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Research Articles

Carbonic anhydrase type II in regenerating retinal pigment epithelium. A histochemical study in the rabbit

G. E. Korte* and J. Smith

Departments of Ophthalmology and Anatomy and Structural Biology, Albert Einstein College of Medicine, Montefiore Medical Center, Bronx, New York (New York 10467, USA)
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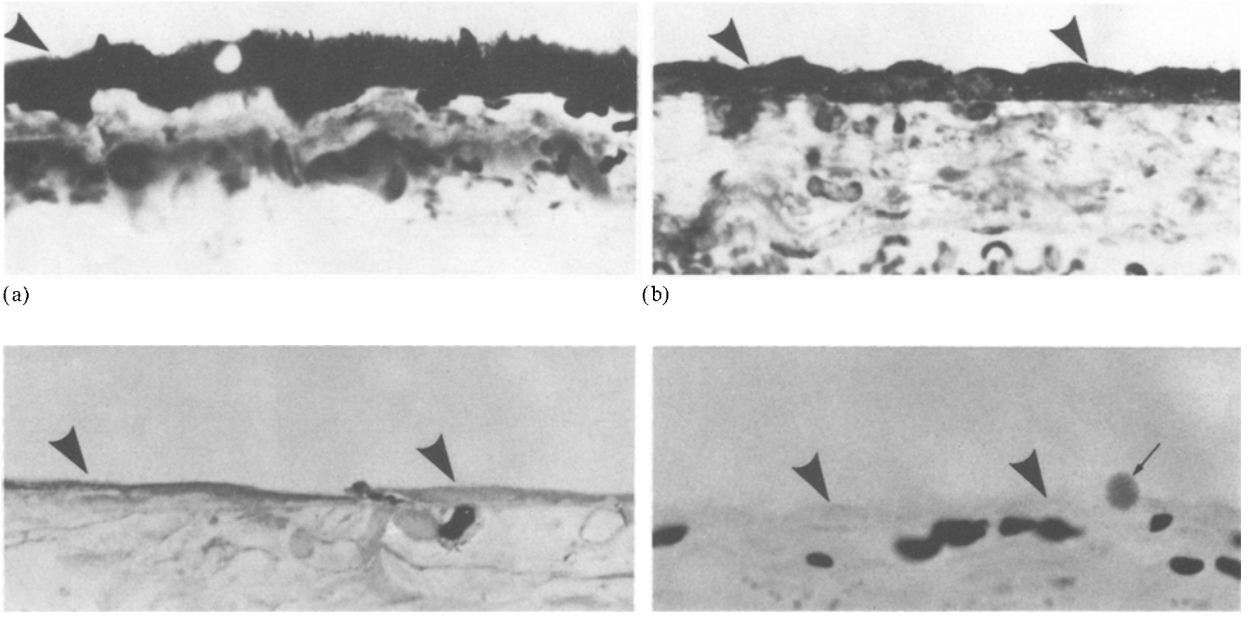
Abstract. Type II carbonic anhydrase (CAII) in the cytoplasm of the retinal pigment epithelium (RPE) may contribute to the transport of water and solutes across the RPE. The activity of this enzyme in RPE during its response to damage, e.g., during regeneration, is therefore of interest in understanding retinal disease. Immunohistochemistry was used to compare CAII activity of normal RPE and RPE experimentally induced to regenerate. In normal rabbits, the RPE stained intensely with a peroxidase-linked antibody specific for human CAII. Regenerating RPE stained less intensely. Within the regenerating epithelium, staining appeared more intense in mature cells than in immature ones, suggesting that CAII activity gradually returns during RPE regeneration.
Key words. Carbonic anhydrase; eye; histochemistry; regeneration; retina; retinal pigment epithelium.

The retinal pigment epithelium (RPE) plays a critical role in controlling the passage of molecules, ions and water into and out of the retina[1]. These transport functions help maintain the ionic and nutritive milieu required for photoreceptor function. If this relationship is upset, e.g., when the photoreceptors are separated from the RPE during retinal detachment, the photoreceptors atrophy and eventually die[2]. Although there is some evidence that RPE transport functions can return with recovery from damage[3,4], to our knowledge there are no studies of the status of transport-related enzymes during RPE healing. We have undertaken studies of this in an experimental model of RPE wound healing, or regeneration – rabbits injected intravenously with sodium iodate[5]. In the case of the plasma membrane enzymes alkaline phosphatase and the sodium potassium ATPase, we observed similar patterns of response, using cytochemical staining procedures[6,7], i.e., normal RPE stained intensely while regenerating RPE stained less intensely. Within the regenerating epithelial sheet the more mature cells stained more intensely than immature ones, suggesting a return towards normal expression of these enzymes. In this report we extend these observations and examine another enzyme involved in transport across the RPE, the cytoplasmic enzyme carbonic anhydrase II (CAII).

Materials and methods

Observations were made in 4 normal and 4 experimental female, albino rabbits weighing 3–4 kg. Normal and experimental animals were processed in pairs, so that normal and experimental tissue processed identically could be compared. The experimental animals received intravenous sodium iodate (50 mg/kg body weight, in 5 ml of saline) to induce necrosis and subsequent regeneration of the RPE[5]. One week later the animals were sacrificed by an overdose of intravenous sodium pentobarbital (145 mg/kg body weight). Animal husbandry and experimentation conformed to the principles and guidelines of the Swiss Academy of Medical Sciences. The eyes were then enucleated into cold saline, the cornea, lens and vitreous removed and the eyecups sliced into quadrants. The neural retina was then peeled away from each tissue piece to expose the RPE, insuring immediate fixation and facilitating exposure of the RPE to antibody (below). In some specimens the neural retina was left attached to serve as an internal, positive control due to staining of the Müller glial cells for CAII[8,9]. The tissue was fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.2 for 1–2 h at 4 °C. Tissue pieces 2–3 mm wide and approximately 5 mm long from the peripheral fundus were then rinsed 3–4 h in 0.01 M phosphate buffer, pH 7.2 containing 0.2% Triton-X-100, and then immersed overnight at 4 °C in sheep polyclonal antibody to human erythrocyte CAII that was linked to horseradish peroxidase (Biodesign International, Kennebunkport, ME). A dilution of 1:500 provided optimal staining and was used for the observations reported herein, the antibody being diluted with 0.01 M phosphate buffer containing 0.2% Triton-X-100. After incubation the tissue pieces were rinsed in 0.1 M phosphate buffer and the peroxidase labelled antibody was localized by the diaminobenzidine procedure[10]. The tissue pieces were then rinsed in buffer, dehydrated and embedded in epoxy resin. Sections 3 μm thick were used for light micro-



(a) (b)

(c) (d)

Figure. Light micrographs of normal and regenerating RPE (denoted by arrowheads) stained with antibody to human CAII. This set of pictures is from normal and experimental animals processed in tandem, and are representative of the differences seen in three other pairs of normal and experimental animals. Space along the top of the pictures is due to peeling off the neural retina during tissue processing. Choroid is at the bottom of each picture.

a Normal RPE stains intensely. Round, white space within RPE is due to a lipid droplet extracted during tissue processing. The space along the bottom of the picture is artifact due to splitting of the choroid during tissue processing (× 720).

b Regenerating RPE that is quite mature, consisting of cuboidal cells like normal RPE, but smaller (cf. fig. a, at same magnification). Stain is less intense than normal RPE but more intense than very immature RPE, as seen in fig. c, (× 720).

c Very immature RPE, characterized by flat, elongated cells. Staining is less intense than more mature RPE seen in fig. b, (× 720).

d Control section of tissue incubated without antibody. RPE does not stain. The cells appear as ghosts above the red blood cells in the underlying choriocapillaris, which stain due to their content of endogenous peroxidase and CAII. Arrow denotes lipid drop in RPE that remained undissolved during tissue processing, (× 720).

scopy, in some cases counterstained with toluidine blue. Control tissue for immunohistochemistry was processed without exposure to the antibody-peroxidase complex. Observations were made on sections from 3–4 blocks of tissue from both eyes of each rabbit.

In an effort to further document differences in staining intensity among immature and mature cells in the regenerating epithelial sheet, one well stained, 3 μm thick section containing stretches of immature and mature epithelium from each of the four experimental animals was subjected to microdensitometry. The sections were examined with a 4 × objective and bright field optics and the image transmitted on a video camera screen and digitized by computer. The grey scale value of a sampling box of 25 × 25 pixels in size was measured, this size box being small enough to sample cytoplasmic areas of regenerating RPE without including nuclei or lipid droplets in it. Measurements were made from 10 cytoplasmic areas of mature cells (comparable to those seen in fig. b) and 10 areas of immature cells (comparable to those seen in fig. c) in each section and the measurements compared in each sample. Measurements among the different slides (i.e., among different animals) were not compared, due to methodologic considerations, e.g., the need to set different baseline grey scale values for each slide.

Results

Observations were made one week after administration of sodium iodate. Previous observations have shown that at this time necrotic RPE has been removed by macrophages and spared RPE cells have produced a new epithelium that migrates along the remnant basement membrane (ref. 5; fig. 1 of ref. 11). This produces a retina that is severely visually compromised, although remnant patches of photoreceptors probably provide some sensitivity to light levels.

Normal RPE stained intensely for CAII, while regenerating RPE stained less intensely (cf. fig. a and b, c). Within the regenerating epithelial sheet immature cells stained less intensely than mature cells (cf. fig. b, c). Microdensitometry measurements supported this observation (cf. table).

The staining was reduced or eliminated in control sections incubated without antibody (fig. d). As described by several other investigators[8,9], the Müller glial cells in the neural retina stained intensely for CAII, serving as an internal positive control.

Table. Microdensitometry of regenerating RPE

Slide #	Mature cells*	Immature cells*
1	88.8 ± 9.2	165.5 ± 11.7
2	38.4 ± 5.4	72.7 ± 4.9
3	13.7 ± 3.1	71.1 ± 4.4
4	82.5 ± 5.3	102.9 ± 5.9

Sampling and measurements as described in 'Materials and methods'.
*Values are mean ± standard deviation. Values from mature and immature cells for each slide are significantly different ($p < 0.0001$, t-test). Note densitometry readings are proportional to amount of light passing through specimen, so immature (i.e., less intensely stained) cells have higher values than mature cells.

Discussion

The observations confirm the presence of cytoplasmic CAII in normal rabbit RPE[12,13] and show that its expression is modulated during experimentally induced regeneration. Within the regenerating RPE sheet there is a gradient in CAII expression, such that activity is less in immature cells than in mature cells. This gradient was documented by microdensitometry. The accuracy of the observations is further supported by identical ones made using another, less specific procedure for staining carbonic anhydrase – the cobalt capture procedure of Hansson[14], which we have reported on previously in abstract form[15].

The observations on CAII are similar to those made on alkaline phosphatase and sodium potassium ATPase[6,7]. They provide additional enzyme histochemical evidence that the RPE can recover function after damage, through regeneration. While the function of CAII in RPE is not well documented, it can be assumed that it contributes hydrogen and bicarbonate ions that are involved in transport mechanisms across the RPE plasma membrane[16,17]. As noted in the introduction, the observations support evidence for the functional recovery of damaged RPE derived from the study of laser lesions and the restoration of the blood-retinal barrier[3,4]. The ability of RPE to recover after damage certainly contributes to the re-establishment of normal-function at the retina-choroid interface, an important site of transport and cellular interactions that support retinal function.

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* Correspondence to Gary E. Korte, Ph.D., Dept of Ophthalmology, Montefiore Medical Center, 111 E. 210th Street, Bronx, New York 10467, USA

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