The role of arachidonic acid metabolites in the mediation of the polymorphonuclear leukocyte response following corneal injury

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The immediate polymorphonuclear leukocyte (PMN) response in the tear fluid was investigated following five different types of corneal injury. All injuries produced significant amounts of PMNs in the tear fluid within the first 6 hr of injury. Histological examination of injured corneas demonstrated that PMNs were attached to the denuded surface but were unable to attach to intact epithelium. Topical arachidonic acid, prostaglandin (PG) PGE₁ and PGE₂, and prostacyclin (PGI₂) induced the arrival of PMNs into the tear fluid of normal rabbit eyes, but topical PGF₂α, PG-6-keto-PGF₁α, and thromboxane did not elicit a PMN response from normal conjunctiva. Tear fluid samples 2 to 4 hr following corneal epithelial denudation demonstrated PGE-type activity. Pretreatment with topical (0.05% and 0.5%) and intraperitoneal (i.p.) indomethacin (100 mg/kg) markedly inhibited the PMN response following partial corneal epithelial denudation. However, an i.p. dose of 5 mg/kg indomethacin potentiated the tear fluid PMN response following corneal injury. The inhibition of the PMN response by indomethacin suggested that arachidonic acid metabolites may be involved in the mediation of PMN chemotaxis following corneal injury.

Key words: corneal injury, PMNs, indomethacin, prostaglandins, chemotaxis

Polymorphonuclear leukocytes (PMNs) invade the corneal stroma in response to various types of corneal injury such as superficial epithelial denudation, deep incision wounds, alkali burns, and the intrastromal injection of clove oil. PMNs arrive at the wound site by migrating from the perilimbal conjunctival blood vessels into the corneal stroma and also by migrating across the conjunctival epithelium into the tear fluid from where they are conducted to the wounded area. Several studies have noted PMNs in peripheral corneal stroma as early as 2 to 6 hr following corneal injury, with a varying peak PMN response in the corneal stroma depending upon the type of corneal injury. On the other hand, reports on the temporal characteristics of tear fluid PMNs following corneal injury have been scant. PMNs were found in the tear fluid 5 hr after an incision-type corneal injury and 1 to 2 hr after complete epithelial denudation. Histological and ultrastructural studies of corneal epithelial wound healing indicated that PMNs adhered to the denuded corneal surface from 3 to 38 hr following epithelial denudation, and recent experiments suggest that surface PMNs may interfere with...
re-epithelialization either by mechanical or chemical means.

The factors that control the release of PMNs after corneal wounding have not been completely elucidated. Weimar found that PMN migration into the corneal stroma could be inhibited by treatment of wounded corneas with sodium salicylate or soybean trypsin inhibitor. She postulated that the activation of a proteolytic enzyme was an essential first step for subsequent PMN invasion. However, the role of prostaglandins (PGs), which are important mediators of ocular inflammation, has not been studied in corneal wound healing.

In this report, we have examined the temporal relationship between tear fluid PMN response and stromal PMN response following several types of corneal epithelial injury. Tear fluid from injured corneas was also bioassayed for PG-like activity, and the ability of normal conjunctiva to mount a tear fluid PMN response following the instillation of arachidonic acid and its metabolites was tested. Finally, the effect of topical and systemic indomethacin, a known PG synthetase inhibitor, on the tear fluid PMN response following corneal denudation was evaluated.

Materials and methods

**Corneal injury and histology.** New Zealand white rabbits (1.5 to 2 kg) were anesthetized by intravenous injection of sodium pentobarbital (Nembutal; Abbott Laboratories, North Chicago, Ill.). All experiments utilized 12 corneas from six rabbits. In experiments 1 and 2, the corneas were completely and partially denuded, respectively, with a round dental stone by a technique previously described. In the third experiment, the corneas were injured in the central region by heat application from the tip (5 mm in diameter) of a heated metal rod (130° C) for 10 sec. In experiment 4, all corneas sustained a central, completely penetrating, incision with a sharp von Graefe knife; corneal perforations were approximately 2 mm in diameter. In the fifth experiment, the corneas were subjected to central burns inflicted by the absorption of alkali from a 5 mm disc of filter paper soaked in 2N NaOH according to a technique described by Matsuda and Smelser. Two rabbits (four corneas) from each group were sacrificed at 6 and 15 hr following injury, and their corneas were prepared for histological study.

**PMN counts.** Tear fluid PMN counts were performed on groups of eight eyes injured by each of the several methods described. Normal saline (50 µl) was instilled in the inferior cul-de-sac of the rabbit eye, and after gentle mixing, 20 µl of tear fluid were withdrawn at 1, 2, 4, 6, 24, and 48 hr following this injury with a Hamilton syringe fitted with a soft plastic tip. The tear fluid was mixed with an equal volume of fixative (Toisson’s), and the PMNs were counted in an improved Neubauer hemocytometer.

**Tear fluid PG assay.** PG-like activity was measured by a bioassay method using the rat stomach (fundus) strip, which is sensitive to PG-like substances. The stomach strip was continuously superfused with oxygenated Krebs’ solution, 10 ml/min at 37° C. The perfusate contained a mixture of pharmacological antagonists (acetylcholine, 5-hydroxytryptamine, catecholamines, and histamine) plus indomethacin (1 µg/ml) to inhibit endogenous PG synthesis of the bioassay organ and render it sensitive to exogenous PG only. The contraction of the rat stomach strip induced by the PG-like substances present in the tear fluid was matched by bolus injection of known amounts of standard PGE2. At 2 and 4 hr after complete corneal denudation, tear fluid from four eyes was pooled and tested for PG-like activity on the rat stomach strip.

**Instillation of arachidonic acid and its metabolites.** Three different doses of sodium arachidonate were instilled in the inferior fornix of rabbit eyes. The doses tested were 5 µg (n = 12), 1 µg (n = 14), and 400 ng (n = 10) made up to a total volume of 50 µl in Tris HCl, pH 7.5. PGE2 was tested in three different doses, 5 ng (n = 12), 50 ng (n = 12), and 500 ng (n = 12). PGE2 was tested at final doses of 50 ng (n = 4) and 500 ng (n = 3) in a volume of 50 µl. Prostacyclin (PGI2) was tested in a dose of 5 ng (n = 8). 6-Keto-PGF1a, a stable metabolite of PGI2, was tested at doses of 5 ng (n = 8) and 500 ng (n = 4). PGF2α was tested in a 50 ng dose (n = 4). Thromboxane A2 (TXA2) was generated by incubating washed rabbit platelets (50 µl) with 400 ng of arachidonic acid at 37°, and the mixture was immediately (within 20 sec) transferred to the normal rabbit inferior cul-de-sac. TXA2 generation was tested by bioassay on superfused rabbit aorta. In these experiments, control rabbit eyes received washed platelets (50 µl) alone or only arachidonic acid and Tris buffer.

**Topical and intraperitoneal administration of indomethacin.** Two different concentrations (0.5% and 0.05%) of topical indomethacin were used. A 50 µl volume of 0.5% indomethacin solution, pH
7.5 to 8.0, was instilled into the inferior cul-de-sac of both eyes of rabbits \((n = 7)\) 1½ hr prior to partial corneal denudation. Control eyes received an equal volume of buffer solution. A 50 \(\mu\)l volume of 0.05% indomethacin was similarly instilled \((n = 8)\). PMN counts were performed on tear fluid samples at 1, 2, 4, and 5 hr after injury.

Indomethacin at concentrations of 5 mg/kg \((n = 8)\), 50 mg/kg \((n = 14)\), and 100 mg/kg \((n = 8)\) was injected intraperitoneally \(i.p.\) into three groups of rabbits 1½ hr prior to partial epithelial denudation. Control animals received an equal volume of saline \(i.p.\) prior to corneal denudation. PMN counts were performed on tear fluid samples at 1, 2, 4, and 5 hr after injury.

Normal rabbit eyes \((n = 8)\) also received 0.5% indomethacin solution to check on conjunctival irritation as a cause of increased PMNs in the tear fluid, and PMN counts were performed at 1, 2, 4, and 5 hr after instillation.

Results

Stromal and tear fluid PMNs after corneal injury. Histological sections of corneas 6 hr after injury showed that PMNs were present in the peripheral corneal stroma following complete corneal denudation (Fig. 1). PMNs were not obvious in the peripheral stroma following partial denudation, incision-type penetrating wound, alkali burn, and thermal cautery. At 15 hr after injury, partially and completely denuded corneas showed some PMNs in the anterior peripheral corneal stroma, but the alkali-induced corneal injury and thermal corneal injury showed only a few PMNs in the periphery of the cornea. Although incised corneas did not contain PMNs at the corneal periphery at 15 hr, a few PMNs were present deep in the corneal stroma adjacent to the incision wound.

Tear fluid PMN counts indicated a marked response in the tear fluid from 2 to 6 hr following all injuries (Fig. 2). In histological sections PMNs were found on the surface of completely denuded corneas at 6 hr after injury, and both completely and partially de-
Fig. 2. Number of PMNs in a sample of tear fluid following various types of corneal injury. Each point on the curve represents the mean ± S.E.M. (n = 8).

Fig. 3. Number of PMNs in a sample of tear fluid from an uninjured eye following the instillation of three different doses of sodium arachidonate. Control eyes received saline. Each point on the curve represents the mean ± S.E.M. (5 µg, n = 12; 1 µg, n = 14; 400 ng, n = 10; control eyes, n = 20).

Nudged corneas demonstrated PMNs on the surface at 15 hr after injury (Fig. 1). Corneas injured by other methods did not demonstrate PMNs on the epithelial surface at 15 hr.

Effect of arachidonic acid and its metabolites on PMN egress from normal conjunctiva. In preliminary experiments the PG content of the tear fluid was determined at 2 and 4 hr after complete de-epithelialization by abrasion. Pooled tear fluid contained 2 to 4 ng of PGE-like activity (n = 3 eyes), whereas tear fluid from uninjured eyes (n = 3) showed no PG-like activity. Acidified (pH 3) tear fluid samples retained PG-like activity which disappeared in alkaline (pH 12) tear fluid sample, indicating that this activity was of the PGE type.

Arachidonic acid in doses up to 5 µg had a good PMN-attracting capacity in normal rabbit eyes (Fig. 3). PGE, at doses of 5 and 50 ng attracted PMNs into the tear fluid; however, the results of a 500 ng dose were not appreciably different from those in control eyes receiving an equal volume of saline. PGE2 similarly exhibited a PMN-attracting capability at the 50 ng dose, but the response to the 500 ng dose was not different from that of the controls (Fig. 4, A and B). PGF2α (50 ng) did not attract PMNs into the tear fluid. PGJ2 at a 5 ng dose gave a good PMN-attracting response (Fig. 5), but its stable metabolite, 6-keto-PGF1α, did not attract PMNs. TxA2 did not elicit a PMN response 30 min after instillation in the fornix, despite obvious signs of conjunctival inflammation such as hyperemia and edema.

Effect of indomethacin on the PMN response following corneal denudation. Tear
Fig. 4. Number of PMNs in a sample of tear fluid from an uninjured eye following the instillation of PGE\(_2\) (A) and PGE\(_1\) (B). Each point on the curve represents the mean ± S.E.M. (n = 12 for all doses of PGE\(_2\); n = 4 for 500 ng PGE\(_1\); n = 3 for 500 ng PGE\(_1\); control eyes n = 8).

fluid PMN counts which were not appreciably different from saline controls confirmed that topical 0.5% indomethacin did not have an irritative effect on normal conjunctiva. Indomethacin (0.5% and 0.05%) inhibited the early tear fluid PMN response to corneal injury in a dose-dependent manner (Fig. 6). Topical 0.5% indomethacin pretreatment effectively inhibited the PMN response, but the 0.05% concentration only partially inhibited the response.

The 100 mg/kg i.p. dose of indomethacin markedly inhibited the PMN response following corneal injury (Fig. 7), in contrast to the PMN response to a 50 mg/kg dose which was not markedly different from that of controls receiving saline injections. However, the 5 mg/kg dose of indomethacin potentiated the PMN response in the tear fluid following corneal injury.

Fig. 5. Number of PMNs in a sample of tear fluid from an uninjured eye following the instillation of PGI\(_2\). Each point on the curve represents the mean ± S.E.M. (n = 8); control eyes (n = 8).

Discussion

The results indicate that tear fluid PMNs enter the wound site faster than stromal PMNs in all types of corneal injuries studied and are able to attach to the injured corneal surface if the epithelium is denuded. In corneal injuries such as thermal cautery and alkali burn, in which the epithelium remained intact for the first 15 hr, the PMNs were unable to attach to the epithelial surface. The rapid mobilization of PMNs from the vascular conjunctiva into the tear fluid to remove the debris of corneal injury is a unique method by which healing is promoted in avascular corneal denudation.

The present experiments indicate that arachidonic acid, PGE\(_1\), and PGE\(_2\) can attract PMNs into the tear fluid from normal rabbit conjunctiva, despite reports\(^{15,16}\) which have suggested that PGs were not chemotactic for PMNs and may, in fact, inhibit PMN chemotaxis.\(^{17}\) Other arachidonic acid metabolites have been reported to also possess chemotactic properties; HETE (12 L-OH-5,8,10,14-eicosatetraenoic acid), a hydroxy fatty acid generated from arachidonic acid, is chemotactic for human PMNs and macrophages.\(^{18}\)

An interesting observation in these experiments is that PGE\(_1\) and PGE\(_2\) promoted PMN migration into the tear fluid in normal eyes at low doses (5 and 50 ng) but did not have an effect on PMN migration at the higher (500 ng) dose. This difference in effect

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between a high and a low dose of PGE₁ and PGE₂ suggests a modulation of PMN migration can be achieved by variations in PGE concentration. Our preliminary results indicated some PGE activity in injured tear fluid at 2 and 4 hr, but further experiments are needed to correlate tear fluid PMN counts with the concentration of PGE in tear fluid.

In the corneal epithelial denudation model, the inhibition of the early PMN response by pretreatment with topical and i.p. indomethacin suggests several mechanisms by which PMNs may be released into the tear fluid following injury. For example, PGs may be directly involved in the initiation of PMN release following corneal injury or alternatively through their effect on cyclic AMP, since indomethacin can inhibit cyclo-oxygenase and phosphodiesterase. Another attractive possibility is that HETE, a known chemoattractant agent, initiates the PMN response following epithelial injury but that its synthesis is blocked by the concentration of indomethacin used in these experiments. In this regard, recent work by Bhattacherjee et al. have confirmed that the conjunctiva has a greater ability to convert arachidonic acid to HETE than the iris.
Although 100 mg/kg indomethacin i.p. is a high dose which conceivably could cause a generalized leukopenia in the experimental animal, the topical doses of 0.5% and 0.05% indomethacin also inhibit the PMN response. Preliminary experiments in our laboratory indicate that the cyclo-oxygenase enzyme is inhibited in the conjunctiva by topical indomethacin at doses of 0.5% and 0.05%.

Our data also suggest that the 5 mg/kg i.p. dose of indomethacin only partially inhibits the cyclo-oxygenase enzyme whereas the 100 and 50 mg/kg i.p. doses almost completely inhibit cyclo-oxygenase. It is not clear at the present time why the low dose of indomethacin potentiated the PMN response following corneal injury. It is plausible that the low dose of indomethacin incompletely blocked the cyclo-oxygenase enzyme to allow the synthesis of other metabolites in the arachidonic acid cascade, such as HETE, which are chemotactic for PMNs.

Thus, although PGs are capable of attracting PMNs from normal conjunctiva and indomethacin pretreatment inhibits the tear fluid PMN response to corneal injury, the role of PGs in the initiation and/or the augmentation of the PMN response following corneal injury remains unknown.

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