Inhibition by Triptolide of Chemokine, Proinflammatory Cytokine, and Adhesion Molecule Expression Induced by Lipopolysaccharide in Corneal Fibroblasts

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PURPOSE. The production of proinflammatory cytokines and chemokines as well as the surface expression of intercellular adhesion molecule (ICAM)-1 by corneal fibroblasts contribute to corneal inflammation. The effects of triptolide on the expression of these proteins induced by lipopolysaccharide (LPS) in human corneal fibroblasts were examined in comparison with those of dexamethasone.

METHODS. The release of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1β, and IL-8 from cultured corneal fibroblasts was measured with assay kits. Surface expression of ICAM-1 on the cultured cells was measured with a whole-cell enzyme-linked immunosorbent assay.

RESULTS. Lipopolysaccharide (LPS) induced the release of the proinflammatory cytokine IL-6 and that of the chemokines G-CSF, MCP-1, MIP-1β, and IL-8 as well as surface expression of ICAM-1 by corneal fibroblasts, whereas IL-1β, TNF-α, and GM-CSF were not detected in the culture supernatants of cells incubated with or without LPS. Triptolide and dexamethasone each inhibited in a concentration-dependent manner the LPS-induced release of IL-6, G-CSF, MCP-1, and IL-8 by corneal fibroblasts. Whereas the inhibitory effect of dexamethasone on LPS-induced IL-6 release was greater than that of triptolide, the inhibitory effect of triptolide on LPS-induced G-CSF release was more pronounced than was that of dexamethasone. Dexamethasone also inhibited LPS-induced MIP-1β release, whereas triptolide did not. Both compounds inhibited the LPS-induced surface expression of ICAM-1.

CONCLUSIONS. Triptolide inhibits the LPS-induced expression of IL-6, chemokines (G-CSF, MCP-1, IL-8), and ICAM-1 in cultured human corneal fibroblasts. This compound might thus be expected to limit the infiltration of immune cells into the cornea.

Materials and Methods

Eagle minimum essential medium (MEM), fetal bovine serum (FBS), and trypsin-EDTA were obtained (Invitrogen-Gibco, Carlsbad, CA), as were 24- and 96-well culture plates (Corning-Costar, Corning, NY), collagena-cose, dispase, dexamethasone, and LPS derived from Pseudomonas aeruginosa (Sigma-Aldrich, St. Louis, MO), triptolide (Alexis Biochemicals, Carlsbad, CA), mouse monoclonal antibody to ICAM-1 (BD Pharmingen, San Diego, CA), horseradish peroxidase–conjugated goat anti-


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tibodies to mouse immunoglobulin G (Chemicon, Temecula, CA), an enzyme-linked immunosorbent assay (ELISA) kit for IL-8 (R&D Systems, Minneapolis, MN), a protein multiarray system and human cytokine assay (Bio-Plex; Bio-Rad, Hercules, CA), and a particle counter (Beckman Coulter, Fullerton, CA). All media and reagents used for cell culture were endotoxin minimized.

Isolation and Culture of Human Corneal Fibroblasts

Human corneas were obtained for corneal transplantation surgery (NorthWest Lions Eye Bank, Seattle, WA). Human tissue was used in strict accordance with the tenets of the Declaration of Helsinki. Corneal fibroblasts were prepared and cultured as described previously.18 In brief, the endothelial layer of the rim of the cornea remaining after transplantation surgery was removed mechanically, and the tissue was then incubated with dispase (2 mg/mL in MEM) for 1 hour. After mechanical removal of the epithelial sheet, the tissue was treated with collagenase (2 mg/mL in MEM) until a single-cell suspension of corneal fibroblasts was obtained. Cells were cultured in MEM supplemented with 10% FBS and were used for experiments after four to seven passages under subconfluent (actively proliferating) conditions. Purity of the corneal fibroblast cultures was judged on the basis of cell morphology and reactivities with antibodies to vimentin and to cytokeratin, as previously described18; all the cells were positive for vimentin and negative for cytokeratin, suggesting that the cultures were not contaminated by epithelial cells. No changes in cell morphology or immunoreactivity were apparent after culture for up to seven passages.

Assay for IL-1β, TNF-α, IL-6, G-CSF, GM-CSF, MCP-1, MIP-1β, and IL-8

Corneal fibroblasts were cultured in 24-well plates until they achieved confluence, after which the culture medium was replaced with serum-free MEM for 1 day. Cells were then incubated first for 1 hour with various concentrations of triptolide (1–30 nM) or dexamethasone (0.1–100 nM) and then for an additional 24 hours in the same medium supplemented with LPS (100 ng/mL) and 0.5% human serum (AB type). The medium was then collected and centrifuged at 120 g for 5 minutes, and the resultant supernatant was frozen at –80°C for subsequent assay of proinflammatory cytokines and chemokines. Cells were detached from the culture plate by exposure to trypsin-EDTA, and their number was determined with a particle counter. The morphology and number of cells were not affected by incubation with LPS, triptolide, or dexamethasone for 24 hours (data not shown). Simultaneous quantification of IL-1β, TNF-α, IL-6, G-CSF, GM-CSF, MCP-1, and MIP-1β in culture supernatants was performed with a human cytokine assay system (Bio-Plex; Bio-Rad). IL-8 concentration was determined with an ELISA kit, as described previously.8

Whole-Cell ELISA for ICAM-1

Whole-cell ELISA for ICAM-1 was performed as described previously.19 In brief, corneal fibroblasts (1 × 10^3 cells per well) were grown for 3 days in 96-well plates in MEM supplemented with 10% FBS, after which the culture medium was changed to serum-free MEM for 1 day. Cells were subsequently incubated first for 1 hour with various concentrations of triptolide (1–30 nM) or dexamethasone (0.1–100 nM) and then for an additional 24 hours in the same medium supplemented with LPS (100 ng/mL) and 0.5% human serum (AB type). The medium was then aspirated, and the cells were washed twice with phosphate-buffered saline (PBS), fixed for 15 minutes at room temperature with PBS containing 1% paraformaldehyde, washed with PBS, and incubated for 1 hour at 37°C with a monoclonal antibody to ICAM-1 (1:10,000 dilution) in PBS containing 1% bovine serum albumin (BSA). After three washes with PBS containing 0.1% BSA, the cells were incubated for 1 hour at room temperature with horseradish peroxidase-conjugated goat antibodies to mouse immunoglobulin G, washed three more times with PBS containing 0.1% BSA, and incubated for 20 minutes in the dark with 100 μL tetramethylbenzidine solution. The reaction was stopped by the addition of 50 μL of 1 M H_2SO_4, and the absorbance of each well was measured at 450 nm with a microplate reader. ICAM-1 expression was calculated as a percentage of the control value.

Statistical Analysis

Data were presented as mean ± SEM and were analyzed by Dunnett multiple comparison test or Student’s unpaired t test. P < 0.05 was considered statistically significant.

RESULTS

Effects of LPS on IL-1β, TNF-α, IL-6, G-CSF, GM-CSF, MCP-1, MIP-1β, and IL-8 Expression in Corneal Fibroblasts

We recently showed that LPS increases the expression of IL-8 and MCP-1 in cultured human corneal fibroblasts.8,9 In the present study, we first examined the effects of LPS on the release of IL-1β, TNF-α, IL-6, G-CSF, GM-CSF, MCP-1, MIP-1β, and IL-8 from these cells. IL-1β, TNF-α, and GM-CSF were not detected in the culture supernatants of cells incubated in the absence or presence of LPS (100 ng/mL) for 24 hours (Table 1). G-CSF was also not detected in the culture supernatant of cells incubated in the absence of LPS, but it was present in that of cells exposed to LPS. Incubation of corneal fibroblasts with LPS also resulted in significant increases in the release of IL-6, MCP-1, MIP-1β, and IL-8.

Effects of Triptolide and Dexamethasone on IL-6, G-CSF, MCP-1, MIP-1β, and IL-8 Expression in Corneal Fibroblasts

We next investigated the effects of triptolide and dexamethasone on the expression of IL-6, G-CSF, MCP-1, MIP-1β, and IL-8 in corneal fibroblasts (Table 2). Cells were incubated with various concentrations of triptolide (1–30 nM) or dexamethasone (0.1–100 nM) for 1 hour before incubation for 24 hours in the additional absence or presence of LPS (100 ng/mL). Triptolide exhibited no effect on the basal expression of IL-6 but inhibited LPS-induced IL-6 release in a concentration-dependent manner; this latter effect was statistically significant at a concentration of 3 nM and maximal (55% inhibition) at 30 nM. Dexamethasone had a small inhibitory effect on basal IL-6 release at concentrations of 10 and 100 nM, and it inhibited LPS-induced IL-6 expression in a concentration-dependent manner; this latter effect was significant at a concentration of 1 nM and maximal (83% inhibition) at 100 nM.

<table>
<thead>
<tr>
<th>Cytokine/Chemokine</th>
<th>None</th>
<th>LPS</th>
</tr>
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<tbody>
<tr>
<td>IL-1β (pg/mL)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IL-6 (ng/mL)</td>
<td>0.05 ± 0.02</td>
<td>14.79 ± 1.39*</td>
</tr>
<tr>
<td>GM-CSF (pg/mL)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>G-CSF (ng/mL)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MCP-1 (ng/mL)</td>
<td>4.70 ± 0.71</td>
<td>11.30 ± 1.37*</td>
</tr>
<tr>
<td>MIP-1β (pg/mL)</td>
<td>3.00 ± 0.41</td>
<td>6.55 ± 0.38*</td>
</tr>
<tr>
<td>IL-8 (ng/mL)</td>
<td>2.58 ± 0.23</td>
<td>36.00 ± 1.49*</td>
</tr>
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Cells were incubated in the absence or presence of LPS (100 ng/mL) for 24 hours, after which the amounts of the indicated proteins in culture supernatants were determined. Data are mean ± SEM of triplicates from an experiment that was repeated three times with similar results. ND, not detected.

* P < 0.05 (Student’s t test) compared with the corresponding value for cells cultured without LPS.
Triptolide inhibited LPS-induced G-CSF release in a concentration-dependent manner; this effect was significant at a concentration of 1 nM and maximal (55% inhibition) at 30 nM. Dexamethasone similarly inhibited the stimulatory effect of LPS on G-CSF expression; this effect was significant at a concentration of 1 nM and maximal (72% inhibition) at 100 nM.

Triptolide induced a small inhibitory effect on basal MCP-1 release at a concentration of 30 nM and inhibited LPS-induced MCP-1 release in a concentration-dependent manner; this latter effect was significant at a concentration of 3 nM and maximal (36% inhibition) at 30 nM. Dexamethasone also inhibited basal and LPS-induced MCP-1 release in a concentration-dependent manner; the latter effect was significant at a concentration of 10 nM and maximal (36% inhibition) at 100 nM.

Triptolide had little or no effect on basal or LPS-induced MIP-1β release at any of the concentrations examined. In contrast, whereas dexamethasone did not significantly affect the basal expression of this chemokine, it inhibited LPS-induced MIP-1β release in a concentration-dependent manner; this effect was significant at a concentration of 0.1 nM and maximal (38% inhibition) at 100 nM.

Triptolide induced a small inhibitory effect on basal IL-8 release at concentrations of 10 and 30 nM, and it inhibited LPS-induced IL-8 release in a concentration-dependent manner; this latter effect was significant at a concentration of 1 nM and maximal (46% inhibition) at 30 nM. Dexamethasone exhibited effects similar to those of triptolide on basal and LPS-induced IL-8 release; the latter effect was significant at a concentration of 0.1 nM and maximal (50% inhibition) at 100 nM.

**Inhibition by Triptolide and Dexamethasone of ICAM-1 Expression in Corneal Fibroblasts**

We recently showed that LPS increases the expression of ICAM-1 in cultured human corneal fibroblasts. Therefore, we next investigated the effects of triptolide and dexamethasone on the surface expression of ICAM-1 in corneal fibroblasts incubated in the absence or presence of LPS. Cells were incubated with various concentrations of triptolide or dexamethasone for 1 hour before incubation for 24 hours in the additional absence or presence of LPS (100 ng/mL). Although triptolide had no effect on basal ICAM-1 expression, it inhibited LPS-induced ICAM-1 expression in a concentration-dependent manner (Fig. 1A); at a concentration of 30 nM, triptolide inhibited the effect of LPS by 35%. Dexamethasone also had no effect on basal ICAM-1 expression, and it inhibited (by 27%) the effect of LPS on ICAM-1 expression only at a concentration of 100 nM (Fig. 1B).

**Discussion**

We have shown that LPS induced the production of IL-6, G-CSF, MCP-1, MIP-1β, and IL-8 and the surface expression of ICAM-1 in human corneal fibroblasts. Furthermore, similar to the action of dexamethasone, triptolide inhibited in a concentration-dependent manner the LPS-induced release of IL-6, G-CSF, MCP-1, and IL-8 from these cells. The inhibitory effect of dexamethasone on LPS-induced IL-6 release was greater than that of triptolide; whereas the inhibitory effect of triptolide on LPS-induced G-CSF release was more pronounced than was that of dexamethasone. Dexamethasone also inhibited MIP-1β expression induced by LPS, whereas triptolide did not. Triptolide and dexamethasone exhibited similar inhibitory effects on LPS-induced surface expression of ICAM-1. Our results thus suggest that triptolide might prove effective in limiting the infiltration of immune cells into the cornea.

LPS is an important trigger for host inflammatory responses during infection of the cornea with Gram-negative bacteria. Fibroblasts play a substantial role in the regulation of inflammation in the infected cornea. We have now shown that LPS stimulates proinflammatory cytokine, chemokine, and ICAM-1 expression in corneal fibroblasts, consistent with our previous observations. We also recently showed that LPS interacts...
with human corneal fibroblasts to induce the phosphorylation and degradation of IκBα in the cytoplasm and the consequent activation of NF-κB. The activation of NF-κB by LPS may thus contribute to the LPS-induced expression of IL-6, chemokines, and ICAM-1 observed in the present study. These effects of LPS likely promote the infiltration and activation of leukocytes associated with corneal inflammation.

Leukocytes play important roles in the pathogenesis of corneal inflammatory diseases. Migration to and infiltration into the tissue of leukocytes and the subsequent activation of these cells are regulated by chemokines and other cytokines. IL-6 promotes the differentiation of B cells, whereas IL-8 and MCP-1 are potent and selective chemoattractants for neutrophils and monocytes–macrophages, respectively. We previously showed that keratocytes release IL-8 and MCP-1 and that this release might facilitate the chemotaxis of leukocytes to the corneal stroma. Triptolide inhibits IL-6 expression in human bronchial epithelial cells stimulated with phorbol 12-myristate 13-acetate, TNF-α, or IL-1β. LPS-stimulated mouse macrophages express ICAM-1 is a 76- to 114-kDa cell surface glycoprotein and a member of the immunoglobulin superfamily of proteins. It contributes to the local infiltration of leukocytes during immune responses by mediating the adhesion and activation of these cells. ICAM-1 is thus selectively expressed in the cornea in areas of inflammation. Furthermore, leukocyte infiltration into tissue is markedly reduced in ICAM-1–deficient mice compared with that in control animals. We have previously shown that exposure of corneal fibroblasts to LPS increases the adherence of neutrophils to these cells, indicating that ICAM-1 is expressed by corneal fibroblasts in response to LPS is biologically active. Inhibition of the expression of ICAM-1 in corneal fibroblasts might thus provide a new therapeutic option in the treatment of corneal inflammatory diseases. We have now shown that, although triptolide significantly inhibited the LPS-induced increase in the surface expression of ICAM-1 in human corneal fibroblasts, the extent of this effect was limited.

Triptolide and dexamethasone each exert various effects on immune cells and on tissue resident cells that participate in inflammatory responses. The similarity in the effects of these two compounds is dependent on cell type, however. We have previously shown, for example, that dexamethasone inhibits not only the synthesis but also the activation of matrix metalloproteinases in rabbit corneal fibroblasts, whereas triptolide inhibits only the synthesis of these enzymes. Dexamethasone and triptolide each inhibit IL-8 and MCP-1 expression in cultured human corneal fibroblasts induced by the proinflammatory cytokines IL-1 or TNF-α. Triptolide has been shown to be effective for the treatment of several autoimmune diseases such as rheumatoid arthritis, lung fibrosis, and uveoretinitis in animal models. Furthermore, it appears to be safe and clinically beneficial in patients with rheumatoid arthritis or psoriasis vulgaris. In the present study, dexamethasone inhibited the LPS-induced release of all the tested proteins from corneal fibroblasts, whereas the inhibitory action of triptolide was more selective. Dexamethasone was more effective at inhibiting LPS-induced IL-6 release than was triptolide, but triptolide was more effective at inhibiting LPS-induced G-CSF release than was dexamethasone. Dexamethasone also inhibited MIP-1β expression induced by LPS, but triptolide did not. Whether triptolide is a viable alternative to steroids for the treatment of corneal inflammation and whether it is devoid of adverse effects remain to be clarified.

We recently showed that neutrophils stimulate collagen degradation by corneal fibroblasts and that triptolide inhibits IL-1–induced collagen degradation by these cells. Interactions of inflammatory cells with resident corneal fibroblasts, therefore, likely play an important role in corneal inflammation. The downregulation by triptolide of the production of chemokines and other cytokines and of the surface expression of ICAM-1 by LPS-stimulated corneal fibroblasts suggests that this compound may be of therapeutic benefit in diseases characterized by corneal stromal inflammation.

References
18. Kumagai N, Fukuda K, Nishida T. Synergistic effect of TNF-
19. Kumagai N, Fukuda K, Fujitsu Y, Nishida T. Expression of func-

2. Thakur A, Willcox MD. Cytokine and lipid inflammatory mediator
profile of human tears during contact lens associated inflammatory

expression of chemokines Gro-alpha (CXCL-1), IL-8 (CXCL-8), and
MCP-1 (CCL-2) in inflated human corneas in vivo. Arch Ophthal-
mol. 2003;121:825–831.

tion in keratocytes and inflammatory cell infiltration into the cor-

5. Cubitt CL, Lausch RN, Oakes JE. Differential regulation of gran-
ulocyte-macrophage colony-stimulating factor gene expression in
2005;46:1141–120.

collagen degradation by corneal fibroblasts. J Leukoc Biol. 2003;
74:412–419.

7. Whitcup SM, Nussenblatt RB, Price FW Jr, Chan CC. Expression of
cell adhesion molecules in corneal graft failure. Cornea. 1993;12:
475–480.

expression of intercellular adhesion molecule-1 and chemokines in
2005;46:4114–120.

lysaccharide-induced chemokine and adhesion molecule expres-
sion in corneal fibroblasts by soluble CD14 or LPS-binding protein.

10. Schultz CL, Morck DW, McKay SG, Olson ME, Buret A. Lipopoly-

389–400.

12. Chang DM, Chang WY, Kuo SY, Chang ML. The effects of tradi-
tional antirheumatic herbal medicines on immune response cells.
J Rheumatol. 1997;24;436–441.

13. Qiu D, Zhao G, Aoki Y, et al. Immunosuppressant PG90 (triptol-
ide) inhibits T-cell interleukin-2 expression at the level of purine-
box/nuclear factor of activated T-cells and NF-κB transcriptional

14. Zhao G, Vazsar LT, Qiu D, Shi L, Kao PN. Anti-inflammatory effects of

wilfordii hook F extracts on induction of cyclooxygenase 2 activ-
ity and prostaglandin E2 production. Arthritis Rheum. 1998;41:
130–138.

16. Lu Y, Fukuda K, Seki K, et al. Inhibition by triptolide of IL-1-
induced collagen degradation by corneal fibroblasts. Invest Oph-

on chemokine expression induced by proinflammatory cytokines
2546–2552.

18. Kumagai N, Fukuda K, Nishida T. Synergistic effect of TNF-α and
IL-4 on the expression of thymus- and activation-regulated chem-
okine in human corneal fibroblasts. Biochem Biophys Res Com-

19. Kumagai N, Fukuda K, Fujitsu Y, Nishida T. Expression of func-
tional ICAM-1 on cultured human keratocytes induced by tumor