Lipoteichoic Acid Selectively Induces the ERK Signaling Pathway in the Cornea

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PURPOSE. To identify signal transduction pathways and gene expression induced by the bacterial cell wall component lipoteichoic acid (LTA) in human corneal keratocytes.

METHODS. Human corneal keratocytes were cultured in the presence of 6.25 to 50 μg/ml LTA from Staphylococcus aureus. Induced DNA-binding of NF-κB was determined by electrophoretic mobility shift assays (EMSA). Activation of MAP-kinase signaling pathways (p38, JNK-1/2, ERK-1/2, Elk 1, MEK-1/2, c-Raf) was evaluated by Western blotting using phospho-specific antibodies. To investigate the effect of LTA signaling on gene expression, keratocytes were transfected with a luciferase reporter gene under the control of serum response elements (SREs). LTA-induced gene expression was quantified using luciferase assays.

RESULTS. Exposure of various concentrations of LTA up to 24 hours did not lead to activation of NF-κB, whereas TNF-α potently induced this transcription factor. A systematic analysis of LTA-activated MAPK pathways revealed no significant effects on JNK and p38, but a dose- and time-dependent phosphorylation of members of the ERK pathway. Analysis of the ERK-activating signaling cascade showed LTA-induced phosphorylation of ERK-1, MEK1/2, and c-Raf. ERK activity resulted in an enhanced transcription of an SRE-controlled reporter gene.

CONCLUSIONS. LTA induces SRE-enhanced gene transcription in corneal keratocytes that is selectively mediated by the ERK pathway. Therefore, it seems possible that components of the bacterial cell wall such as LTA can alter the transcriptional program within the corneal stroma and thereby trigger an inflammatory response. (Invest Ophthalmol Vis Sci. 2002;43: 2272-2277)

Bacterial keratitis often leads to localized inflammation associated with cellular injury and tissue destruction. Bacteria can directly invade the cornea and generate proteolytic enzymes as well as toxins. However, inflammation and tissue destruction can also occur without the presence of bacteria in the cornea, such as in catarrhal infiltrates that are complications of long lasting blepharocconjunctivitis due to staphylococci and streptococci.1,2 Early investigations of the pathomechanism of this disease have shown that topical application of devitalized staphylococci can cause keratitis and conjunctivitis.3 Corneal lesions contain neutrophils but no bacteria and are thought to be the result of an antigen-antibody reaction.4,5 The inflammation is believed to be initiated by focal breakdown of the epithelial barrier that allows staphylococcal antigens to reach the corneal stroma.4,5 Alternatively, toxins from staphylococcus may be causative for blepharocconjunctivitis and inflammatory infiltrates.6,7 Injection of alpha toxin in the stroma has recently been shown to cause severe corneal damage by initiating apoptosis and necrosis of corneal epithelial cells.6,9 This suggests that various secreted products but also components of bacteria such as the cell wall could be implicated in the mechanism of stromal infiltrates.

The corneal wall of Gram-positive bacteria consists of peptidoglycan connected by peptide chains and cross-bridges. Lipoteichoic acid (LTA) is a component of the cell wall in most Gram-negative bacteria.10 It represents a phosphate-containing polymer that consists of a hydrophobic glycolipid moiety and a hydrophilic chain. Numerous functions have been described for LTA and its antigenic properties as well as the activation of complement seem to be most relevant for inflammation.10,11 However, LTA has been shown to also directly activate inflammation by modulating the function of inflammatory cells such as macrophages or monocytes. LTA can induce the secretion of various cytokines such as interleukin (II)-1β, II-6, or tumor necrosis factor (TNF)-α.12-14 Furthermore LTA affects various chemokines that represent peptide mediators with potent effects on leukocyte chemotaxis and activation. Macrophage inflammatory protein (MIP)-1α has shown to be upregulated in samples from human endocarditis secondary to Staphylococcus aureus, and MIP-1α mRNA was upregulated by LTA.15 These data suggest that LTA can selectively modify gene transcription in various cell types and thereby augment and possibly initiate tissue inflammation.

A prerequisite for the activation of gene transcription is the induction of signal transduction cascades that activate transcription factors. The identification and characterization of signal transduction pathways induced by inflammatory processes is of great interest to develop new therapeutic agents targeting these signaling components. In an effort to learn about signal transduction in the context of corneal inflammation and to further study the mechanism of catarrhal corneal infiltrates, we systematically investigated the LTA inducibility of signaling pathways that are frequently involved in the inflammatory process: The mitogen-activated protein kinases (MAPKs) p38, JNK (cJun N-terminal kinase), and ERK (extracellular-signal-regulated kinase) and the inducible transcription factor NF-κB. Here we show that LTA triggers activation of the Raf/MEK-1/2/ERK-1/2/Elk 1 cascade, thus resulting in elevated transcription of reporter genes controlled by SREs.

MATERIALS AND METHODS

Cell Culture and Stimulations

Experiments were performed in adherence to the Declaration of Helsinki. Human corneas stored for <24 h in Likorol (Chauvin-Opsia, Labège Cedex, France) at 4°C came from donors ranging in age between 30 and 65 years. Stromal keratocytes were cultured as outgrowth cultures at 37°C and 5% CO2 in Dulbecco’s modified Eagle medium (DMEM) containing 10% (vol/vol) heat-inactivated fetal calf

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serum (all from Gibco BRL Life Technologies, Paisley, Scotland) as described before. For analysis, preconfluent cells (passage 2–4) grown in serum-free medium for 24 hours were stimulated as specified. LTA from *Staphylococcus aureus* (Sigma, St. Louis, MO) was used at concentrations between 6.25 and 50 μg/ml and TNF-α (Roche Molecular Biochemicals, Mannheim, Germany) at concentrations of 2000 U/ml.

**Electrophoretic Mobility Shift Assays and Western Blotting**

Cells were lysed in TOTEX buffer (20 mM Hepes/KOH, pH 7.9, 0.35 M NaCl, 20% (vol/vol) glycerol, 1% (vol/vol) NP-40, 1 mM MgCl₂, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, and 0.5 mM sodium vanadate) and incubated on ice for 30 minutes. After centrifugation, equal amounts of supernatant were tested for DNA binding activity to a double-stranded, 32P-labeled oligonucleotide. The sense sequence of the oligonucleotide was as follows: 5’-AGTTAGGGGACTTTCCGAGGC-3’. The NF-κB binding site is underlined. The binding reaction was performed as described, and subsequently the free and complexed oligonucleotides were separated by electrophoresis on a native 4% polyacrylamide gel. The gel was dried after electrophoresis and exposed to an x-ray film (Amersham Hyperfilm; Amersham, Braunschweig, Germany).

For Western blotting, equal amounts of protein were separated on a reducing SDS polyacrylamide gel. Subsequently the proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA) using a semidy blotting apparatus (Bio-Rad Laboratories, Munich, Germany) as described. After blocking of the membrane in TBST buffer (25 mM Tris/HCl, pH 7.4, 137 mM NaCl, 5 mM KCl, 0.7 mM CaCl₂, 0.1 mM MgCl₂, 0.05% [vol/vol] Tween-20) containing 5% (wt/vol) skim milk powder, the membrane was incubated in a small volume of TBST containing various dilutions of the primary antibodies. The following antibodies were used: phospho-specific antibodies recognizing the phosphorylated forms of p38, JNK, ERK-1/2, Elk1, MEK1/2, and c-Raf (New England Biolabs) as well as phospho-FAK (Biosource, Camarillo, CA). The respective proteins were detected with an appropriate secondary antibody coupled to horseradish peroxidase. Secondary antibodies were visualized by enhanced chemiluminescence according to the instructions of the manufacturer (Amersham).

**Transfections and Luciferase Assays**

The (SRE)2-Luc reporter plasmid was transfected to cultured corneal keratocytes (CCK) using the Lipofectamine Plus reagent from Gibco BRL Life Technologies according to the manufacturer’s instructions. Transfected cells were then incubated in serum-free medium, followed by the addition of LTA (50 μg/ml) for 18 hours in the presence or absence of 100 μM PD 98059. Harvested cells were lysed in reporter lysis buffer (25 mM Tris-phosphate, 2 mM DTT, 2 mM EDTA, 10% [vol/vol] glycerol, 1% [vol/vol] Triton X-100). Luciferase activity was determined in a luminometer (Duo Lumat LB 9507; Berthold) by injecting 50 μl of assay buffer (40 mM Tricine, 2.14 mM (MgCO₃)₄ Mg(OH)₂× 5 H₂O, 5.34 mM MgSO₄, 0.2 mM EDTA, 66.6 mM DTT, 540 μM CoA, 940 μM luciferin, 1.06 mM ATP) and measuring light emission for 10 seconds.

**RESULTS**

**NF-κB Is Not Induced by LTA in Cultured Corneal Keratocytes**

To characterize the inflammatory process elicited by exposure of human keratocytes to LTA from *Staphylococcus aureus* at the molecular level, we investigated signaling pathways that are frequently involved in inflammation. Because the inducible transcription factor NF-κB controls the expression of a plethora of immunologically relevant target genes and therefore is a key regulator of the inflammatory response, we tested the impact of LTA treatment on NF-κB activation in stromal keratocytes from human corneas. Cells were incubated with LTA (50 μg/ml) for various periods ranging from 15 minutes to 4 hours. Electrophoretic mobility shift assays (EMSA) revealed that LTA failed to activate NF-κB in corneal cells at all time points tested (Fig 1). In contrast, administration of TNF-α strongly triggered the activation of NF-κB, confirming that corneal keratocytes contain NF-κB in its activable form.

**Identification of the LTA-Inducible MAPK Pathway in Corneal Keratocytes**

Besides the NF-κB pathway, inflammation-induced cellular response involves also other signal transduction pathways such as activation of MAPKs. To identify the LTA-regulated MAPK(s) on a systematic basis, human corneal keratocytes were stimulated for various periods with LTA (50 μg/ml) and subsequently analyzed for phosphorylation and thus activation of the various MAPKs by immunoblotting using phospho-specific antibodies. These experiments revealed a LTA-triggered phosphorylation of ERK-1 and ERK-2 (also called p44 and p42 MAPK; Fig. 2A). Basal ERK-2 phosphorylation was significantly augmented 10 minutes after the addition of LTA and reached its maximum after an incubation period of 1 hour. Phosphorylation of ERK-1 became detectable after 10 minutes and
peaked, similar to the phosphorylation of ERK-2, 1 hour after the addition of LTA. In parallel, extracts prepared from LTA-stimulated cells were also tested for the phosphorylation of p38 and JNK1/2 using phosphospecific antibodies. The experiments revealed that these MAPKs are not significantly induced in response to LTA, thus indicating that the ERK pathway but not JNK or p38 is activated in the context of the LTA-induced corneal cellular response.

Although the time-course experiments had shown that LTA-induced ERK phosphorylation was maximal after 1 hour, we subsequently determined the dose dependence of LTA-mediated ERK activation (Fig. 2B). LTA at a concentration of 50 µg/ml resulted in a maximal activation of ERK-1/2. Corneal keratocytes were stimulated for 1 hour with the specified concentrations of LTA and analyzed for ERK-1/2 phosphorylation by Western blotting. Representative experiments are shown.

Characterization of the LTA-Induced ERK Activation Pathway

ERK kinases are activated by a G-protein, which in turn stimulates an activation module consisting of three kinases: a MAPK kinase kinase (MAPKKK), that phosphorylates and activates a MAPK kinase (MAPKK), which then phosphorilates the MAPK ERK. The ERK activating G-protein is Ras, the MAPKKK is Raf, which in turn stimulates the MAPKs MEK-1 and MEK-2.22 Interestingly, the ERK pathway can also be activated by FAK (focal adhesion kinase), a key mediator for multiple signals derived from membrane receptors and the extracellular matrix.23

To test which level of the ERK activating signaling cascade is affected by LTA, human corneal keratocytes were treated for various periods with this bacterial cell wall component and the phosphorylation status of c-Raf and MEK-1/2 was analyzed by immunoblotting using phosphospecific antibodies (Fig. 3). These experiments revealed a LTA-triggered phosphorylation of c-Raf and MEK1/2, indicating that LTA affects these early events in the ERK signaling cascade. Interestingly, phosphorylation of MEK-1/2 and c-Raf occurred with distinct kinetics, because MEK-1/2 phosphorylation was already maximal 20 minutes after administration of LTA, whereas phosphorylation of c-Raf reached its maximum after 1 hour.

To extend the analysis of the ERK signaling pathway to substrates of ERK-1/2, we measured LTA-mediated phosphorylation of Elk1, one of the numerous ERK substrate proteins. These experiments revealed that LTA leads to a time-dependent increase in Elk1 phosphorylation (Fig. 3), showing that the stimulatory effects of LTA are transmitted to the level of ERK effector proteins.

To confirm that LTA induces the activation of the ERK pathway through MEK, we used the MEK-inhibitor PD 98059. Cultured stromal cells were starved in serum-free medium overnight and incubated with 100, 10, or 1 µM PD98059 in the presence of 50 µg/ml of LTA for 1 hour followed by protein isolation and Western blots. The result as shown in Figure 4...
LTA strongly depend on the microorganism from which it was isolated. For example LTA from *Streptococcus pyogenes* has no effect on the expression of cell adhesion molecule (CAM), but causes an increase in IL-8 expression. In contrast, LTA from *S. aureus* inhibits lipopolysaccharide (LPS)-induced CAM expression and reduces LPS-triggered IL-8 expression.

The signaling pathways mediated by LTA remain incompletely defined. Like LPS, which is a component of the cell membrane of Gram-negative bacteria, LTA isolated from Gram-positive bacteria can elicit expression of inflammatory cytokines, which lead to most of the clinical manifestations of bacterial infection. Although the direct toxic effect of bacterial exoproteins such as alpha toxin in *Staphylococcus* blepharitis is well established, the role of inflammatory cytokines needs to be determined.8,9,29 Both staphylococci as well as LPS have been shown to induce cytokines such as interleukin-1 in corneal epithelial cells.30 Phagocytosis of formalin fixed staphylococci can significantly enhance the number of Langerhans cells in the cornea and this effect seems to be mediated by interleukin-1.51 In addition stromal cells can upregulate cytokine production in response to inflammatory stimuli such as TNF-α.52 Here we report that LTA induces signaling pathways in the cornea. Furthermore, preliminary experiments suggest that LTA can induce inflammatory cytokines in human corneal stroma that could initiate stromal keratitis (data not shown).

The initial event in signal transduction is receptor binding. LTA binds to the glycosphosphatidylinositol-anchored membrane protein CD14 and mediates cellular activation by toll-like receptors (TLR). Macrophages without TLR-4 lack the response to LTA,53 showing that LTA signals via this receptor. Previous results suggesting the involvement of TLR-2 in the LTA response54 are due to impurities within the LPS.55 The intracellular domain of the TLRs resembles that of the IL-1 receptor (IL-1R), thereby defining the Toll/IL-1R superfamily.56 Signals transduced by Toll/IL-1R family members result in the activation of protein kinases, but the kinases induced by LTA remain poorly defined. Pharmacological evidence points to the involvement of tyrosine kinases, because their inhibition prevents the LTA-induced expression of inducible NO synthase (iNOS).57 Tyrosine phosphorylation may also be important for the activation of the Raf/MEK/ERK signaling pathway, because the catalytic activation of c-Raf involves tyrosine phosphorylation events.58 Alternatively it may be possible that the Raf/MEK/ERK cascade is activated by NO produced on LTA stimulation.59 NO is known to activate Ras, which results in the recruitment and activation of c-Raf.60 This pathway can also be activated by the highly reactive oxygen species peroxynitrite,11 which is formed via a radical coupling reaction of NO with superoxide. Activated c-Raf signals to the dual specificity kinases MEK-1/2, which activate the ERKs via phosphorylation of a Thr-Glu-Tyr motif in the activation loop. Our data indicate that moderate concentrations of LTA preferentially activate ERK-1. This is particularly interesting, because most transfection and gene transfer experiments suggest that ERK-1 and ERK-2 are functionally equivalent.22 It is so far unclear why two kinases exist, but the fact that both kinases are generally coexpressed and the genes encoding ERK-1 and ERK-2 are even found in worms may indicate functional diversification of the two kinases.

Although the importance of JNK and p38 for the inflammatory process is well documented, there is growing evidence that also the ERK signaling pathway—which is mainly associated with central functions such as proliferation, cell survival, and differentiation—contributes to inflammatory events as shown by the following examples: IL-5 stimulation of cosinophils results in the ERK-dependent biosynthesis of leukotrienes.52 The involvement of ERK for the arachidonic acid metabolism is also evident from pharmacological ap-
proaches, because the ERK inhibitor PD 98059 prevents induced leukotriene C4 production in immunologically activated human basophils.43 ERK is known to participate in the expression of inflammatory cytokines. The ERK inhibitor PD 98059 negatively interferes with CD40-mediated induction of IL-1β and TGF-α production in monocytes44 and platelet-factor-mediated upregulation of the cytokine RANTES.45 The relevance of the ERK signaling pathway for the inflammatory process is also revealed by a physiological inflammation model: Phorbol ester-induced edema and swelling of the mouse ear is prevented by topical treatment of ears with the MEK-1/2 inhibitor U0126.46 Future studies must reveal whether selective inhibitors of the Raf/MEK/ERK cascade will be useful therapeutics to interfere with LTA-mediated inflammatory processes in the eye.

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References

37. Kengatharan M, De Kimpe SJ, Thiemermann C. Analysis of the signal transduction in the induction of nitric oxide synthase by


