Role of Leukocytes in Ocular Inflammation of Tyrosinemia II

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In the animal model of tyrosinemia II only corneas from tyrosine (tyr)-fed rats produce chemoattractants in organ culture. To study the role of neutrophils (PMNs) in production of these chemoattractants, leukocytes (WBCs) were depleted using i.p. cyclophosphamide (CP). Saline (SAL)-treated rats maintained 18,375 ± 894 WBC/mm³ (mean ± SEM) with 4168 ± 424 PMNs. Rats receiving CP (150 mg/kg day 0, 75 mg/kg day 4) has 1565 ± 170 WBC (565 ± 129 PMN) on day 3, and 398 ± 68 WBC (19 ± 5 PMN) on day 8. Rats ate a low-protein ±5% tyr diet on days 4-8. Only SAL-treated tyr-fed rats developed plaque-like gray epithelial lesions; histopathology showed corneal epithelial necrosis, stromal edema, and epithelial and stromal PMN infiltration. Control and CP-treated tyr-fed rat corneas showed no inflammation. On day 8 corneas were cultured in RPMI 1640 + 5% heat-inactivated fetal bovine serum. After 3 days, supernatants were assayed for chemotactic activity (leading front method); data were expressed as the percentage of peritoneal PMN migration relative to 5% zymosan-activated rat serum. The mean total migration toward 75% supernatant from SAL-treated, tyr-fed rat corneas was 79%, whereas migration toward corneal supernatants from controls and CP-treated tyr-fed rats ranged from 42-48%. Corneal extracts were assayed for proteolytic activity. WBC depletion prevented the increase in cathepsin B- and D-like activities present in tyr-fed corneas, suggesting that PMNs were a major source of these enzymes. The data suggest that WBC depletion reduces both corneal inflammation in vivo and the production of chemotactic activity by tyr-fed corneas in culture. Invest Ophthalmol Vis Sci 27:926–931, 1986

Tyrosinemia II is a human autosomal recessive metabolic disorder involving a deficiency of hepatic tyrosine aminotransferase.1 Clinical presentation includes palmar and plantar hyperkeratosis, corneal inflammation with subsequent neovascularization and scarring, and increased plasma tyrosine (tyr).2 A syndrome similar to tyrosinemia II can be reproduced in young rats fed a high tyr diet.2 Early corneal epithelial lesions contain needle-shaped crystalline deposits that are limited to areas of corneal damage and are thought to be tyr.3 Later lesions contain dense infiltrates of polymorphonuclear leukocytes (PMNs).4 Little is known about inflammatory mediators in the avascular cornea that are responsible for PMN accumulation seen in this and other inflammatory keratopathies.

The current hypothesis of ocular inflammation in tyrosinemia II suggests that tyr crystals develop intracellularly and disrupt lysosomal and cellular membranes, thus initiating an inflammatory response.3,5 In support of this, tyr crystals injected intradermally or intraperitoneally (i.p.) do not produce an inflammatory response, and tyr alone is not a chemoattractant in vitro.1 Tyr crystals can lyse hepatic lysosomes and erythrocyte membranes.6,7 Intralesional corneal epithelial cells in this rat model contain increased lysosomal activity,3 but protease levels8 have not been measured. These enzymes could generate chemotactic factors locally, since proteases released by tissue damage or by PMNs can generate chemoattractants via the complement pathway in other systems,9 and complement components are present in normal corneas.10 Organ culture supernatants from tyr-fed rat corneas with Stage II-III keratopathy2 contain heat-labile, trypsin-sensitive chemotactic activity that on molecular sieve chromatography fractionates into four peaks of activity.11 We have utilized this model of corneal inflammation to study the effects of cyclophosphamide-induced leukopenia on the production of chemotactic activity in corneal organ culture supernatants. We report that leukocyte (WBC) depletion reduces both corneal inflammation in vivo and the production of chemotactic activity by tyr-fed rat corneas in culture. Furthermore, WBC depletion prevents the increase in cathepsin B- and D-like activities present in tyr-fed rat

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corneas, suggesting that the PMN is a major source of these enzymes detected at this stage of tyr keratopathy.

Materials and Methods

Animal Model

Young male Sprague–Dawley rats (80–100 g) (King Animal Laboratories; Oregon, WI) were WBC-depleted using cyclophosphamide (CP) (Neosar, Adria Laboratories, Inc.; Dublin, OH) injected i.p. at 150 mg/kg and 75 mg/kg on days 0 and 4, respectively. Non-WBC-depleted rats were injected with sterile 0.9% NaCl (SAL) at an equivalent vol/kg on days 0 and 4. WBC counts and differentials were performed on posterior tail blood. Tyr-fed rats were fed a protein-poor diet supplemented with 5% tyr by weight (ICN Nutritional Biochemicals; Cleveland, OH)2 on days 4–8. Control laboratories, Inc.; Dublin, OH) injected i.p. at 150 mg/kg using cyclophosphamide (CP) (Neosar, Adria Laboratories, Inc.; Dublin, OH) injected i.p. at 150 mg/kg and 75 mg/kg on days 0 and 4, respectively. Non-WBC-depleted rats were injected with sterile 0.9% NaCl (SAL) at an equivalent vol/kg on days 0 and 4. WBC counts and differentials were performed on posterior tail blood. Tyr-fed rats were fed a protein-poor diet supplemented with 5% tyr by weight (ICN Nutritional Biochemicals; Cleveland, OH)2 on days 4–8. Control rats were fed a protein-poor diet without added tyr. Eyes were examined biomicroscopically with a slit lamp (Haag-Streit 900; Bern, Switzerland) by a blinded observer (R.A.H.). The forms of keratitis were recorded as the mean distance ± standard error of the mean (SEM) in triplicate samples, using a semi-automated method.11 Because of day-to-day variability of peritoneal PMN response, data were expressed as percent relative migration,11 with the response to ZAS for each day normalized to 100% by the formula:

Per cent migration

\[
\frac{\text{Experimental} - \text{Negative Control}}{\text{Positive Control} - \text{Negative Control}} \times 100
\]

The SEM was always ≤5 μm, equivalent to ≤11%.

Protease Determinations

Corneas were homogenized in 50 mM Tris, pH 7.5, containing 0.15 M NaCl, 0.1% Triton X-100, and 0.2% NaN3 (1 ml/4 corneas) using a ground glass homogenizer. Homogenates set at 4°C overnight before centrifugation (27,000 × g × 30 min). The supernatants were stored frozen (−20°C) until determination of enzyme content. Histologic examination of the pellets confirmed the complete destruction of cellular and stromal matrix structures. Acid protease content was determined from pH 1.5–5.5 using a hemoglobin assay17 and the Lowry procedure to detect cleaved peptides.18 Buffers used were 0.5 M phosphate for pH 1.5–3.0, formate for pH 3.0–4.5, and acetate for pH 4.5–5.5. Pepstatin (Sigma) (final concentration 10 μM; added in ethanol) was added to test for the presence of carboxyl proteases.19 Cathepsin B-like activity was assayed using the substrate carbobenzoxy-alanyl-arginyl-arginyl-methoxy-β-naphthylamide (CBZ-Ala-Arg-Arg-MNA) at pH 5.8 in the presence of 10 mM dithiothreitol and 10 mM ethylenediaminetetraacetate.20 Specificity was tested using the inhibitor from Streptomyces, leupeptin (Sigma), at a final concentration of 32 mM. Neutral and alkaline protease contents were determined using FITC-casein as a substrate21 with Tris-maleate buffers for pH 5.0–9.0.
of 777/mm³ ± 66 (mean ± SEM; 1565 ± 170 on day 3 and 398 ± 68 on day 7); PMNs averaged 140/mm³ ± 33 (565 ± 129 on day 3 and 19 ± 5 on day 8). SAL-treated control rats (n = 6) maintained a WBC count of 18,375/mm³ ± 894 and a PMN count of 4168/mm³ ± 424. There were no significant differences in WBC or PMN counts between tyr-fed and control-fed animals within the CP-treated or the SAL-treated group.

Corneal Lesions

A diffuse gray mosaic pattern and fine punctate epithelial erosions (PEE) were seen biomicroscopically in SAL-treated rats fed control diet (no added tyr), as well as CP-treated (leukopenic) rats fed either the control or tyr diet (Fig. 1A). PEE have often been seen in normal and control rats and normal rabbits, and represent nonspecific changes probably associated with decreased blink rates [R.A.H.: Unpublished observations]. In one experiment, two of three CP-treated control-fed rats and one of three CP-treated tyr-fed rats had bilateral severe PEE, keratinization, and low to absent tear menisci, compared to the SAL-treated, control-fed rats; however, in all experiments corneal histology of both CP-treated groups was identical to that of SAL-treated rats fed control diet (vide infra). In contrast, only SAL-treated rats fed the tyr diet demonstrated a keratitis with a spectrum that included the previously described gray inflammation, and linear granular opacities that evolved into plaque-like epithelial lesions. This keratopathy was associated with a low-grade anterior stromal inflammation or infiltration. Some tyr-fed, SAL-treated rats had a herpetic-like dendrito-geographic ulceration that had been described previously in humans with tyr keratopathy (Fig. 1B).

Sections of corneas from CP- or SAL-treated rats on control diet and from CP-treated rats on the tyr diet showed normal histology and no PMN infiltration (Fig. 2A). Only SAL-treated, tyr-fed rat corneas showed epithelial necrosis and stromal edema, as well as epithelial and stromal PMN infiltration (predominantly directly beneath the corneal epithelium; Fig. 2B), typical of tyr keratopathy. Gram stains of similar sections showed no bacteria (data not shown).

Chemotactic Activity in Organ Culture Supernatants

Culture supernatants at 50 and 75% (n = 7) were assayed for chemotactic activity, since these concentrations showed maximal migration previously. Mean migration toward 75% supernatant from SAL-treated tyr-fed rat corneas was 79% (Fig. 3), significantly higher than that toward 75% supernatant from SAL-treated control-fed rats (42%), CP-treated control-fed rats (48%) and CP-treated tyr-fed rats (46%) (P < 0.02, Student's
two-tailed, paired t-test). There were no significant differences among SAL-treated control-fed, CP-treated control-fed and CP-treated tyr-fed rats. Identical results were obtained using 50% supernatant; mean migration toward 50% SAL-treated tyr-fed rat corneal supernatants (70%) was significantly higher than toward supernatants from SAL-treated control-fed (34%), CP-treated control-fed (42%), and CP-treated tyr-fed rats (35%) \((P < 0.01)\). No significant difference was seen comparing chemotactic activity of SAL-treated control-fed, CP-treated control-fed and CP-treated tyr-fed rats. Corneal culture supernatants from CP-treated tyr-fed and SAL-treated tyr-fed rats \((n = 5\) experiments) were chromatographed on a Sephadex G-75 column; only peak A' contained a small amount of chemotactic activity \((Fig. 4)\).

**Protease Content**

Corneal extracts were assayed for proteolytic activity over the pH range of 1.0–5.5 using hemoglobin as the substrate, and over the pH range of 5.5–9.0 using FITC-casein. At higher pH values \((7.0–9.0)\), very little protease activity was detected with FITC-casein. At lower pH values, two peaks of activity were observed, one at
**Table 1. Acid protease activity of rat corneas**

<table>
<thead>
<tr>
<th>Rat cornea</th>
<th>Cathepsin D-like* activity</th>
<th>Cathepsin B-like† activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n eq tyrosine released/min/cornea</td>
<td>n moles MNA released/min/cornea</td>
</tr>
<tr>
<td>SAL-treated, control diet</td>
<td>2.55 ± 0.12$</td>
<td>0.019 ± 0.001</td>
</tr>
<tr>
<td>CP-treated, control diet</td>
<td>2.93 ± 0.10</td>
<td>0.016 ± 0.001</td>
</tr>
<tr>
<td>SAL-treated, tyr diet</td>
<td>3.53 ± 0.16</td>
<td>0.029 ± 0.001</td>
</tr>
<tr>
<td>CP-treated, tyr diet</td>
<td>2.03 ± 0.10</td>
<td>0.019 ± 0.002</td>
</tr>
</tbody>
</table>

* Cathepsin D-like activity was determined at pH 3.0 using the hemoglobin assay of Anson$^7$ and the Lowry reaction$^{18}$ to detect trichloroacetic acid soluble peptides. A tyr standard curve was used to relate Lowry color to the cleaved peptide content. (The tyr content of the cornea was negligible compared to that of the cleaved peptides.)

† Cathepsin B-like activity was determined at pH 5.8 using CBZ-Ala-Arg-Arg-MNA. A standard MNA curve was used. All activity was inhibited by leupeptin.

$\dagger$ Mean value ± S.E.M. for two separate experiments, each with three separate determinations. There were significant differences ($P < 0.01$) among the means, using the one-way analysis of variance; therefore, the Scheffe Comparison Procedure was used to test for individual contrasts. Both cathepsin activities of SAL-treated, tyr-fed rat corneas were significantly different ($P < 0.01$) from the other groups.$^{23}$

neas (Table 1). Treating the tyr-fed rats with CP prevented the increase in these enzyme activities. CP treatment had no effect on enzyme levels measured in CP-treated control-fed rat corneas.

**Discussion**

The biochemical mechanism(s) of corneal epithelial and stromal inflammation seen in tyrosinemia II remains poorly characterized. Although later lesions contain dense PMN infiltrates, little is known about the humoral factors responsible for this local accumulation in the avascular cornea. Heat-labile, trypsin-sensitive chemotactic activity has been demonstrated in corneal culture supernatants$^1$ from tyr-fed rats with Stage II-III keratopathy. Several known humoral mediators may be responsible for the inflammatory response of tyrosinemia II. Candidates include: (1) lysosomal proteases released by local tissue damage or by PMNs, with subsequent C5a generation locally (injected C5 and C5a produce PMN migration into the cornea$^{24,25}$; (2) products of the arachidonic acid cascade, particularly leukotrienes$^{26,27}$; and (3) a chemotactic factor released by PMNs after crystal phagocytosis.$^{28}$ Studies of these potential mechanisms are currently in progress.

The lysosomal system has been implicated in the pathology of tyr-fed corneas. Using histochemical methods, Gipson and Anderson$^5$ showed increased lysosomal enzymes (aryl sulfatase and acid phosphatase) associated with autophagic vacuoles and multivesicular bodies in injured corneal epithelial cells 60 hr after introduction of the high tyr diet. At 84 hr the enzymes were associated additionally with PMNs. As expected, in the present study the lysosomal proteases cathepsins B and D were elevated in the tyr-fed rats. Both of these
enzymes, although acid proteases, potentially contribute to stromal degradation since (1) PMNs release H\(^+\) ions [30]; (2) the pH maximum for cathepsin B is only slightly lower at 5.8 than the pH of the cornea, 6.73; and (3) the active pH range for cathepsin D is increased by the presence of proteoglycans such as those in the cornea.

The data presented here suggest that WBC depletion inhibits the formation of tyr keratopathy and the production of chemotactic activity by tyr-fed rat corneas in culture. WBC depletion also prevents the increase in cathepsin B- and D-like activities observed in the corneas of tyr-fed rats, suggesting that WBCs, mainly PMNs, are a possible source of these activities. The control corneas contain substantial levels of these enzymes as reported previously for the rabbit cornea. Neutral proteases known to be present in PMN lysosomes were not detected in the corneal extracts, most likely due to the presence of protease inhibitors.

Characterization of these humoral factors in a rat model of keratopathy may lead to increased understanding of corneal inflammation. Pharmacologic intervention of the responsible mediators may be developed to prevent ulceration and eventual blindness in tyrosinemia II and may be extended to ocular inflammation of other causes.

**Key words:** chemotaxis, keratopathy, leukocytes, proteases, tyrosinemia

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**References**