Localization of Alkaline Phosphatase on Basolateral Plasma Membrane of Normal and Regenerating Retinal Pigment Epithelium

A Cytochemical Study in Rabbits

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Ultrastructural cytochemistry was used to analyze the polarized distribution of alkaline phosphatase (AP) on the plasma membranes of normal and regenerating retinal pigment epithelium (RPE) and the ciliary body epithelium in rabbits. The AP activity was concentrated on the basolateral plasma membrane in normal RPE. In regenerating RPE (after intravenous administration of sodium iodate to damage the RPE), there was a differential expression of AP activity according to the site on the regenerating epithelial sheet. At the edge of the sheet, where cells were undifferentiated and immature (ie, without the polarized distribution of basolateral and apical plasma membrane specializations seen in normal RPE), no plasma membrane AP activity was observed. The AP activity was reexpressed more proximal in the regenerating sheet. It was first evident where contiguous cells formed junctional complexes and cytologic polarization again became apparent. New AP activity was restricted to the basolateral plasma membrane. Eventually, the polarized distribution of AP activity seen in normal RPE was reestablished. In the ciliary body, AP activity was localized to the basolateral plasma membranes of the outer, nonpigmented epithelial cells. It was concluded (1) that cytochemical activity for nonspecific AP is concentrated on the basolateral plasma membrane domain of normal RPE and the nonpigmented ciliary epithelium and (2) that, during RPE regeneration, the normal pattern of AP activity is lost initially and then reexpressed. Invest Ophthalmol Vis Sci 32:3187–3197, 1991

Several molecules have a polarized distribution over the plasma membrane of the retinal pigment epithelium (RPE). The sodium–potassium adenosine triphosphatase, receptors for retinol binding proteins, mannose-6-phosphate and mannose binding sites, proteoglycans, integrin, fibronectin, and endocytosis sites (coated pits) were localized to the apical or basolateral plasma membranes or discrete portions of them. Their polarized distribution is important for retinal function, eg, the transport of molecules and ions across the retina–choroid interface.

We describe the polarized distribution of another molecule associated with the plasma membrane—nonspecific alkaline phosphatase (AP)—over the surface of normal rabbit RPE and its redistribution during regeneration. First, we wanted to clarify the distribution of AP over the surface of normal RPE after reports that these cells stain for AP by light-microscopic histochemistry and biochemical studies suggesting its enrichment on the basolateral plasma membrane. Second, we wanted to obtain more information on the redistribution or reexpression of a plasma membrane molecule during RPE responses like those seen during retinal disease, in this case, during experimentally induced regeneration. This phenomenon has been observed for sodium–potassium adenosine triphosphatase, found on the basolateral plasma membrane and the apical plasma membrane (where it normally is located) when the tight junctions between RPE of dystrophic rats break down. Third, we also examined the distribution of AP on the ciliary body epithelium. This has not been described previously to our knowledge, and we wondered if the inner epithelium, which is continuous with the RPE, would exhibit similar activity for AP.

Our study used rabbits that received sodium iodate intravenously. Sodium iodate poisons the RPE over
Materials and Methods

Observations were made on two albino and two pigmented normal, mature female rabbits and three pigmented and three albino rabbits that received sodium iodate (sodium iodate-induced retinopathy is expressed similarly in pigmented and albino rabbits: GK, unpublished observations). The drug was administered intravenously in a single dose of 50 mg/kg in 10 ml of saline over approximately 10 min. The rabbits were killed 1–2 weeks later by an overdose of sodium pentobarbital (145 mg/kg intravenously), and their eyes were enucleated into cold saline. The cornea and lens were removed and the vitreous expressed. The eyeballs were then immersed in fresh cold saline, where the neural retina was peeled away to expose the RPE sheet. The remaining eyecup, lined with RPE, was immersed for 2 hr in cold 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. The eyecups were sliced into pieces 1–2 mm wide, in the central-to-peripheral direction. This provided pieces of tissue for histologic examination in which the spared RPE at the far periphery could be traced into continuity with the regenerating epithelial sheet arising from it when the embedded tissue slices were cut lengthwise. Tissue from normal rabbits was obtained from sites throughout the fundus, but favoring the far periphery where the spared RPE giving rise to regenerating RPE was examined. The ciliary body also was examined in normal rabbits, using identical procedures.

After rinsing in buffer overnight at 4°C, the slices of tissue were incubated in media for AP localization. The former procedure uses an incubation medium consisting of 80 mM calcium chloride, 40 mM sodium barbiturate, 10 mM sodium glycerophosphate, and 5 mM magnesium chloride, at pH 9. The tissue is rinsed in distilled water briefly and then in 0.05 M lead nitrate solution for 2–5 min. The latter procedure uses an incubation medium consisting of 28 mM Tris HCl buffer, 20 mM sodium glycerophosphate, 15 mM magnesium sulfate, and 2 mM lead citrate, at a final pH of 9.2–9.4. This procedure gave the most consistent staining with the least background of precipitate. Control slices of tissue were incubated in media containing levamisole (2.5 mM; Sigma, St. Louis, MO) or in the absence of sodium beta-glycerophosphate. Enzyme activity at a site was considered specific when, by comparison with control tissue, the activity was reduced greatly or eliminated. Remnant precipitate or that with no apparent difference between experimental and control specimens was considered nonspecific activity.

Slices of tissue were reacted at room temperature for 10–40 min. We found that incubation of razor-cut slices of tissue (compared with sections cut on a cryostat or tissue chopper) permitted reagents to penetrate and maintain the geographic orientation important for analyzing the regenerating epithelial sheet. Other investigators also used this procedure for cytochemistry of the RPE and adjacent tissues. After incubation, the slices were rinsed in 0.1 M sodium cacodylate buffer, immersed for 1 hr in 1% osmium tetroxide in cacodylate buffer, and then dehydrated and embedded in epoxy resin. Thick sections (2–3 μm) were stained with toluidine blue, and thin sections were stained with lead citrate and uranyl acetate. Unstained thin sections often were examined to control for artifactual deposition of lead or uranyl salts on the section surface during staining. This could be confused with the precipitate at sites of AP localization.

The animals were treated according to the ARVO Resolution on the Use of Animals in Research.

Results

Our findings for normal rabbit RPE are described first, followed by those for regenerating RPE of experimental animals. Our observations of the normal rabbit ciliary body epithelium then are presented.

Normal RPE

The basolateral plasma membrane of normal RPE stained intensely for AP (Figs. 1A, B). The apical plasma membrane generally did not, although in some cells sparse activity was observed on the apical plasma membrane (Fig. 1B, inset). This distribution of precipitate was observed in pigmented and albino rabbits and at all sites sampled in the fundus. The activity was reduced or eliminated in control sections of normal and regenerating RPE incubated in the absence of substrate or the presence of levamisole (Fig. 2).

Regenerating RPE

The distribution of AP differed on the plasma membranes of regenerating cells at or near the leading edge and on more mature cells located proximal in the regenerating epithelial sheet. After sodium iodate...
administration, the RPE migrated along the basement membrane denuded by removal of necrotic RPE during the first few days after administration of the drug. During the time window of 1–2 weeks after administration of the sodium iodate, the leading edge of the regenerating epithelial sheet had similar histologic findings. It consisted of flat, undifferentiated cells at the distal edge with maturing cells more proximal. These cells, in turn, gradually became fully mature, redifferentiated RPE. The histology of the regenerating RPE sheet in this model briefly was described elsewhere.16'17'23

Cells at or near the edge of the regenerating epithelial sheet: The cells at the edge of the epithelial sheet and several cells more proximal characteristically were flat and undifferentiated (Fig. 3A). There was no unequivocal AP activity of their plasma membranes, even after incubations as long as 40 min, except for scattered sites where some faint activity was apparent (Figs. 3A–C). The lack of activity in this region probably was not a result of failure of the histochemical procedure because sites more proximal in the regenerating sheet of the same specimens showed intense AP activity.

Cells more proximal in the regenerating epithelial sheet: When electron microscopic montages were examined of the epithelial sheet beginning at the distal edge, extending proximally, and including more differentiated cells than those found at the edge, a gradient of AP activity was observed. In cells more proximal, and thus more differentiated (eg, with well-developed junctional complexes, more prominent basal and apical plasma membrane specializations, and more numerous attachments to the basement membrane), the staining was extensive along the basolateral plasma membrane and appeared similar to that observed on normal RPE (Figs. 4A, B). In the most mature regenerated RPE, the staining pattern was identical to that of normal RPE (Fig. 5).

Ciliary Body Epithelium

In the ciliary body epithelium, activity was observed only on the basolateral plasma membranes of the nonpigmented epithelium of the pars plicata and pars plana (Fig. 6). This activity also was lost in control tissue (Fig. 6, inset).

Discussion

Our observations clarify the distribution of AP over the surface of normal RPE and ciliary epithelium and provide some new observations on its redistribution during RPE regeneration.

Normal RPE

The distribution of AP in normal RPE confirmed (1) light microscopic observations that RPE contains this enzyme and (2) biochemical ones suggesting it is enriched in the basolateral plasma membrane.8'11 Our observations confirm that the AP in the "b" membrane fraction11 was derived from the basolateral plasma membrane. These investigators also found some AP (about sevenfold less than on the basolateral plasma membrane fraction) on a fraction derived from the apical plasma membrane. Whether this is a contamination of their specimens or corresponds to the faint apical plasma membrane activity seen in some of our preparations cannot be determined without further observations. Additional findings also are required to determine if the apical staining seen in our preparations was nonspecific activity resulting, for example, from phosphates bound to the extracellular matrix.
Fig. 1. (B) Black precipitate indicating AP is localized to the basolateral plasma membrane (eg, that of basal folds [bf], here sectioned somewhat tangentially). Apical plasma membrane (in upper left) beyond the region of the junctional complex (arrow) does not stain or stains only sporadically (deposits of stain encircled in the inset). Bruch's membrane, including the basement membrane of the RPE, and the choriocapillaris are at the bottom of this and subsequent pictures (×14,500; inset ×22,000).
Because sodium–potassium adenosine triphosphatase is enriched on the RPE apical plasma membrane,111 this tissue shows a polarized distribution (or selective enrichment) of these enzymes seen in other epithelial cells.24–27 The RPE distribution of AP and sodium–potassium adenosine triphosphatase were similar to that observed in the choroid plexus epithelium, where these enzymes are enriched on the basolateral and apical plasma membranes, respectively.28

Regenerating RPE

During regeneration, AP activity was not expressed on the most immature (presumably migrating) cells located at the distal edge of the epithelial sheet. More proximal, as the cells matured and formed well-developed intercellular junctions, extensive basal folds, and apical projections, the AP activity was reexpressed in the polarized distribution seen in normal RPE. The new activity first was seen unequivocally where contiguous cells formed junctional complexes and numerous attachments to the basement membrane and basal folds, events that occur simultaneously during the maturation of cells in the regenerating epithelial sheet.

The redistribution of AP activity during RPE regeneration was similar to other experimental systems where AP modulation was observed. In rabbit corneal epithelium after denudation, the AP activity was lost and then reexpressed as the epithelium regenerated.29 In cultured LLC-PK1 cells (an epithelial cell line derived from pig kidney), plasma membrane AP activity differed in exponentially growing and confluent cultures.30 No AP activity was seen in low-density growing cultures, but it returned with confluency and attachment of the cells to the substrate, a situation comparable to that seen in regenerating RPE. Furthermore, as in regenerating RPE, the reexpression of AP occurred simultaneously with or after intercellular junctional complex formation. These observations suggest that possible signals for AP reexpression in regenerating RPE include the formation of junctional...
complexes and interaction with the substrate. The former possibility was supported by observations on MDCK cells, in which the return of plasma membrane aminopeptidase activity first occurred where cells were contiguous and then spread over the rest of the surface. The latter possibility was supported by the observation that AP expression increased with increased attachment of cultured LLC-PK1 cells to their substrate. The functional significance of the modulation of
Fig. 4. Cells from the more proximal part of the regenerating RPE sheet, showing AP activity of formative basolateral plasma membrane on maturing RPE, from a rabbit obtained 11 days after iodate. (A) The plasma membrane on the choroidal side of the junctional complex (arrow) is heavily stained and contains focal sites of basal fold formation (open arrows) that, as the cells mature and remodel, will come to face the basement membrane, as seen in (B). Staining is less intense or absent on plasma membrane facing the basement membrane (bm). The cell whose plasma membrane is stained in the tissue beneath the basement membrane is a fibroblast; their plasma membranes do not normally stain for AP except when activated (eg, during wound healing) (×20,750). (B) AP activity of basolateral plasma membrane of a more mature cell than that seen in (A). The cells are taller, have foci of well developed basal folds (open arrows) that actually face the basement membrane, prominent apical projections and junctional complexes (jc). Some activity occurs on the apical plasma membrane (encircled) of the cell at right, but not on the cell at left. Stain occurs on basal folds (bf) as well as on some unfolded plasma membrane at far left (arrow) (×12,500).

AP expression during RPE regeneration is unclear because the function of this enzyme in RPE, as in other cells, is speculative. For example, modulation of AP expression and its activity was correlated with changes in calcium transport and levels of vitamins A and D in other tissues, but the relationship in RPE (which transports calcium and is a target for vitamin A) has not been examined to our knowledge.
Fig. 5. AP activity of regenerated RPE cells more proximal in the regenerating epithelial sheet than those illustrated in Figure 4, and from the same animal. Note more discrete cytologic polarization (eg, all the basal folds now face the basement membrane, and none are found on the lateral membrane, as they are in Fig. 4). Apical projections are better developed but are not as numerous or as long and slender as in normal RPE cells (cf, Fig. 1). As in normal RPE, stain precipitate is located on the plasma membrane of the basal folds (large arrow) and on the lateral plasma membrane up to the level of the intercellular junctional complexes (small arrow). Some scattered precipitate (encircled) occurs on the apical plasma membrane. The cells contain some melanosomes because the tissue is from a pigmented rabbit. Some nonspecific activity (seen also in the control tissue of Fig. 2) is seen in the cytoplasm bordering melanosomes. Endothelium of choriocapillaris below does not stain, but some deposition is observed on the surface of red blood cells (×14,500).

However, the finding that AP is a marker of the RPE basolateral plasma membrane makes it a useful probe of the reorganization of this membrane and the targeting of a major plasma membrane component to it during regeneration.

Ciliary Body Epithelium

Our observations clarified light microscopic findings indicating that the ciliary body epithelium contains AP but not identifying which layers stain for it.8,9 We showed the outer nonpigmented epithelium stained for AP instead of the inner pigmented epithelium, although we expected the latter to stain because it is in continuity with the RPE and is ontogenetically related to it. Similarly, in the rat, the ciliary epithelium does not stain for cellular retinol binding protein, but the RPE does.38

Like the RPE, the basolateral plasma membrane of
Fig. 6. AP activity of ciliary body epithelium, here from the pars plicata. Activity is located over the basolateral plasma membranes up to the level of intercellular junctional complexes (not illustrated here) that connect the outer, nonpigmented epithelial cells. Posterior chamber is at lower left. Inset: In specimens incubated in the presence of levamisole the activity is lost (both pictures, ×29,000).

the unpigmented ciliary body epithelium also stained for AP. Unlike the RPE, however, this is the same membrane that contains sodium-potassium adenosine triphosphatase. The ciliary body epithelium is thus an example of the copolarization, or enrichment, of these two enzymes on the same plasma membrane.
domain. Other procedures are necessary to identify sodium-potassium adenosine triphosphatase because this enzyme cannot use beta-glycerophosphate as a substrate. This observation and the effectiveness with which the levamisole control eliminated activity indicate that the activity described on the ciliary epithelium was a result mainly of AP.

Key words: alkaline phosphatase, eye, plasma membrane, retinal pigment, epithelium, ultrastructure

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