSA (PBS-BSA), washed three times with PBS-BSA, and incubated in PBS-BSA for 1 hour at 37°C with horseradish peroxidase–conjugated goat antibodies to mouse IgG. After three washes with PBS-BSA, the cells were incubated for 15 minutes in the dark with 100 μL of TMB solution, and the reaction was then terminated by the addition of 50 μL of 1 M H_2SO_4. The absorbance of each well was determined at a wavelength of 450 nm with a microplate reader (MPR AHI, Tosoh, Tokyo, Japan).

Immunofluorescence Analysis of ICAM-1 Expression

Corneal fibroblasts (5 × 10^5) were transferred to eight-well culture slides and cultured for 72 hours. The medium was changed to serum-free MEM, and the cells were cultured for an additional 24 hours, after which they were incubated for 24 hours in MEM containing LPS derived from E. coli, S. minnesota, and P. aeruginosa (10 ng/mL) in the absence or presence of 0.5% human serum (AB type). The cells were washed twice with PBS and then fixed for 15 minutes at room temperature with PBS containing 1% paraformaldehyde. After three washes with PBS-BSA, the cells were incubated in PBS-BSA for 1 hour at room temperature with a mouse mAb to ICAM-1 (1:1000 dilution) or normal mouse IgG (control), washed three times with PBS-BSA, and incubated for 1 hour with Alexa Fluor 488–conjugated goat antibodies to mouse IgG (1:500 dilution). The cells were washed with PBS and then observed by fluorescence microscope (Axioskop 50; Carl Zeiss, Oberkochen, Germany).

ELISA for IL-8 and MCP-1

The concentrations of IL-8 and MCP-1 in culture supernatants were determined by ELISA, as previously described, with measurement of absorbance at 450 nm. The limit of detection was 5 pg/mL. Given that the morphology and number of cells were not affected by incubation with LPS for 24 hours, the concentrations of chemokines in the culture supernatants were normalized by expression as nanograms of chemokine per 1 × 10^6 cells.

Reverse Transcription and Quantitative Real-Time Polymerase Chain Reaction

The medium of corneal fibroblasts cultured in 100-mm dishes was changed to serum-free MEM, and the cells were cultured for an additional 24 hours. After incubation for a further 6 hours in MEM supplemented with LPS (10 ng/mL) and 0.5% human serum (AB type), the fibroblasts were washed with PBS, and total RNA was extracted with the use of a kit. The extracted RNA was subjected to RT also with the use of a kit, and the abundance of specific cDNAs was then quantified by real-time PCR with a thermocycler (LightCycler; Roche Molecular Biochemicals, Indianapolis, IN), as described previously. The size of the PCR products was verified by electrophoresis on a 4% agarose gel, and the PCR products were visualized with ethidium bromide (1 μg/mL) and DNA molecular size markers (Marker 11) were from Nippongene (Toyama, Japan), and agarose was from FMC Bioproducts (Rockland, ME).

Effects of LPS on Corneal Fibroblasts 115
Control fibroblasts contained a small amount of ICAM-1 mRNA, the abundance of which was increased slightly by incubation of the cells with LPS or human serum alone (Fig. 2). Stimulation of the cells with *E. coli*, *S. minnesota*, or *P. aeruginosa* LPS in the presence of serum induced ~35-, ~28-, and ~11-fold increases, respectively, in the abundance of ICAM-1 mRNA compared with control values.

Effect of LPS on Surface Expression of ICAM-1 by Human Corneal Fibroblasts

The effect of LPS on the expression of ICAM-1 at the surface of human corneal fibroblasts was examined by whole-cell ELISA after culture of the cells for 24 hours in the presence of various concentrations of LPS derived from *E. coli*, *S. minnesota*, or *P. aeruginosa*. In the absence of human serum, LPS at concentrations as high as 100 ng/mL induced only a small increase in ICAM-1 expression (Fig. 3). In the presence of 0.5% human serum (AB type), however, LPS induced a marked dose-dependent increase in the amount of ICAM-1 at the cell surface, with the maximum effect apparent at a concentration of 1 ng/mL of *E. coli* and *S. minnesota* LPS and at >100 ng/mL of *P. aeruginosa* LPS. The increase in ICAM-1 expression was statistically significant at concentrations of ≥0.1 ng/mL of *E. coli* and *S. minnesota* LPS and of ≥1 ng/mL of *P. aeruginosa* LPS. We next examined the dose dependence of the facilitatory effect of human serum on the induction of ICAM-1 expression by LPS. In the absence of LPS, human serum induced a slight increase in ICAM-1 expression (Fig. 4). In the presence of LPS (10 ng/mL), however, human serum induced a marked dose-dependent increase in ICAM-1 expression that was at its maximum at a serum concentration of 0.1%. Human serum had no effect on the induction of ICAM-1 expression by TNF-α. Similar effects of LPS and human serum on the expression of ICAM-1 were

![Graph](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933230/)
observed with corneal fibroblasts isolated from three additional donors (data not shown). No relation between the extent of ICAM-1 expression and the age or sex of the corneal donors was apparent. Given that the responses of the cells from the four different donors were virtually identical, we performed subsequent experiments with corneal fibroblasts isolated from one donor.

The effect of LPS (10 ng/mL) in the presence of 0.5% human serum on surface ICAM-1 expression in corneal fibroblasts was time dependent, with statistically significant increases being apparent at ≥6 hours with E. coli, S. minnesota, or P. aeruginosa LPS (Fig. 5). We also examined the expression of ICAM-1 in corneal fibroblasts by immunofluorescence analysis. Although no specific fluorescence was detected in cells incubated without LPS and serum, cells exposed to E. coli, S. minnesota, or P. aeruginosa LPS (10 ng/mL) in the absence of serum or those exposed to serum in the absence of LPS exhibited a low level of ICAM-1–specific immunofluorescence (Fig. 6). In the presence of human serum, LPS derived from each of these bacteria induced a marked increase in ICAM-1 immunofluorescence. The fluorescence was most prominent around the edges of the cells, indicative of surface expression of ICAM-1.

**Effects of LPS on the Abundance of Chemokine mRNAs in Human Corneal Fibroblasts**

We next examined the effects of LPS on the abundance of IL-8 and MCP-1 mRNAs in corneal fibroblasts by RT and quantitative real-time PCR analysis. Corneal fibroblasts were cultured for 6 hours with LPS (10 ng/mL) in the absence or presence of human serum (0.5%), after which total RNA was isolated and subjected to RT-PCR. Consistent with the chemokine release data, corneal fibroblasts constitutively expressed the IL-8 (Fig. 7A) and MCP-1 (Fig. 7B) genes. The abundance of both IL-8 and MCP-1 mRNAs was increased by stimulation of the cells with LPS in the presence of human serum. LPS derived from E. coli, S. minnesota, or P. aeruginosa induced ~112-, ~121-, and ~35-fold increases in the amount of IL-8 mRNA and ~35-, ~42-, and ~22-fold increases in the amount of MCP-1 mRNA, respectively. Again, the effects of LPS on chemokine mRNA abundance were smaller in the absence of human serum.

**Effects of LPS on Chemokine Release by Human Corneal Fibroblasts**

Finally, we examined the effects of LPS on the release of the CXC chemokine IL-8 and the CC chemokine MCP-1 from human corneal fibroblasts. The cells were cultured for 24 hours with LPS (10 ng/mL) derived from E. coli, S. minnesota, or P. aeruginosa, in the absence or presence of 0.5% human serum, and the amount of IL-8 or MCP-1 in the culture supernatant was then determined by ELISA. Corneal fibroblasts constitutively released small amounts of IL-8 (Fig. 8A) and MCP-1 (Fig. 8B) into the culture medium. The concentration of these cytokines in culture supernatants was greatly increased, however, by the presence of LPS and human serum. LPS exhibited smaller
effects on the release of IL-8 and MCP-1 in the absence of human serum.

**DISCUSSION**

We have demonstrated the expression of LPS receptor genes by human corneal fibroblasts. In the presence of low concentration of human serum, LPS derived from three different bacterial species induced the expression of ICAM-1 at the surface of human corneal fibroblasts and increased both the release of the chemokines IL-8 and MCP-1 and the abundance of the corresponding mRNAs in these cells. These results suggest that corneal fibroblasts detect the presence of bacteria as a result of their expression of LPS receptors and that they may regulate the local infiltration of leukocytes through their expression of adhesion molecules and their production of chemokines. The marked facilitatory effect of human serum on both the potency and efficacy of LPS with regard to its actions in corneal fibroblasts suggests that the presence of LBP in normal serum is essential for the activation of these cells via their LPS receptors.

We showed that human corneal fibroblasts express the genes for the LPS receptor component TLR-4 and MD-2, the latter of which is essential for the proper subcellular distribution of TLR-4. We also verified that these cells express the CD14 gene. These observations indicate that human corneal fibroblasts express an LPS receptor complex composed of TLR-4, CD14, and MD-2, similar to that expressed by immune cells such as dendritic cells. Stimulation with LPS derived from three different species of Gram-negative bacteria (E. coli, S. minnesota, and P. aeruginosa) increased the expression of ICAM-1, IL-8, and MCP-1 by corneal fibroblasts, indicating that these cells are activated by LPS regardless of its bacterial origin. Given that the chemotactic activities of IL-8 and MCP-1 as well as adhesion to ICAM-1 in structural cells mediate the local infiltration and activation of neutrophils and monocytes, our results suggest that the activation of corneal fibroblasts by direct stimulation with LPS may be an important step in the pathogenesis of bacterial infection of the cornea. The effects of LPS on corneal fibroblasts were greater for that derived from E. coli or S. minnesota than for that isolated from P. aeruginosa, probably at least in part because LPS from P. aeruginosa has a smaller lipid A component and is substantially less toxic than LPS from the other two bacterial species.

We previously demonstrated that corneal fibroblasts, but not corneal epithelial cells, express the CC chemokines eotaxin (CCL11) and TARC (CCL17) as well as vascular cell adhesion molecule (VCAM)-1 in response to stimulation with Th2 cytokines. Corneal fibroblasts thus may play an im-

---

**FIGURE 6.** Immunofluorescence analysis of the effect of LPS on ICAM-1 expression by human corneal fibroblasts. Cells were incubated for 24 hours in the absence (A–D) or presence (E–L) of 0.5% human serum and either without (A, E, I) or with LPS (10 ng/mL) derived from E. coli (B, F, J), S. minnesota (C, G, K), or P. aeruginosa (D, H, L). The expression of ICAM-1 was then examined by immunofluorescence analysis with antibodies specific for this protein (A–H). As a negative control, cells were stained with normal mouse IgG (I–L). Scale bar, 50 µm.

**FIGURE 7.** Effects of LPS on the abundance of chemokine mRNAs in human corneal fibroblasts. Cells were incubated for 6 hours, with or without LPS (10 ng/mL) from the indicated bacterial species, in the absence (□) or presence (■) of 0.5% human serum. The abundance of IL-8 (A) and MCP-1 (B) mRNAs in the cells was then determined by RT and quantitative real-time PCR. Data, normalized on the basis of the abundance of GAPDH mRNA, are expressed in arbitrary units (with 1 AU corresponding to the value for cells incubated without both LPS and serum) and as the mean ± SEM of quadruplicates from an experiment that was repeated three times with similar results. *P < 0.05 (unpaired Student’s t-test) versus the corresponding value for cells incubated in the absence of LPS.
important role in the corneal destruction associated with ocular allergic diseases by promoting the infiltration of leukocytes into the corneal stroma. Our present results further indicate the importance of fibroblasts in corneal defense and inflammation. These cells are thus likely to induce the infiltration of leukocytes into the cornea not only in response to inflammatory cytokines but also on direct interaction with bacteria. Fibroblasts express different structural and functional properties depending on their location within the body and local stimuli, and these different phenotypes are maintained even after prolonged culture in vitro. We thus showed that TARC expression is regulated differentially by cytokines in fibroblasts derived from human cornea, skin, and lung. The responses of fibroblasts to LPS also appear to differ according to the origin of the cells. For example, lung fibroblasts express MCP-1 but not IL-8 in response to stimulation with LPS, but, as we have now shown, the expression of both of these chemokines was induced by LPS in corneal fibroblasts. The different responses of fibroblasts to LPS, as well as to other inflammatory mediators, may contribute to the distinct characteristics of inflammatory reactions in different organs.

References


