Lipid Mediators of Tumor Necrosis Factor-α-Induced Uveitis

Lloyd N. Fleisher, Jenny B. Ferrell, Maribeth G. Smith, and M. Christine McGahan

The authors tested the hypothesis that platelet-activating factor (PAF) and cyclooxygenase metabolites of arachidonic acid mediate the ocular inflammatory response to intravitreally injected tumor necrosis factor-α (TNFα). Rabbits were treated with the PAF receptor antagonist SRI 63-441, the cyclooxygenase inhibitors indomethacin and naproxen, or SRI 63-441 and indomethacin. At 24 hr after intravitreal injection of TNF (20,000 U), the severity of inflammation was assessed based on iridal hyperemia, aqueous humor leukocyte number and aqueous humor protein, immunoreactive-prostaglandin E (I-PGE), and leukotriene B4 (LTB4) concentrations. Although all of the treatments significantly reduced the severity of anterior uveitis, SRI 63-441 plus indomethacin was the most effective, indomethacin and naproxen were intermediately effective, and SRI 63-441 was the least effective. The results of this study are consistent with an important role for cyclooxygenase metabolites of arachidonic acid in the inflammatory response to TNFα, particularly with respect to dilation of iridal blood vessels. Although naproxen was nearly as effective as indomethacin in reducing aqueous humor I-PGE levels, indomethacin conferred significantly more protection to the blood-aqueous barrier as shown by lower protein levels in the aqueous humor. Thus, although cyclooxygenase inhibition may partially explain the protection afforded the blood-aqueous barrier by these nonsteroidal anti-inflammatory agents, the data suggest that indomethacin may also exert anti-inflammatory effects that are independent of cyclooxygenase inhibition. Furthermore, the data are consistent with TNFα releasing PAF in the eye and PAF acting both directly, by increasing vascular permeability, and indirectly, by promoting release of cyclooxygenase and lipoxygenase metabolites of arachidonic acid. Invest Ophthalmol Vis Sci 32:2393-2399, 1991

Tumor necrosis factor-α (TNFα) is a cytokine that is released by monocytes/macrophages in response to bacterial lipopolysaccharide and other stimuli.1-2 In nonocular tissues, TNFα is a pluripotent inflammatory mediator because of its ability to enhance neutrophil and monocyte chemotaxis,3,4 neutrophil-endothelial cell adherence,5,6 endothelial cell permeability,7 endothelial cell procoagulant properties,8 and neutrophil and monocyte/macrophage degranulation, respiratory burst, and cytotoxicity.9-12 In the eye, effects of TNFα are just being defined. TNFα produces uveitis when it is injected into the vitreal chamber of the rabbit eye, as characterized by hyperemia of iridal blood vessels, disruption of blood-ocular barriers, infiltration of leukocytes, and elevation of prostaglandin (PG) levels in aqueous humor.13,14 TNFα stimulates the production of platelet-activating factor (PAF)15,16 and cyclooxygenase17,18 and lipoxygenase19,20 metabolites of arachidonic acid (eicosanoids) in nonocular tissues. We hypothesized that the ocular inflammatory effects of intravitreally injected TNFα might be due to the release of PAF and eicosanoids. To test this hypothesis, uveitis was induced in rabbit eyes by intravitreal injection of TNFα. The effects of the cyclooxygenase inhibitors, indomethacin and naproxen, and the PAF receptor antagonist, SRI 63-441, on the severity of inflammation were assessed by biomicroscopic, cellular, and biochemical criteria.

Materials and Methods

Induction of Inflammation and Drug Treatments

All experiments were conducted in accordance with the ARVO Resolution on the Use of Animals in Research. Male 2-kg New Zealand white rabbits were anesthetized with intramuscular injections of xylazine (12 mg/kg) and ketamine (60 mg/kg). Human recombinant TNFα (0.1 ml; 20,000 U; 1 μg protein;
Genzyme Corp., Boston, MA) was injected into the right eye; the left eye received an equal volume of sterile vehicle that consisted of phosphate-buffered saline that contained 0.1% low-endotoxin bovine serum albumin (Fraction V; Sigma Chemical Co., St. Louis, MO). Injections were made through a 30-g needle that was attached to a syringe, approximately 3 mm posterior to the limbus. Animals were divided into five treatment groups: group I animals received injections of drug vehicles. The schedule and route of administration of vehicle injections corresponded to those given to the four groups of drug-treated animals (groups II–V). Group II animals received intraperitoneal injections of the cyclooxygenase inhibitor indomethacin (10 mg/kg) at 0.5 hr before TNFα and 7, 14, and 23 hr after TNFα injection. Indomethacin was delivered as the trihydrate after reaction with equimolar amounts of sodium carbonate. Group III animals received intraperitoneal injections of the cyclooxygenase inhibitor naproxen sodium (50 mg/kg) 0.5 hr before TNFα and 7, 14, and 23 hr after TNFα injection. Group IV animals received intravenous injections of the PAF receptor antagonist SRI 63-441 (10 and 20 mg/kg) 0.5 hr before and 2, 4, and 6 hr after TNFα injection. Group V animals were treated with indomethacin and SRI 63-441 as described for groups II and IV. SRI 63-441 was initially given at a dose of 20 mg/kg.21–23 Because this dose produced hemolysis in three of six animals, it was subsequently reduced to 10 mg/kg. SRI 63-441-induced hemolysis has also been reported in equine erythrocytes.24–26 The results reported are the combined means of four animals that received the lower dose and three (in which hemolysis did not occur) that received the higher dose of SRI 63-441. The same dose of indomethacin was used as that reported to reduce PGE2 levels in aqueous humor after intravitreal injection of bacterial lipopolysaccharide.26 However, we found that two injections over 24 hr reduced the PGE2 levels in aqueous humor after intravitreal injection of bacterial lipopolysaccharide (aqueous humor PGE2 levels: indomethacin treatment, 0.18 ± 0.31 ng/ml, n = 3; indomethacin vehicle treatment, mean: 20.17, range = 12.10–28.23, n = 2) as effectively as the three doses given in the previous study. To determine the proper dose regimen for naproxen, a dose–response experiment was performed (Table 1).

An inflammatory index was designed to facilitate the comparison of the effects of each drug treatment on the severity of the inflammatory response. For each inflammatory parameter, the drug treatment that produced the greatest effect was assigned a rating of 4, and the treatment that produced the least effect was assigned a rating of 1. Drug effects on vitreous protein concentration were not included in the computation of the inflammatory index because this concentration was not affected by intravitreal injection of TNFα (see Results section).

The inflammatory index was designed to provide a simple way to visualize the effects of several drug treatments on multiple inflammatory parameters. It is meant to complement the actual results that are summarized in Table 3, not substitute for them.

### Table 1. Effect of dosage regimen of naprochen sodium on I-PGE levels in aqueous 24 hr after intravitreal injection of E. coli lipopolysaccharide

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Injections/24 hr</th>
<th>Aqueous humor I-PGE (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2-4</td>
<td>6.55</td>
</tr>
<tr>
<td>20</td>
<td>2</td>
<td>19.98</td>
</tr>
<tr>
<td>40</td>
<td>2</td>
<td>9.98</td>
</tr>
<tr>
<td>50</td>
<td>2</td>
<td>7.89</td>
</tr>
<tr>
<td>50</td>
<td>4</td>
<td>13.62</td>
</tr>
<tr>
<td>50</td>
<td>4</td>
<td>9.70</td>
</tr>
<tr>
<td>50</td>
<td>4</td>
<td>0.99</td>
</tr>
<tr>
<td>50</td>
<td>4</td>
<td>0.90</td>
</tr>
</tbody>
</table>

Data represent individual values for each animal (n = 2).

Protein Concentration and Leukocyte Number in Aqueous Humor

Protein concentration in aqueous humor was determined with the use of the Lowry method that was modified for reduced volumes. Total leukocyte number in aqueous humor was determined with the use of a hemacytometer.
Radioimmunoassay (RIA) of Eicosanoids

Extraction of Aqueous Humor and Radioimmunoassay (RIA) of Eicosanoids

Aqueous humor supernatant was acidified to pH 3.5 with 5 μl H₂PO₄ (0.49 M), extracted into six volumes of ethyl acetate (twice), dried under vacuum in a Speed-Vac Concentrator (Savant, Hicksville, NY), and reconstituted in 500 μl methanol. The efficiency of extraction, based on recovery of [³H]-PGE₂ and [³H]-LTB₄, was 63.8% and 74.2%, respectively. Aliquots of the methanol reconstitute were dried under vacuum, reconstituted in 300 μl of a modified Krebs—Henseleit buffer (pH = 7.4), and PGE and LTB₄ were measured by RIA. Details of the PGE RIA have been described. The antiserum used in this assay does not distinguish between PGE₁ and PGE₂. Therefore, PG levels are referred to as immunoreactive PGE (I-PGE). LTB₄ levels were measured with the use of a highly specific commercially available RIA kit (Amersham, Inc., Arlington, IL).

Data Analysis

Effects of drug treatment on TNFα-induced uveitis were evaluated using one-way ANOVA. Multiple means were compared with the use of Tukey’s Omega test. Differences between TNFα-treated and vehicle-treated eyes were determined with a paired student t-test. Means were considered significantly different at P < 0.05. Data are expressed as mean ± SEM.

Results

Effects of TNFα in Animals That Received Drug Vehicles

No inflammation was seen in any eyes injected with TNFα vehicle. In group I animals, comparison of TNFα-injected eyes with the paired vehicle-injected eyes showed significant uveitis in the anterior segment based on hyperemia of iridal blood vessels and increases in aqueous humor leukocyte number and I-PGE and LTB₄ concentrations (Table 2). However, based on vitreous humor protein concentration, the posterior segment blood–ocular barrier was not affected at 24 hr post-TNFα injection. These results are consistent with those of a previous report.

Table 2. Effects of intravitreal injections of TNFα and TNFα vehicle in animals that receive systemic treatment with drug vehicles

<table>
<thead>
<tr>
<th>Intravitreal injection</th>
<th>Iridal hyperemia</th>
<th>Aqueous cells (μl)</th>
<th>Aqueous protein (μg/μl)</th>
<th>Vitreous protein (μg/mL)</th>
<th>Aqueous I-PGE (ng/ml)</th>
<th>Aqueous LTB₄ (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFα</td>
<td>2.1*</td>
<td>1882 ± 160*</td>
<td>11.1 ± 1.4*</td>
<td>0.8 ± 0.1</td>
<td>3.78 ± 0.62*</td>
<td>0.154 ± 0.030*</td>
</tr>
<tr>
<td>TNFα vehicle</td>
<td>0.0</td>
<td>0.0</td>
<td>0.9 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>0.66 ± 0.12</td>
<td>0.050 ± 0.012</td>
</tr>
</tbody>
</table>

* Significantly different from TNFα vehicle (P < 0.05). Mean ± SEM (n = 10–12).
Table 4. Relative effectiveness of drug treatments on the inflammatory response to intravitreally injected TNFα

<table>
<thead>
<tr>
<th>Drug therapy</th>
<th>Inflammatory index</th>
<th>Indomethacin</th>
<th>Naproxen</th>
<th>SRI 63-441</th>
<th>Indomethacin SRI 63-441</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug vehicle</td>
<td>0.0</td>
<td>3.2**</td>
<td>2.4*</td>
<td>2.0*</td>
<td>3.6**</td>
</tr>
</tbody>
</table>

* Significantly different from drug vehicle treatment (P < 0.05).
† Significantly different from naproxen and SRI 63-441 treatments (P < 0.05).

Data represent the mean rank for five inflammatory parameters.
nism, but to a lesser extent than with indomethacin or naproxen. This finding has several implications. First, it is consistent with TNFα-induced release of PAF in the eye, a process that has been shown in nonocular tissues. Second, because PAF is generally not a vasodilator, it is not likely to directly induce iridal hyperemia. However, PAF could act indirectly by releasing vasodilatory PGs. Indeed, PAF-induced release of cyclooxygenase metabolites has been shown in nonocular tissues and in rabbit iris. Third, because SRI 63-441 only partially reduced aqueous humor I-PGE levels, compared with indomethacin and naproxen, PAF-induced PG release can account for only part of the total PG production that is induced by TNFα. The remainder of PG release is presumably caused by a direct action of TNFα on ocular tissues and/or on leukocytes. This dual mode of action for TNFα-induced stimulation of cyclooxygenase metabolite production is consistent with the effects of drug treatments on I-PGE concentration in aqueous humor (Table 3). Compared with group I, the blockade of PAF receptors with SRI 63-441 reduced aqueous humor I-PGE levels by 57%, whereas inhibition of cyclooxygenase activity with indomethacin or naproxen reduced these levels by 84% and 75%, respectively. However, PAF receptor blockade plus cyclooxygenase inhibition (indomethacin plus SRI 63-441) reduced I-PGE levels by 94%.

PAF potently alters vascular permeability as shown by its ability to increase cutaneous and conjunctival vascular permeability. Furthermore, PAF has been implicated in the increased uveal permeability that accompanies laser irradiation of rabbit iris and the increased vascular permeability that accompanies endotoxin-induced uveitis and paracentesis in rabbits. The decrease in aqueous humor protein concentration after treatment with SRI 63-441 may be due to antagonism of the effects of PAF on vascular permeability. However, antagonism of PAF receptors with SRI 63-441 did not reduce aqueous humor protein concentration as effectively as indomethacin. Indomethacin-induced decreases in iridal vasodilation that are secondary to the inhibition of vasodilatory PG production would contribute to reductions in aqueous humor protein concentration. However, the findings suggest that processes that are independent of cyclooxygenase inhibition may also be involved in indomethacin-induced preservation of the blood-aqueous barrier. Despite similar reductions in aqueous humor I-PGE concentrations, naproxen was less effective than indomethacin in reducing the aqueous humor protein concentration. Furthermore, we have reported that although another cyclooxygenase inhibitor, flunixin meglumine, was as effective as indomethacin in reducing aqueous humor PGE2 levels during endotoxin-induced uveitis, only indomethacin significantly reduced iridal hyperemia and aqueous humor protein concentration.

The reduction in aqueous humor LTB4 levels after PAF receptor antagonism is consistent with the blockade of PAF-induced release of 5-lipoxygenase metabolites of arachidonic acid. PAF has also been implicated in the stimulation of 5- and 12-hydroxyeicosatetraenoic acid release in a rabbit model of corneal inflammation. Because LTB4 levels in aqueous humor from TNFα-vehicle injected eyes (Table 2) were not different from those in SRI 63-441-treated animals (group IV from Table 3; student's t-test, P > 0.3), TNFα-induced stimulation of LTB4 release may be secondary to the release of PAF. The decrease in aqueous humor LTB4 levels that is induced by the cyclooxygenase inhibitors is not likely due to a direct effect on the lipoxygenase pathway, but may be a reflection of the reduction in ocular inflammation. Also, nonsteroidal anti-inflammatory drugs, of which indomethacin and naproxen are representatives, inhibited neutrophil functions through effects on membrane function that is independent of PG formation.

Leukocyte number in aqueous humor was most effectively reduced by a combination of cyclooxygenase inhibition plus PAF receptor antagonism; cyclooxygenase inhibition was immediately effective; PAF receptor antagonism was the least effective (see Table 3). We have shown that the leukocyte response 24 hr after TNFα injection was largely monocytic (67%); neutrophils represented approximately 24% of the leukocytes that were present at this time. Although LTB4 is chemotactic for neutrophils, the chemotactic factors for monocytes during uveitis are poorly understood. Therefore, it was not surprising that the blockade of PAF receptors, which was accompanied by maximal inhibition of LTB4 production, did not reduce leukocyte infiltration more dramatically. Because cyclooxygenase metabolites are not believed to possess significant chemotactic properties, the association between cyclooxygenase inhibition and reduced leukocyte number in aqueous humor is more difficult to explain. This could be an indirect effect that is secondary to the anti-inflammatory action of these agents.

Further investigation is required to elucidate the mechanisms by which eicosanoids and PAF mediate TNFα-induced uveitis. Based on the results shown, it is clear that these potent autacoids are important mediators of the ocular inflammatory response to intravitreally injected TNFα.

**Key words:** tumor necrosis factor, platelet-activating factor, eicosanoids, SRI 63-441, uveitis
Acknowledgments

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