Cellular Retinoic Acid-Binding Protein in Rat Lacrimal Gland

Katsuhiro Yamaguchi,*† Vinod P. Gaur,† Richard W. Young,†‡ and Andrew J. Sweatt*†§

We employed a monoclonal antibody to cellular retinoic acid-binding protein (CRABP) to assess the presence and localization of this retinoid-binding protein in the lacrimal gland of the rat. Immunoblots of extracts of rat lacrimal gland showed specific immunostaining of lacrimal CRABP in the region 14–16 kDa. Sections of rat lacrimal glands that were stained with anti-CRABP antibodies showed reaction product in the cytoplasm of the acinar cells. Retinoic acid may play a role in maintaining the proper function of lacrimal gland cells.

Vitamin A (retinol) and its metabolites play essential roles in the normal development and maintenance of many tissues. In the corneal epithelium, retinoids are vital for maintenance of normal growth and function. Vitamin A deficiency can result in keratinization of the corneal epithelium (xerophthalmia), and may lead to ulceration and melting of the corneal stroma (keratomalacia). In addition, in experimental vitamin A deficiency, the acinar cells of the lacrimal gland are adversely affected, showing loss of secretory granules and their glycosaminoglycan (PAS-positive) contents, abnormal nuclear morphology, and obliteration of secretory lumina.1 Topical application of retinoic acid reverses corneal keratinization associated with xerophthalmia in human vitamin A deficiency2 and expedites healing of experimental corneal wounds.3 Topical application of retinoic acid or retinol also promotes healing of corneal lesions in experimental vitamin A deficiency.4 The lacrimal glands of vitamin A-deficient animals also respond to systemic resupply of vitamin A, showing recovery of some aspects of normal morphology.1

Ubels and associates56 provided evidence that the lacrimal gland is the major source of retinol in rabbit tear fluid, although the conjunctival vasculature may be a source of blood-borne retinol. A compromise of vitamin A delivery by lacrimal gland could have severe consequences for the cornea and may be integral to the development of xerophthalmia in vitamin A deficiency. Hassel and Newsome7 have documented a requirement for retinoic acid in the synthesis of corneal glycoproteins, although the source of retinoic acid has not been identified.

Endogenously, cellular retinoic acid-binding protein (CRABP) is associated with all-trans retinoic acid,8 which implies an active participation of this protein in retinoic acid metabolism. However, CRABP has not been localized in the lacrimal gland or in tear fluid. To understand the metabolism of vitamin A derivatives in the lacrimal gland, we employed a recently developed monoclonal antibody to CRABP9 to assess the presence and localization of this retinoid binding protein in the lacrimal gland of the rat.

Materials and Methods

Animals

Postnatal day 16 and young adult male Sprague-Dawley rats were used and sacrificed in accordance with the ARVO Resolution on the Use of Animals in Research. The rats were maintained on a 12-hour light/12-hour dark cycle and light was provided by cool-white fluorescent lights at 6–7 foot candles at the cage level.

Antibody

The antibody used in these studies, MAb C-1, was raised in mice against purified bovine retinal CRABP, as described in detail in an earlier publication.9 In that work, through competition with purified CRABP, MAb C-1 was shown to be specific for a retina-derived...
proteins were separated by SDS-PAGE using 10\% acrylamide minigels, and transferred to polyvinylidene fluoride membranes (Immobilon P; Millipore Corp., Bedford, MA) using standard methods.10

The blots were probed using a blot-manifold system (Miniblot; Immunetics, Inc., Cambridge, MA), which allows several probes to be applied to each transferred gel lane. The buffer for immunoblot procedures was 50 mM Tris-buffered saline containing 1 mM each MgCl2 and CaCl2, pH 7.3. For blocking of nonspecific binding sites and for dilution of antibodies, this buffer was supplemented with 0.05\% Tween-20 (Sigma, St. Louis, MO). Blots were exposed to primary antibody (1:100 dilution) for 60 min, rinsed, exposed to peroxidase-conjugated goat anti-mouse IgG (1:250; E-Y Laboratories, San Mateo, CA) for 60 min, rinsed, incubated with 0.01% H2O2 and peroxidase substrate (diaminobenzidine) for 10 min, rinsed, and dried. Control treatments included substitution of buffer for the primary antibody.

Immunocytochemistry for CRABP

For CRABP immunocytochemistry, rat lacrimal glands were fixed in 4\% paraformaldehyde in PBS for 4 hours at room temperature. Tissues were dehydrated in ethanol and embedded in paraffin following standard techniques. Paraaffin-embedded tissues were sectioned at 10 \(\mu\)m and sections were mounted on gelatin-coated slides. These sections were deparaffinized then rehydrated prior to immunocytochemical treatment. Sections were treated with 0.3% hydrogen peroxide to eliminate endogenous peroxidase activity, then treated with 20% normal goat serum to block nonspecific antigenic sites. Sections were incubated sequentially with mouse monoclonal anti-bovine CRABP (1:100) and goat anti-mouse IgG secondary antibody—horseradish peroxidase conjugate (1:250). Incubations in primary and secondary antibodies were overnight at 4\(^\circ\)C, followed by rinses in 10 mM PBS, pH 7.4. After exposure to the peroxidase substrate, (0.025\% diaminobenzidine + 0.01\% hydrogen peroxide in 50 mM Tris-HCl, pH 7.6), the sections were dehydrated, cleared in xylene, and mounted in Permount.

For demonstration of lacrimal gland morphology, some tissues were fixed in 1% glutaraldehyde/1% paraformaldehyde in 0.086 phosphate buffer, dehydrated in ethanol, and embedded in LR White (Polysciences, Warrington, PA). Semi-thin sections (1–2 \(\mu\)m) were stained with toluidine blue. All specimens were examined and photographed with a Zeiss light microscope.

Results

Immunoblots of extracts of rat lacrimal gland, retina, and testis showed specific staining of CRABP in the region 14–16 kDa (Fig. 1). Control lanes (no primary antibody) showed no specific staining. Homogenates of rat liver, which has one-tenth the level of CRABP in testis,11 had barely detectable immunostaining near 14–16 kD (data not shown). In addition, both testis and liver had an immunostained band near 34 kDa, as noted in the earlier work in which the antibody was characterized.9

Light micrographs of rat lacrimal gland are shown in Figure 2. The typical arrangement of acinar cells in the lacrimal gland is seen in Figure 2A. While no staining was observed in control sections (Fig. 2B), tissues that were incubated with anti-CRABP had peroxidase reaction product in the acinar cells (Fig. 2C). Peroxidase reaction product was predominant in the perinuclear region of lacrimal gland acinar cells (examples are indicated by arrowheads in Fig. 2C).

Discussion

Based on recent studies of experimental vitamin A deficiency,1 the health of the lacrimal gland would seem to depend on a supply of vitamin A. Lacrimal glands of vitamin A-deficient rats show loss of secretory granules and other structural abnormalities that are indicative of impaired function. In light of these recent findings, the role of retinoic acid in maintaining normal function of the lacrimal gland acinar cells should be considered. While there is no conclusive evidence to suggest participation of retinoic acid in the visual cycle, retinoic acid is thought to play an important role in growth, morphogenesis, and differentiation. Demonstration of a retinoid gradient in areas undergoing vigorous growth seems to indicate a role for retinoic acid as a morphogen and inducer of differential gene switching.12,13 Recent characterization and localization of retinoic acid receptors in epithelial cells suggests a role in epithelial differentiation.14
Fig. 1. Immunoblot of extracts of rat lacrimal gland (Lanes 1, 2), retina (Lanes 3, 4), and testis (Lanes 5, 6). Primary antibody was replaced with buffer for lanes 1, 3, and 5, while anti-CRABP MAb C-1 was used to probe lanes 2, 4, and 6. Arrowhead indicates position of specific immunostaining of CRABP in the region 14–16 kD. Migration positions of molecular weight standards (kD) are shown at left of Figure.

The mechanism of action of retinoic acid is similar to that of steroid hormones—retinoic acid interacts with nuclear receptors.\textsuperscript{15,16} CRABP is considered to be important for the transfer of retinoic acid from the cytoplasm to a nuclear receptor.\textsuperscript{17} This mechanism of translocation of retinoic acid is supported by the fact that CRABP-deficient mutant cells do not respond to retinoic acid.\textsuperscript{18,19} Our clear demonstration of immunoreactive CRABP in the cytoplasm of acinar cells lends support to the hypothesis that retinoic acid plays a role in maintaining the health of the lacrimal gland.

It also should be noted that the corneal epithelium depends on a steady supply of vitamin A, and that the

Fig. 2. (A) Light micrograph of rat lacrimal gland, embedded in LR White, sectioned at 1 \( \mu \)m, and stained with toluidine blue. Several acini are shown, each composed of columnar epithelial secretory cells arranged around a central lumen. The cells have basal, darkly stained nuclei, and lightly staining apical regions consisting of endoplasmic reticulum and secretory vesicles. Scale bar: 25 \( \mu \)m. (B) Control paraffin section for immunoperoxidase procedure, for which buffer was substituted for anti-CRABP. Reaction product is not seen in control sections. (C) Immunostained paraffin section of lacrimal gland. Reaction product (arrowheads) is found in the cytoplasm of all the epithelial secretory cells. Scale bar for (B) and (C): 50 \( \mu \)m.
lacrimal gland is the major source of retinol in the tear fluid. Ubels and Edelhauser reported that topically applied retinol is metabolized to retinoic acid and more polar compounds in rat cornea. Because this is the common route of intracellular isomerization of exogenous retinol, a similar series of transformations may occur for vitamin A derivatives in the lacrimal gland. The subsequent metabolism of retinoic acid in the lacrimal gland could involve effects on nuclear receptors and incorporation into secreted retinoids that act on the cornea. Based on our localization of CRABP in the lacrimal gland, we suggest that retinoic acid metabolism in the lacrimal gland may be important for maintaining normal lacrimal gland function. Based on the role of the lacrimal gland in the production of tear fluid components, retinoic acid metabolism in the lacrimal gland also may be important for the health of the corneal epithelium.

Key words: cellular retinoic acid binding protein, lacrimal gland, retinoic acid, vitamin A, immunocytochemistry

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References