A Neutrophil Chemoattractant Is Released From Cellular and Extracellular Components of the Alkali-Degraded Cornea and Blood

Roswell R. Pfister and Jeffrey L. Haddox

Purpose. A tripeptide chemoattractant(s) for neutrophils has been shown to release from alkali-degraded cornea. This study is designed to determine the source of the chemoattractant(s).

Methods. Whole corneas were degraded in 1 N NaOH for 10 minutes, 30 minutes, 1 hour, 4, 8, 24, and 48 hours to determine an optimal duration of alkali exposure for production of the chemoattractant. Whole cornea, cornea minus epithelium, cornea minus endothelium, and stroma alone were degraded in 1 N NaOH for 4 hours to determine the relative contribution of each corneal layer. In a separate experiment, epithelium alone, endothelium alone, cultured keratocytes alone, or bovine corneal collagen were treated separately in 1 N NaOH for 4 hours. Finally, human plasma, platelets, polymorphonuclear leukocytes (PMNL), and red blood cells were treated separately in 1 N NaOH for 4 hours to determine if a similar chemoattractant was released from alkali-treated noncorneal tissue. Neutralized suspensions of all samples were ultrafiltered and dialyzed. The chemotactic potential of each sample was determined in the polarization assay.

Results. Activation of the PMNL polarization response increased with the duration of exposure of corneal tissue to alkali, peaking at 6 hours. Release of the chemoattractant from alkali-degraded corneal tissue showed a significant decrease when the epithelium was removed from the stroma. All tissue layers showed a PMNL polarization response when treated with alkali. The response decreased from epithelium > endothelium > cultured keratocytes > collagen. Alkali degradation of human blood components, including plasma, showed significant polarization responses ranked in the following order: plasma > PMNL > tendon collagen > platelets = red blood cells.

Conclusions. This study demonstrates that the tripeptide chemoattractant(s) is released from all layers of the cornea after alkali injury. The substance released from blood components is of similar size and biologic activity as that found in the cornea, but its exact molecular composition is yet to be determined. Timed response of alkali degradation determined the optimal conditions for generation of the chemoattractant(s) including clinically relevant time intervals. Invest Ophthalmol Vis Sci. 1996;37:230–237.

Polymorphonuclear leukocytes (PMNL) rapidly invade injured tissues, responding to the release of chemoattractants from the damaged organ. These are usually low molecular weight agents that rapidly diffuse to the local capillary system. Vascular endothelial cells are activated and become sticky for marginating PMNL, already traveling at a reduced speed in vessels conducting blood through the edematous tissues. Neutrophils that become adherent to the vascular endothelium diapedese through the vessel wall, moving toward the source of the chemoattractant gradient. The impact of PMNL accumulation in corneal tissue is destructive, allowing the release of a variety of hydrolytic enzymes and oxygen-free radicals designed to degrade damaged tissues. Destructive PMNL activities are a major contributor to the development of these ulcers in the alkali-injured cornea.1–3

The model for the alkali-injured rabbit eye produces a reproducible PMNL inflammation with a con-
Sistente percentage of corneal ulcers. The generation of neutrophil chemoattractants is likely to be a critical step in triggering the acute inflammatory response after alkali injury. In previous experiments, the neutrophil chemoattractants, N-acetyl-Pro-Gly-Pro and N-methyl-Pro-Gly-Pro, were obtained from fresh or previously frozen corneas treated with alkali for only 30 minutes and as long as 24 hours. Eighty-four or previously frozen corneas treated with alkali for 10 minutes, 30 minutes, 1 hour, 4, 8, 24, and 48 hours and treated as noted in the Alkali Treatment section.

Isolation of Bovine Corneal Tissue

Whole corneas were excised from frozen bovine eyes. Five corneas were used for each sample. Corneas were rinsed and blotted. The whole cornea sample was not scraped. Alternatively, only one surface of the cornea (either epithelial or endothelial) was removed by scraping with a scalpel blade. Finally, both surfaces of some corneas were scraped, and the remaining stromas were combined to comprise the corneal stroma sample.

Isolation of Bovine Corneal Cells

Corneal Epithelium and Endothelium. Whole corneas were excised from frozen bovine eyes, rinsed, and blotted. Epithelia and endothelia were collected separately by scraping the surfaces of 60 corneas with a scalpel blade.

Cultured Corneal Keratocytes. Ten confluent secondary cultures of bovine corneal keratocytes (14 days) were grown by the method of Perlman and Baum as detailed in Whikehart and Fletcher. The coexistence of two or more cell types in a given culture plate was eliminated by the combination of periodic microscopic examinations and the use of specific types of media. Each culture plate was rinsed five times with HBSS. After the last rinse, excess HBSS was drained from the plates, and each cell type was collected by scraping with a rubber policeman. Separate samples were centrifuged at 800g for 10 minutes to form cell pellets.

Separation of Cell Types From Human Whole Blood

This study was approved by the institutional review board of Brookwood Medical Center, and the tenets of the Declaration of Helsinki were followed. All blood donors signed consent forms explaining the nature and possible consequences of the study. Whole blood was collected in heparinized tubes. Blood was layered on Hypaque-Ficoll with a density of 1.114 and centrifuged at 300g for 45 minutes. Techniques described in this article were developed in this laboratory to obtain specific human blood cell types. The percentage of cells present was determined by microscopy.
and/or a microcellcounter model CC-130 (Sysmex; Toa Medical Electronics, Los Alamitos, CA).

**Plasma and Platelets.** Whole heparinized blood was obtained from each of four donors (84 ml/donor). Eight milliliters of Hypaque–Ficoll (density = 1.114) was added to each of 48 test tubes. Seven milliliters of blood was layered on each tube and centrifuged for 35 minutes at 300g. The top 500 μl of each plasma layer was removed, mixed, and centrifuged at 15,000g for 15 minutes. The supernatant fraction from this plasma was essentially free of cells and platelets.

The next 2.5 ml of each plasma layer, above the mononuclear leukocyte band, was removed for platelet isolation. These samples were centrifuged for 5 minutes at 15,000g. The supernatant was discarded, and another aliquot of plasma was placed in these same eppendorf tubes until all the plasma was centrifuged. The final pellet was rinsed twice by resuspending in HBSS and centrifuging for 5 minutes at 15,000g. All pellets were combined into one tube using 1.0 ml of HBSS. This platelet fraction was essentially free of contaminating cells.

**Polymorphonuclear Leukocytes.** As previously noted the blood was placed on Hypaque-Ficoll and the plasma was removed. The PMNL cell layers were collected and washed by adding 1.5 ml of cells to 13.5 ml of HBSS and mixing. Samples were centrifuged at 200g for 10 minutes twice, each time discarding the supernatant. Pellets of isolated PMNL were resuspended in small volumes of HBSS and combined into a single 1-ml sample. Contaminating cell types made up less than 5% of this sample.

**Red Blood Cells.** Blood was layered on Hypaque–Ficoll and centrifuged as noted earlier. Plasma with platelets, mononuclear leukocyte, and PMNL bands were removed. Red blood cell (RBC) pellets were collected and washed twice with 10 ml of HBSS by centrifuging at 300g for 7 minutes, producing an RBC fraction essentially free of contaminating cell types.

**Collagen (Type 1)**

Type 1 collagen from bovine Achilles tendon was treated with alkali (0.25 gm/3 ml) as noted in the Alkali Treatment section. Soluble bovine corneal collagen type 1 was purified by the technique of Davison et al.12 and treated with alkali (0.25 gm/3 ml) as noted in the Alkali Treatment section.

**Alkali Treatment**

Cellular and extracellular tissue samples were alkali treated on a dry weight-to-wet weight basis, determined by preliminary studies. The amount of water in the cell pellets was calculated from these numbers and used to adjust the amount of alkali. The final NaOH concentration was 1 N in all cases. The ratio of tissue dry weight to alkali volume was 1:12 (wt/vol). All samples were incubated at 35°C for 4 hours, unless otherwise stated. Neutralization was accomplished by titration with 1 N HCl to pH 7.4. Crude suspensions were centrifuged at 15,000g for 15 minutes, and the supernatant fractions were collected.

The supernatant was then placed in a 30,000 MWt cutoff centrifprep system (Amicon, Beverly, MA) to be centrifuged at 1500g for 30 minutes. The resultant filtrate was placed into an Amicon MFS-1 micropartition system with a YMI Diaflo membrane (1000 MWt cutoff) and centrifuged for 30 minutes at 2500g. One-milliliter aliquots of this final ultrafiltrate were dialyzed (100 MWt cutoff; Spectra/Por CE; Spectrum Medical, Los Angeles, CA) in 500 ml of distilled water for 4 hours. The water was changed once per hour. Final molecular size of the chemoattractant was between 100 and 1000 MWt. The pH and osmolality of all samples were measured and adjusted, if necessary, to pH 7.3 and 280 to 320 mOsm by adding varying amounts of HBSS or distilled water. These samples were then tested for activity in the polarization assay.

An experiment was performed to determine the percentage of activity that each corneal layer contributes to the total activity of the cornea. Four separate samples (whole cornea, cornea minus epithelium, cornea minus endothelium, or stroma) were treated with an identical volume of 1 N NaOH (final concentration) for 4 hours at 37°C. Samples were neutralized and centrifuged, and the supernatants were purified by ultrafiltration and dialysis as above. Volumes were kept constant throughout the procedure to make valid comparisons of total activity.

A control experiment was performed to determine whether the chemoattractant released from alkali-degraded corneas is endogenous to the uninjured cornea and capable of extraction by rinsing. Previously frozen corneas were treated with HBSS or alkali for 1 hour at 37°C because exposure in buffer for 4 hours might pose a risk for bacterial contamination, cellular disintegration, or both. Two separate groups of six corneas were pooled, rinsed, blotted, and treated with 6 ml of HBSS or 1 N NaOH (final concentration) at 37°C for 1 hour. In each case, this yielded a dry weight to final volume ratio of 1:12. Samples were titrated to pH 7.4 with HBSS or 2.0 N HCl, respectively, while stirring. After centrifugation of each neutralized sample (300g for 15 minutes), the supernatants were purified by ultrafiltration and dialysis as above.

**Homogenization and Sonication of Corneal Epithelium**

Corneal epithelium was obtained from 30 thawed bovine eyes as previously described and placed in HBSS (1:18, dry wt:vol). Tissue was ground for 5 minutes by manual crushing in a tapered glass tissue grinder. The sample was disintegrated further by a 15-minute pe-
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period of high-frequency sonication (6 mm probe) at 0°C to 4°C with Vibra Cell VC50 (Sonics and Materials, Chicago, IL). This step reduced the entire sample to subcellular particles as determined by microscopy. The sample was centrifuged at 200g for 5 minutes to remove large particulate material. The supernatant fraction was divided into two equal aliquots for treatment with either 1 N NaOH (final concentration) or HBSS for 1 hour at 37°C and then neutralized with acid or HBSS, respectively. After centrifugation of each neutralized sample (15,000g for 15 minutes), the supernatants were purified by ultrafiltration and dialysis as above. Samples were then collected to measure the polarization response.

Polymorphonuclear Leukocyte Isolation for Polarization Assay

Neutrophils were isolated from fresh human whole blood by centrifugation on Hypaque-Ficoll (density = 1.114). Isolated PMNLs were resuspended in HBSS, containing 500 μM Ca2+ and 600 μM Mg2+, to a purity of 80.0% ± 1.6% (mean ± SEM, n = 8) and 96% to 99% viability. The remaining percentage consisted of RBCs and less than 5% platelets, lymphocytes, and eosinophils.

The polarization assay of Haston and Shields was used to quantitate the shape change that occurs after the exposure of PMNL to a chemoattractant. Briefly, 1 × 106 PMNL were suspended in HBSS for each sample to be tested. Each incubation mixture (total volume = 250 μl) was then exposed to a sample in a stirred reaction chamber at 35°C for 5 minutes. At the end of the incubation period, each cell suspension was mixed with an equal volume of 4% glutaraldehyde. In each sample, PMNLs were observed microscopically and 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 PMNL for each sample were added, producing a total score that was converted to a polarization index by subtracting the negative control values (PMNL in HBSS only). One unit of activity was defined as the amount of sample required to generate 50% polarization. Specific polarization activity was calculated as U activity/mg tissue dry weight. Total polarization activity was calculated as U/cornea.

We have shown that N-acetyl-Pro-Gly-Pro (312 MWt) or N-methyl-Pro-Gly-Pro (283 MWt) are the only known PMNL chemoattractants created directly by alkali degradation of corneal tissue. These biologically active tripeptides also activate the polarization response of PMNL. It is well known that the change in cell shape, known as polarization, is only the first event of PMNL chemotaxis. It is inaccurate to refer to polarization activity as chemotaxis when investigating non-corneal tissues with unknown activity. In these instances, polarization activity is referred to as chemotactic potential. In the current study of low molecular weight samples (100 to 1000 MWt) from cornea, we used the polarization response as representative of chemotaxis because we have previously demonstrated the chemotactic activity of the N-acetyl and N-methyl tripeptides.

Statistics

Data from the alkali-treated samples were evaluated by a Bonferroni correction of analysis of variance, using a two-tailed t-test, unless otherwise noted. Results from alkali-treated fresh or frozen whole corneas were evaluated by the Student’s t-test. The single-sample Student’s t-test was used to determine whether the timed response data from alkali-degraded corneas differed significantly from zero.

RESULTS

Table 1. Comparison of Polarization Activity From Corneal Layers

<table>
<thead>
<tr>
<th>Tissue</th>
<th>% Activity</th>
<th>Total Activity</th>
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</thead>
<tbody>
<tr>
<td>Whole cornea</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>Epithelium</td>
<td>25.4</td>
<td></td>
</tr>
<tr>
<td>Endothelium</td>
<td>6.8</td>
<td></td>
</tr>
<tr>
<td>Stroma</td>
<td>66.1</td>
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</table>

Total polarization activity (U/cornea) generated by the chemoattractant from alkali-degraded whole cornea was 36.6 ± 3.1 (mean ± SEM, n = 5). Total activity (mean ± SEM, n = 5) was decreased when the epithelium (27.3 ± 1.3), endothelium (34.1 ± 3.6), or both (24.2 ± 1.4) were removed from the stroma (0.01 < P < 0.02). Based on the whole cornea releasing 100% of the total polarization activity, it was determined that each corneal layer released the following portions of total activity: epithelium (25.4%), endothelium (6.8%), and stroma (66%) (Table 1).

Alkali degradation of each corneal component showed a decreasing response of PMNL polarization as follows: epithelium (P < 0.01) > endothelium (NS) > cultured keratocytes (0.02 < P < 0.05) > corneal collagen (Table 2). Alkali degradation of human blood components, including plasma, showed significant PMNL polarization responses (Table 2). Plasma or PMNL generated a significantly greater response than platelets or RBCs (0.02 < P < 0.05). Results from tendon collagen are not significantly different from any other sample. Alkali-treated fresh whole corneas (specific activity = 0.76 ± 0.13 U/mg dry wt, mean ± SEM, n = 5) produced a higher polarization response.
TABLE 2. Comparison of Polarization Activity

<table>
<thead>
<tr>
<th>Alkali-Degraded Cells and Extracellular Matrices</th>
<th>Specific activity (U/mg dry weight) [mean ± SEM (n = 5)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine corneal cells and corneal collagen</td>
<td></td>
</tr>
<tr>
<td>Epithelium</td>
<td>1.54 ± 0.23</td>
</tr>
<tr>
<td>Endothelium</td>
<td>1.02 ± 0.10</td>
</tr>
<tr>
<td>Cultured keratocytes</td>
<td>0.84 ± 0.08</td>
</tr>
<tr>
<td>Collagen (type 1)</td>
<td>0.40 ± 0.05</td>
</tr>
<tr>
<td>Human blood and tendon collagen</td>
<td></td>
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<tr>
<td>Plasma</td>
<td>1.60 ± 0.40</td>
</tr>
<tr>
<td>Polymorphonuclear leukocytes</td>
<td>1.53 ± 0.04</td>
</tr>
<tr>
<td>Collagen (type 1)</td>
<td>1.00 ± 0.17</td>
</tr>
<tr>
<td>Platelets</td>
<td>0.54 ± 0.05</td>
</tr>
<tr>
<td>Red blood cells</td>
<td>0.54 ± 0.08</td>
</tr>
</tbody>
</table>

compared to frozen whole corneas (specific activity = 0.53 ± 0.03 U/mg dry wt, mean ± SEM, n = 5), but the difference was not significant.

Alkali degradation of cornea (previously frozen) for 1 hour shows a strong polarization response compared to virtually no response from the same tissue immersed in HBSS alone (Fig. 1).

Corneal epithelium, lysed to subcellular particles by homogenization and sonication, showed little activation of the PMNL polarization response. However, the same sample incited strong polarization after exposure to alkali (Fig. 2).

Activation of the PMNL polarization response significantly increased with the duration of exposure of corneal tissue to alkali, reaching a plateau at 6 hours and remaining for the duration of the 48-hour experiment (Fig. 3).

DISCUSSION

Alkali injury to the eye immediately begins to degrade numerous corneal proteins, releasing a heterogeneous group of peptides. One resultant amino acid sequence, Pro-Gly-Pro, found in the N-acetylated and N-methylated form, acts as a PMNL chemoattractant. The current study shows that these PMNL chemoattractants are released from all layers of the cornea. Although the epithelium represents approximately 7% of the corneal mass, it contributes approximately 25% of the chemoattractant. Epithelium and endothelium contribute one third of the total polarization activity, whereas the stroma accounts for the remaining two thirds. These experiments, comparing whole corneas to corneas with specific layers removed, are verified by the specific activity measurements of each layer individually.

The specific activity of each corneal layer can be ranked in the following order: epithelium > endothelium > keratocytes > collagen, indicating that certain cells release more Pro-Gly-Pro sequences than others. Furthermore, cells release more of these sequences than the extracellular matrix, based on specific activities. In spite of these considerations, there are two overriding facts that emphasize the importance of the stroma as a major source for the chemoattractant in
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FIGURE 2. Corneal epithelium was lysed to subcellular particles by homogenization and sonication. An aliquot of this sample was then incubated with Hanks' balanced salt solution (HBSS), producing little activation of the polymorphonuclear leukocyte polarization response (solid circles). However, another aliquot of the same sample incited a significant polarization response after exposure to alkali ($P < 0.001$) (solid squares). Both samples were treated in exactly the same manner except for the use of HBSS or 1 N NaOH. Data points represent the mean ± SEM; $n = 5$.

The anterior corneal stroma is most heavily invaded by PMNL and most vulnerable to ulceration after alkali injury for two possible reasons. First, epithelial remnants still attached to stroma might release the chemoattractants, exciting an inflammatory response as long as they remain attached to the tissue. Second, results of confocal microscopy of normal corneas have demonstrated a concentration of 26.3 keratocytes per unit area beneath the epithelium, declining to a minimum of 15.2 cells in the deep posterior stroma. The sum of these considerations is a significantly higher concentration of cells in or on the anterior stroma subject to the release of larger amounts of inflammatory mediators when exposed to alkali.

A vast array of proteins exist in the mammalian cornea, but not all have been identified. A handful of proteins comprise the large majority of the corneal dry weight, and most of these have been sequenced. Although only a few proteins contain the Pro-Gly-Pro sequence, they comprise approximately 75% of the corneal dry weight. The following major proteins were identified by a nonredundant blast search (National Center for Biotechnology Information, The National Institute of Health; Swissprot, PIR, and Genpept databases; Jan. 31, 1994, updated daily) to contain the Pro-Gly-Pro sequence: types I to XII collagen (55% to 68% corneal dry weight), proteoglycans (5% to 14% corneal dry weight), fibronectin, laminin, ICAM-1, integrin, procollagenase, and Na+K+-ATPase. There may be other Pro-Gly-Pro containing proteins in the cornea that have not yet been identified.

Various human plasma and cellular components of blood elicit a polarization response after alkali degradation. The similar activity and molecular weights of these noncorneal active samples, when compared to the corneal samples, suggests that the tripeptide chemoattractants might be released on exposure to alkali anywhere in the body. It might reasonably be presumed, then, that this chemoattractant participates in triggering the inflammatory cycle initiated by alkali wherever the injury occurs. As a corollary, N-acetylated and N-methylated Pro-Gly-Pro might be two of the inflammatory mediators released from other tissue-destructive processes that are not alkali related.

The duration of alkali exposure determines the relative polarization activity of PMNL. Specific polarization activity of PMNL in response to whole cornea degraded in alkali over time increased to an asymptotic slope at 48 hours. Clearly, more chemoattractant is released as the exposure to alkali is lengthened, possibly accounting for more severe PMNL infiltrations in cornea as the alkali injury worsens. The fact that chemoattractant release was demonstrated in the current experiment in as little as 10 minutes shows that this brief exposure to alkali does correlate with...
the short alkali exposure occurring to the human eye. In fact, although the apparent alkali exposure in accidents to humans appears to be minutes in length, alkali has been shown to persist in the tissues for far longer.19

In these experiments, the release of the chemotactants is driven by alkali degradation of corneal tissue. Exposure of whole cornea or subcellular fragments of corneal epithelium to Hanks’ balanced salt solution over a period of 1 hour did not result in any PMNL polarization activity. The same time exposure to alkali resulted in a 70% or 60% polarization index, respectively. It is our experience that attempts to study previously frozen corneal tissue in HBSS for significantly longer than 1 hour has questionable validity because of contamination with bacteria or interference with polarization activity if antimicrobials are used. These difficulties are not attendant when the tissue is degraded in alkali because such treatment renders the tissue sterile.

These studies show that the chemotactants do not exist in a free state intracellularly or in the extracellular matrix. We conclude that there is no preformed endogenous chemotactant in whole cornea and that it must be released from a larger protein through its alteration or cleavage by alkali. The N-terminal blocking group (acetyl or methyl) is most likely created during the alkali exposure, but some may already exist in the native protein. It follows, therefore, that the chemotactants are the direct product of alkali degradation and not simply released, secreted, or synthesized.

There are relevant clinical implications to be gleaned from these studies. If dead or devitalized epithelial tags remain attached to the corneal surface after an alkali injury, they should be scraped immediately to prevent these remnants from releasing PMNL chemotactants. Sharp excision of dead and devitalized conjunctiva should also reduce the mediator load. For the procedure to influence the outcome of the injury, time is of the essence. Indeed, the findings of this study give validity to the procedure of lamellar keratectomy during the acute phase of alkali injury to excise the bulk of nonviable stromal tissue that can give rise to significant amounts of inflammatory mediators.20

Key Words
alkali-degraded cornea, chemotactant, corneal cells, inflammatory mediator, polarization, polymorphonuclear leukocytes

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