Injection of Chemoattractants into Normal Cornea: A Model of Inflammation after Alkali Injury

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Purpose. The objective of this study was to establish and characterize the invasion of polymorphonuclear leukocytes (PMNs) into a normal cornea after intrastromal injection of the tripeptide chemoattractants generated from alkali-degraded corneas.

Methods. The following samples were injected into the midstroma of normal rabbit corneas: ultrafiltered tripeptide chemoattractants (N-acetyl-proline-glycine-proline and N-methyl-proline-glycine-proline) generated from alkali-degraded corneas, synthetic N-acetyl-PGP, positive control (leukotriene B4 [LTB4]), or negative control (Hanks' balanced salt solution [HBSS]). Timed responses of PMN infiltration were established for effective concentrations of LTB4 or the ultrafiltered chemoattractants.

Results. All intrastromal injections resulted in the immediate development of an edematous disc that was 10 mm in diameter. The lesion essentially had cleared in the HBSS-injected eyes by 8 hours, and histologic sections revealed minimal numbers of PMNs in the cornea or limbal tissue. The injection of LTB4 or the ultrafiltered tripeptide chemoattractants induced peak numbers of PMNs within the stroma at 8 hours, subsiding by 16 hours.

Seventy units of ultrafiltered chemoattractants yielded a strong PMN response, similar to 1 × 10⁻⁵ M LTB₄. The highest concentration of ultrafiltered chemoattractants (350 U) produced a severe PMN response that was characterized by a solid sheet of neutrophils surrounding the injection site. The injection of synthetic N-acetyl-PGP (2 × 10⁻⁶ M) produced a marked PMN response.

Conclusions. PMN invasion of the normal cornea after the injection of the ultrafiltered tripeptide chemoattractants or the synthetic N-acetyl-PGP mimicked early PMN infiltration in the alkali-injured eye, confirming the importance of this chemoattractant as an inflammatory mediator. (Invest Ophthalmol Vis Sci. 1998;39:1744-1750)

Alkali-injury to the cornea is characterized by a marked infiltration of polymorphonuclear leukocytes (PMNs), which leads to corneal ulceration and perforation.¹ ² The direct release of neutrophil chemoattractants from hydrolyzed corneal proteins is likely to be a critical step in triggering the acute inflammatory response to the alkali-injury. Previous in vitro studies have shown that alkali-degradation of viable or nonviable corneal tissue directly generates two chemoattractants,³ N-acetyl-PGP and N-methyl-PGP, from all layers of the cornea.⁴ The chemotactic activity of these compounds was firmly established in vitro by the polarization and collagen gel-visual chemotactic assays.³

The PGP chemoattractant sequence is found in a relatively small number of commonly occurring proteins, which are represented in the extracellular and cellular corneal tissue. The proteins containing this amino acid sequence, which can be identified through national protein databases, are collagen, proteoglycans, fibronectin, laminin, intracellular molecule 1, integrin, and Na⁺ K⁺-ATPase.⁵ One or more of these potential sources of the tripeptide chemoattractants, N-acetyl-PGP and N-methyl-PGP, are located in all layers of the cornea. The synthetic versions of these chemoattractants have demonstrated biologic activity, in vitro, similar to the purified chemoattractants.⁵

The purpose of this study was to demonstrate that the ultrafiltered and synthetic tripeptide chemoattractants, which...
have been proved to have chemotactic activity in vitro, can be injected into the normal cornea to mimic early PMN infiltration into the alkali-injured eye.

**Materials and Methods**

**Preparation of Samples**

CaCl₂ and MgCl₂ were purchased from Sigma Chemical (St. Louis, MO). Hanks’ balanced salt solution (HBSS) was purchased from Gibco Laboratories (Chagrin Falls, OH) and was used as a negative control. Leukotriene B₄ (LTB₄; Biomol Research Laboratories, Plymouth Meeting, PA) was dissolved in HBSS and used as a positive control. Synthetic N-acetyl-PGP (Research Genetics, Huntsville, AL) was dissolved in HBSS and was used as a positive control. Synthetic N-acetyl-PGP (Research Genetics, Huntsville, AL) was dissolved in HBSS and was used as a positive control.

All solutions were prepared by dissolution of the chemotactic compound in HBSS, supplemented with 500 µM calcium and 600 µM magnesium, and titration with 1 N HCl or 1 N NaOH to a pH of 7.4 and an osmolality of 280 to 320 mosm.

Tripeptide chemoattractants were obtained from alkali-degraded rabbit corneas. Corneal buttons were excised from rabbit eyes (Pel-Freez Biologicals, Rogers, AR) using an 11-mm trephine. Based on an average dry weight of 11 mg/cornea in a preliminary experiment, corneas were placed in a known amount of 1.0 N NaOH (83.34 mg corneal dry weight/ml of alkali, 1:12) for 24 hours at 37°C. The resultant suspension was titrated to pH 7.4 with 1.0 N HCl. This yielded a crude extract containing 41.67 mg corneal dry weight/ml of neutralized alkali. Briefly, the purification technique involved ultrafiltration (30,000, 3,000, and 1,000 MWt cutoff membranes in sequence) and dialysis (100 MWt cutoff membrane) of this crude extract. Ultrafiltered tripeptide chemoattractants were prepared for intrastromal injection by lyophilizing the final ultrafiltered and dialyzed fraction. The lyophilized powder was stored at −10°C and was dissolved in HBSS on the day of injection.

According to a previous study, the ultrafiltered chemoattractant sample was composed of small peptides between 100 and 1000 MWt. The only chemoattractants in this ultrafiltrate were N-acetyl-PGP and N-methyl-PGP, and the specific activity of N-acetyl-PGP was found to be superior.

**Isolation of Human Polymorphonuclear Leukocytes**

These experiments followed the tenets of the Declaration of Helsinki and were approved by the human research committee at Brookwood Medical Center. All donors signed written consent forms that explained the nature and possible consequences of the study. Each day, neutrophils from one person were isolated from fresh whole blood by centrifugation on Hypaque (Winthrop Pharmaceuticals, New York, NY)-Ficoll (Sigma Chemical) at a density of 1.114. Isolated PMNs were resuspended in HBSS, which contained 500 µM Ca²⁺ and 600 µM Mg²⁺, to a purity of 90.0% and a viability of 96% to 99%. The remaining percentage consisted of red blood cells and less than 5% platelets, lymphocytes, and eosinophils.

**Polarization Assay**

The polarization assay of Haston and Shields was used to quantitate the frequency and degree of cellular shape change that occurs after the exposure of PMNs to a chemoattractant. Briefly, incubations were performed at 35°C for 5 minutes by placing 1 × 10⁶ PMNs in reaction chambers and by adding increasing volumes of the ultrafiltered chemoattractant sample for a total volume of 250 µl. At the end of the incubation period, each cell suspension was mixed with an equal volume of 4% glutaraldehyde. PMNs in each sample were observed microscopically and were assigned scores of 0 (resting, spherical with a smooth membrane), 1 (activated, irregular with uneven membranes), or 2 (polarized, length ≥ width × 2). The scores of 100 PMNs for each sample were added, which produced a total score that was converted to a polarization index by subtracting the negative control values (PMNs in HBSS only).

The specific activity of the ultrafiltered chemoattractants was determined before intrastromal injection. One unit of activity was defined as the amount (milligrams) of corneal dry weight (originally degraded in alkali) required to generate a polarization index of 50%.

**Intrastromal Injections**

All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Twenty-nine New Zealand Dutch strain albino rabbits (Myrtles Rabbitry, Thompson Station, TN), weighing between 2.0 and 2.5 kg, were anesthetized with ketamine (12 mg/kg intramuscularly), xylazine (7.5 mg/kg intramuscularly), and topical proparacaine (0.5%). The peripheral corneas of 45 eyes received 80 µl midstromal injections 3 mm from the limbus, in line with the superior rectus muscle. Injections were performed under microscopic observation, using a 26-gauge, 2-point-style needle (KEL-F hub needle; Hamilton, Reno, NV) attached to a 1-ml tuberculin syringe (Monoject; Sherwood Medical, St. Louis, MO). In these experiments, 16 rabbits received either the injection of a high concentration of chemoattractant in one eye and a low concentration of the same chemoattractant or a negative control in the contralateral eye, or identical injections in both eyes. These animals were killed 8 hours after intracorneal injection. Thirteen animals received a unilateral injection of a chemoattractant. These included all rabbits that were killed 2 or 16 hours after intracorneal injection and one rabbit injected with the synthetic peptide.

The eyes were evaluated subsequently in a double-blind fashion by dissecting microscopy and slit lamp microscopy (every 2 hours on the first day and once a day thereafter) for clinical signs of inflammation. Corneal cloudiness and edema were ranked using a 0 to 4 classification system. After euthanizing the rabbits with Nembutal (100 mg/kg intravenously), eyes were collected and fixed in 10% formaldehyde for histologic analysis at the termination of the experiment. Histologic sections were taken directly through the center of the lesion, stained with hematoxylin and eosin, and examined in a double-blind fashion. The severity of PMN infiltration was ranked using a 0 to 4 classification system, evaluated by Kruskal-Wallis one-way analysis of variance, and tested for specific differences by Dunn’s multiple comparisons test. The photographs and micrographs presented in the Results section were representative of their respective groups. Analogous regions of histologic sections were selected when comparing a series of samples.

All groups, including each substance, each chemoattractant concentration, and each time interval, were composed of three separate corneas (n = 3). To obtain time responses, corneas were collected at 2, 8, and 16 hours after injections of 1 × 10⁻⁵ M LTB₄ or the ultrafiltered chemoattractants (350 U).
Figure 1. Histologic sections demonstrating the time response of polymorphonuclear leukocyte (PMN) infiltration in the peripheral cornea, approximately 0 to 4 mm from the limbus (L), after the injection of 350 U of ultrafiltered chemoattractants at (A) 2 hours (1.5+ PMN infiltration), (B) 8 hours (4+ PMN infiltration forming a solid sheet of PMNs around the needle tract [nt]), and (C) 16 hours (2+ PMN infiltration). PMNs are the most abundant and darkest staining cells in the stroma. Scale bar, 500 μm.
To determine the most effective chemoattractant concentration, 80 μl of the following concentrations of each substance were injected, and these corneas were collected at 8 hours: LTB4 (1 × 10^{-6} M, 1 × 10^{-7} M, 1 × 10^{-6} M, and 1 × 10^{-5} M) or ultrafiltered chemotactants (7 U, 70 U, and 350 U). HBSS (negative control) or synthetic N-acetyl-PGP (2 × 10^{-4} M) were injected, and the corneas subsequently were collected at 8 and 4.5 hours, respectively.

RESULTS

Specific Activity Units in Ultrafiltered Chemoattractant Samples

The specific activity of the ultrafiltered chemoattractant sample was determined using the PMN polarization assay on 3 successive days (n = 3). Increasing volumes (microliters) of the ultrafiltered chemoattractants were added to the incubation mixtures, which created a dose-response for polarization (mean ± SD) based on the following original corneal dry weights: 0.04 mg, 10% ± 1% polarization; 0.20 mg, 54% ± 7% polarization; 0.30 mg, 71% ± 9% polarization; 0.40 mg, 81% ± 11% polarization; and 0.60 mg, 93% ± 10% polarization. Fifty percent polarization and 1 U activity was interpolated from the dose-response curve as 0.19 mg corneal dry weight (originally degraded in alkali). Using this computation, 80-μl injections of the ultrafiltered chemoattractant samples were calculated to contain 7 U, 70 U, and 350 U. It is important to note that the intrastromal injection of 70 U (15.35 mg of original corneal dry weight degraded in alkali) of the ultrafiltered chemotactants is approximately equivalent to the chemotactants obtained from one alkali-degraded cornea.

Intrastromal Injections

Intrastromal injection of HBSS or of any of the chemoattractants resulted in the immediate development of a circular, opaque cloudiness in the cornea, approximately 10 mm in diameter, which was probably the consequence of fluid distortion of the lamellar orientation of the collagen fibrils. The disc of edema was positioned eccentrically so as to extend from the central cornea and to overlap the sclera by approximately 3 mm.

A time response was constructed for corneas injected with 1 × 10^{-5} M LTB4 or with 350 U ultrafiltered chemotactants (Fig. 1A, 1B, 1C) and, in both cases, showed peak numbers of PMNs within the stroma at 8 hours, subsiding by 16 hours. Subsequent studies, therefore, used 8 hours to reveal maximum responses.

Corneas injected with HBSS (negative control) cleared substantially over a period of approximately 4 hours with only minor edema and cloudiness visible. The lesions had essentially cleared by 8 hours (Fig. 2A). Corneas injected with low con-
FIGURE 3. Histologic sections demonstrating the polymorphonuclear leukocyte (PMN) response in the peripheral cornea, approximately 1 mm to 3 mm from the limbus (L), 8 hours after the intrastromal injection of (A) Hank's balanced salt solution (0.5+ PMN infiltration), (B) 1 × 10^{-7} M leukotriene B_4 (2+ PMN infiltration), and (C) 350 U of ultrafiltered chemoattractants (4+ PMN infiltration forming a solid sheet of PMNs around the needle tract [nt]), and 4.5 hours after the injection of (D) 2 × 10^{-4} M synthetic N-acetyl-PGP (3+ PMN infiltration). PMNs are the most abundant and darkest staining cells in the stroma. Scale bar, 100 μm.

centrations of an LTB_4 positive control (1 × 10^{-6} M or 1 × 10^{-7} M) seemed clinically similar to HBSS at 8 hours. Intracorneal injections of the highest concentration of LTB_4 (1 × 10^{-5} M) showed moderate cloudiness and edema at 8 hours (Fig. 2B). The injection of 7 U of the ultrafiltered chemoattractant sample produced clinical responses in the corneas indistinguishable from HBSS at 8 hours. The injection of 70 U of chemoattractant yielded a moderate corneal edema, and the injection of 350 U (Fig. 2C) produced severe edema at 8 hours. Corneal cloudiness and edema reached a peak at 4.5 hours after intrastromal injection of 2 × 10^{-4} M synthetic N-acetyl-PGP (Fig. 2D).

Histologic sections of corneas, 8 hours after the injection of the HBSS negative control, revealed no PMNs in the central part of each lesion (3 mm from the limbus) and only a minimal number of PMNs in the peripheral cornea, 1 mm from the limbus and in the limbus proper (Fig. 3A). Corneas injected with low concentrations of LTB_4 positive control (1 × 10^{-6} M and 1 × 10^{-7} M) seemed histologically similar to HBSS at 8 hours. The injection of 1 × 10^{-6} M LTB_4 produced a 1+ PMN response at 8 hours. Histology of the corneas 8 hours after the injection of 1 × 10^{-5} M LTB_4 revealed a 2+ PMN infiltration extending from the limbus to the injection site (Fig. 3B). Three hundred and fifty units of the ultrafiltered chemoattractant produced a statistically significant PMN response at 8 hours (P < 0.01), characterized by a solid sheet of neutrophilic cells at the injection site (Fig. 3C). Histology revealed a marked PMN response in the corneas 4.5 hours after intrastromal injection of 2 × 10^{-4} M synthetic N-acetyl-PGP (Fig. 3D).

The injection of increasing concentrations of the ultrafiltered chemoattractant increased the severity of the PMN response. The injection of 7 U of the ultrafiltered chemoattractant (Fig. 4A) produced PMN responses at 8 hours that were similar to HBSS. The injection of 70 U of the ultrafiltered chemoattractant (Fig. 4B) yielded a 2+ PMN response, and the injection of 350 U (Fig. 4C) produced a 4+ response at 8 hours.

**DISCUSSION**

The PMN chemotactic properties of the ultrafiltered tripeptides obtained from alkali-degraded corneas were confirmed by injection into the normal cornea. When the concentration of the ultrafiltered chemoattractant was increased, the numbers of PMNs found in the corneal stroma increased significantly. Intrastromal injection of 70 U, approximately
FIGURE 4. Histologic sections showing polymorphonuclear leukocyte (PMN) invasion into the peripheral cornea, approximately 0 to 3 mm (or 3.5 mm) from the limbus, 8 hours after intrastromal injection of the following concentrations of the ultrafiltered chemoattractants: (A) 7 U, 0+ PMN infiltration, the injection site is at or just past the edge of the photograph; (B) 70 U, 2+ PMN infiltration, the injection site is at or just past the edge of the photograph, and (C) 350 U, 4+ PMN infiltration, the photograph was aligned 0.5 mm off the limbus (arrow) so as to include the overwhelming PMN response throughout the area of the injection site. PMNs are the most abundant and darkest staining cells in the stroma. Note the gathering of PMNs in the limbal tissues of the less concentrated chemoattractants, even though the corresponding peripheral corneas have dramatically fewer PMNs. The limbus (L) is labeled in each photograph for orientation and comparison purposes. Scale bar, 500 μm.
equivalent to the chemoattractants released from one alkali-degraded cornea, achieved an inflammatory response consistent with early neutrophil invasion in the alkali-injured eye. These findings confirm that the tripeptide chemoattractants are inflammatory mediators in the alkali-injured eye, thereby creating a mimetic model for early PMN infiltration into the alkali-injured cornea.

The previous discovery of the release of tripeptide chemoattractants from corneal proteins, cleaved by alkali exposure, was a unique finding. The chemoattractants were characterized and purified from the cornea using ultrafiltration, dialysis, and high-pressure liquid chromatography. The purified triptides, N-acetyl-PGP and N-methyl-PGP, which were identified by mass spectrometry and amino acid analysis, subsequently were synthesized. The purified and synthetic forms of the peptides were shown to produce similar PMN polarization and chemotactic responses in vitro. In the present study, intrastromal injection of the ultrafiltered or synthetic chemoattractants produced a similar PMN infiltration into the normal cornea, adding to the foundation of knowledge concerning these chemoattractants and providing further evidence that these substances are one and the same.

The invasion of PMNs into the normal cornea after injection of the ultrafiltered or synthetic tripeptide chemoattractants compared favorably to the neutrophil invasion observed after the injection of LTB4 in the same experiment, and formyl-methionyl-leucyl-phenylalanine in an earlier study. In the present study, the injection of the ultrafiltered chemoattractants or LTB4 created a gradient that attracted a self-limiting PMN infiltration, reaching a maximum at 8 hours and significantly dissipated by 16 hours. Injection of the chemotactants, 3 mm from the limbus, caused the injectant to spread beyond the limbus into the adjacent sclera, bringing the attractants into direct contact with the vascular supply. The peripherality of the injection would have a more dilute chemotactant concentration compared with the center. Under these conditions, neutrophils would follow the gradient toward the source until the concentration of the chemoattractant exceeded the threshold level for chemotaxis. Because a single injection was given, the gradient was not maintained and, in the absence of any other inflammatory mediators, the neutrophilic invasion and accumulation subsided. It is, therefore, clear that for this inflammatory invasion to progress to a destructive process other inflammatory mediators must be present.

In a clinical setting, the magnitude of the initial assault by PMNs would be dependent on the concentration of the tripeptide chemoattractants released by the alkali-injury. When the severity of the injury escalates, prior in vitro data suggest that a larger amount of the chemoattractants would be released. Clinical findings confirm that severe injury results in the attraction of huge numbers of PMNs to the stroma. Simultaneously with the release of the tripeptide chemoattractants, alkali-injury of corneal tissue directly releases a stimulant of the respiratory burst of PMNs. Once the respiratory burst is activated, PMNs elaborate their own self-recruiting chemoattractants, thereby expanding the scope of the inflammatory process. The secondary release of PMN chemoattractants in the presence of respiratory burst stimulants, amid a denatured corneal extracellular matrix, creates the ideal conditions for ulcer formation or even for the conversion of the cornea into a necrotic sequestrum.

The present study substantiates the role of alkali-generated tripeptide chemoattractants in triggering early neutrophil response in the alkali-injured cornea. Other low-molecular-weight chemoattractants, such as platelet-activating factor, are known to be present in the experimental alkali-injured eye. Although these and other chemoattractants play a role during different time periods in the inflammatory process, they are probably active in the further recruitment and accumulation of neutrophils, not as the primary or initiating event in the alkali-injured cornea.

The importance of identifying PMN chemoattractants lies in the opportunity it affords for intervention by antagonists. The concept of developing inhibitors that bind to chemoattractants is novel, but it also shows great promise. The ability to chemically nullify the effects of chemoattractants soon after their formation, but before their attraction of PMNs, represents an opportunity to control inflammation from the very beginning. The investigation of a variety of approaches to uncover antagonists for these chemoattractants is currently underway in our laboratory.

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References