Twenty-five human eyes of various ages from eye bank donors and surgical enucleations were obtained for ultrastructural cytochemical demonstration of acid phosphatase (AcPase) and arylsulfatase B (ASB) in the retinal pigment epithelium (RPE) and Bruch's membrane. Results with post-mortem (<10 hr) tissues were comparable to those of fresh specimens. Vigorous reactivity was demonstrated in lysosomes of RPE and choriocapillary endothelium but no reactive sites were found in Bruch’s membrane, although many lysosome-like dense bodies occurred in eyes >20 yr of age. Granular drusen of 30–70-yr-olds contained no reactive bodies. In eyes >80 years old blebs of RPE basal cytoplasm protruding into Bruch’s membrane contained reactive lysosomes. We conclude that the RPE ordinarily does not extrude or exocytose active lysosomes (ie, phagolysosomes, other secondary lysosomes, residual bodies, lipofuscin) or lysosomal enzymes. Aged RPE, however, extrudes cytoplasm with active lysosomes into Bruch’s membrane. The possible impact of this process on the extracellular connective tissue is discussed, particularly with regard to age-related deterioration of Bruch’s membrane and neovascularization. Invest Ophthalmol Vis Sci 28:1138–1147, 1987

A retinal pigment epithelial (RPE) cell is known to possess an extremely active lysosomal system capable of degrading thousands of outer segment disks per day.1 With time, indigestible residues of this phagocytic activity appear to accumulate in the RPE as residual bodies (lipofuscin granules). The life history of lipofuscin pigment granules and their ultimate fate are unknown. Some investigators suggest that residual bodies are released into Bruch’s membrane2–3 and others claim that active RPE lysosomes4 and even phagosomes5 reach Bruch’s membrane. If release of phagolysosomal organelles regularly occurs, then their lytic enzyme contents might be dispersed with possible damaging impact. Enzymatic modification of Bruch’s membrane connective tissue has been suggested as a mechanism facilitating neovascular invasion of Bruch’s membrane in age-related macular degeneration.6

Accumulation of abnormal material in Bruch’s membrane is one of the hallmarks of the aging eye. Some of the accumulated materials resemble cytoplasmic constituents such as vesicles, membrane-bound lysosome-like dense bodies, etc., whereas others resemble normal extracellular components such as collagen, basement membrane, plasma proteins, etc. (Fig. 1). The mechanisms underlying the age-related accumulation of these components in Bruch’s membrane are beginning to be more clearly delineated. The bulk of the materials that resemble cytoplasmic components appear to originate in the RPE cell; a bolus of basal cytoplasm appears to be sequestered and jettisoned into Bruch’s membrane where subsequently the limiting membrane of the bolus apparently breaks, scattering the contents. In order to evaluate further the hypothesis that the debris which accumulates in Bruch’s membrane is of RPE origin, we have analyzed this material for the presence of lysosomal enzymes.

Materials and Methods

Table 1 lists the age, sex, post-mortem delay time and cause of donors’ death for the eyes used in this study. Two eyes were fixed immediately following surgical enucleations for malignant melanoma; other eyes were generally placed in cold fixative by eye bank personnel after removal of the cornea and then were further dissected in our laboratory. Eyes of the donors age 1, 22, 69, 78, 84, 85 and 98 were dissected prior to fixation. The anterior segment, lens, vitreous and sensory retina were removed and the remaining posterior eye cup immersed in fixative. During 1-hr fixation, small (2 mm × 5 mm) pieces of RPE-cho-
roid from macular, posterior non-macular, equatorial, and peripheral areas were cut. The fixative was 1.5% glutaraldehyde in 0.1 M Na cacodylate buffer, pH 7.2, containing 1% sucrose. In several cases tissue was chopped after fixation on a Smith-Farquhar tissue chopper (DuPont-Sorvall, Newton, CT), but this was not essential for obtaining adequate penetration of reagents; moreover, drusen contents were lost when these slices were incubated. Tissue was washed three times in 0.05 M acetate buffer plus 7% sucrose at pH 5.2 then incubated for 1 hr at pH 5.2 in Novikoff's cytidine monophosphate medium (Sigma).

Table 1. Twenty-five eyes of 18 individuals used in this study

<table>
<thead>
<tr>
<th>Age</th>
<th>Sex</th>
<th>Post-mortem/enuel time (hr)</th>
<th>AcPase</th>
<th>ASB</th>
<th>Comments</th>
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<td>M</td>
<td>0.1</td>
<td>OD</td>
<td>OS</td>
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<td>OD</td>
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<tr>
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</tr>
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Chemical Co., St. Louis, MO) for the demonstration of acid phosphatase (AcPase). Additional pieces of tissue were incubated in the presence of 5 mM ouabain to lessen ATPase-related precipitation of lead reagent at the apical side of the RPE cells. Also, deletion of substrate or addition of 10 mM NaF to the incubation medium were additional controls for specificity. In four cases, tissue was incubated in medium containing para-nitrocatechol sulfate (Sigma), pH 5.5, for demonstration of aryl sulfatase B (ASB). Controls consisted of deletion of substrate or addition of tauroxycholate to the incubation medium. Following the incubation procedures tissues were rinsed, post-fixed in osmic acid, dehydrated and embedded in epoxy resin. Semi-thin sections were stained and examined by light microscopy to locate sites of interest in Bruch's membrane. Although young eyes had few or no drusen or other obvious alterations of Bruch's membrane, each block of tissue from each area of retina was sectioned at several levels and examined with ×100 oil immersion optics for debris sites; when found, these were thin-sectioned for electron microscopic examination. Initially, thin sections were examined and photographed unstained by transmission electron microscopy (TEM) to document the distribution of the cytochemical reaction product and then were stained with uranyl acetate and lead to improve tissue contrast. Generally, the reaction product was found to be unaltered by the staining procedure. Serial sections were examined in all possible cases to assess the fidelity of the phosphatase-induced lead phosphate precipitate from one section to another and to partially reconstruct three-dimensional structures. Unincubated specimens of all of the eyes were also prepared and photographed by conventional TEM.

Results

Lysosomes of RPE cells, choriocapillary endothelial cells, leukocytes in the lumen of choroidal blood vessels, and various cells of the choroid were positive for AcPase, that is, they contained the lead precipitate characteristic of this histochemical reaction. In RPE cells the AcPase reaction was located as a thin, interrupted cortical layer around phagosomes, around some lipofuscin granules (residual bodies of the lysosomal system) and similarly in some melanosomes (Figs. 1-4). The reaction product in other bodies smaller than lipofuscin granules, interpreted as primary and secondary lysosomes, usually covered most of the area of the organelle (Figs. 3-8). In some specimens reaction product was seen at sites not identified with a lysosome, eg periodic deposits along lateral plasma membranes and fine precipitates internal to the plasma membrane (Figs. 1, 4-6, 8). These are interpreted as sites where enzymes have leaked from lysosomes and now are situated in the cytosol; this finding and the specific localizations described above are consistent with the results in fresh animal tissue reported in the literature. Localization of reaction product in the choriocapillaris is confined to small distinct bodies (ie, lysosomes) of endothelial cells (Fig. 6), pericytes, and leukocytes that occasionally lie in the capillary lumen.

The ASB reaction yielded fewer reaction sites in all cells examined than did the AcPase method (Fig. 9). The reported enhanced demonstration of vascular endothelial cell lysosomes by this method was not observed in this study.

Bruch's membrane, despite the frequent presence of bodies resembling lysosomes, failed to show positive reactions in these bodies (Figs. 1, 4, 6-9). The possibility that the failure was due to lack of penetration of substrate or other reactants could be dismissed when adjacent choriocapillary endothelial cells contained lysosomes that were positive (Fig. 6). Bruch's membrane sites that contained lysosome-like bodies were always examined for evidence of adequate pen-
Fig. 3. Surgical specimen, 60-yr-old. Chopping of tissue before incubation separated the RPE and its BM from Bruch's membrane. Note reaction product forms a rim around phagosome (Ph), and most lipofuscin granules (Lf): it fills other primary and secondary lysosomes (Ly) at the apex and base of the cells. Some non-specific staining is seen, principally in the apical cytosol, however none is seen on apical microvilli. Ouabain incubation medium (×19,000).

Fig. 4. RPE-Bruch's membrane of an 84-yr-old donor, 4.5 hr post-mortem. The distribution of AcPase reaction product in the RPE is similar to that of the surgical specimens, Figs. 1 and 3. Bruch's membrane contains various bodies (B) that resemble lysosomes, but none of these shows enzyme activity. Electron-dense calcified structures are distinguishable from the lead precipitate (arrows). Unchopped specimen: ouabain incubation (×11,000).

eration of reactants, and in most cases satisfactory verification of this could be found in the choroid. Therefore we feel confident that the negative result was not due to some technical or methodologic failure. We conclude that these extracellular lysosome-like bodies originate in the RPE but their enzymatic...
activity is either lost or masked when they become dispersed in Bruch’s membrane.

The youngest eye in which a focal cytoplasmic debris site was found in the inner collagenous zone of Bruch’s membrane was the 17-yr-old specimen. Three sites were found in the 22-yr-old specimen (Fig. 6). The frequency of these sites appeared to increase with the age of the donor. No intact blebs of RPE basal cytoplasm were found in Bruch’s membrane of these young eyes, however.

The only instances in which AcPase-positive lysosomes were seen within Bruch’s membrane were where a bag of cytoplasm, still attached to the RPE cell by a narrow isthmus, protruded through the RPE basement membrane into the inner collagenous zone (Fig. 5). In our sample of eyes such instances were found only in specimens of 84- and 85-yr-old donors. Two such sites were found in non-macular specimens of each eye, but an exhaustive search for more was not conducted. At sites where the limiting membranes of presumably comparable bags of cytoplasm had apparently ruptured, the lysosome-like bodies were negative (Fig. 6).

Drusen of the granular type (ie, those containing dense bodies, vesicles, etc., as opposed to drusen with homogenous non-granular contents) when incubated for AcPase and ASB, likewise showed no reaction in the bodies or granules (Figs. 7, 9), even though these structures often resemble morphologically the lysosomes of the overlying RPE cell.

The post-mortem tissue used in this study gave results surprisingly comparable to that of fresh tissue. Although it is often assumed that lysosomes release their content of hydrolytic enzymes soon after death, we did not find this to be the case with RPE of the eye bank eyes used in this study. Evidence of some enzyme leakage, that is, reaction product scattered in the cytosol, was found in the RPE of the rapidly fixed surgical specimens (Figs. 1 and 3) as well as the eye bank eyes (Figs. 4, 8, 9). Leaked enzyme was gener-
ally retained within cell borders. Even the 28-hr post-mortem specimen had vigorous and appropriate enzyme localization, with leakage confined to intracellular sites of intact cells; however, there were more ruptured RPE in this specimen than in those with shorter post-mortem times. As in the other specimens, Bruch's membrane was negative for AcPase in this donor (data not shown). We find consistently in this and other studies that the tissue of old donors retains better post-mortem ultrastructural integrity than tissue of young donors.

Discussion

We felt that the question of possible extrusion of lysosomes into Bruch's membrane was of sufficient importance to warrant a study using post-mortem eyes, despite the customary avoidance of any but freshly harvested tissue for ultrastructural enzyme localization. Comparison of results obtained from fresh surgical specimens to those from eyes obtained post-mortem indicate that the latter are suitable for lysosomal enzyme cytochemical studies when obtained up to 10 hr after death. Artifactual staining of non-lysosomal sites is often obtained with the enzyme cytochemical techniques employed in this study. However, since the major finding was that extracellular lysosome-like structures in Bruch's membrane did not stain, the customary concern over the presence of artifactual-positive sites is not germane.

The possibility of artifactual-negative sites in Bruch's membrane can be dismissed for practical purposes, if there is evidence of adequate penetration of substrate and lead into Bruch's membrane. Reaction product being visible by light microscopy allowed selection of areas with positive sites on both sides of Bruch's membrane for ultrastructural examination. Based on findings in this sample of 25 eyes, we conclude that the RPE does not exocytose active...
lysosomes (ie, phagosomes, primary or secondary lysosomes, residual bodies or lipofuscin granules) into Bruch's membrane despite the presence of morphologically similar bodies in this layer in virtually all human specimens after the first decade of life.\textsuperscript{18} By contrast, however, aged, 9th-decade specimens had occasional sites along Bruch's membrane where blebs of RPE cytoplasm that contained active lysosomes emanated from the basal surface of the cell. The appearance of these blebs suggests that fragments...
Fig. 8. Small subclinical druse. 84-yr-old. 5 hr PM: AcPase reaction. Compare to morphology of young surgical specimen. Fig. 1. RPE organelles and cytosol intensely positive. Bruch’s membrane shows no reaction in lysosome-like bodies. Bodies and vesicles near the RPE BM (enclosed area, enlarged in inset) have precipitate resembling that of RPE cytosol (X 10,000; inset X 15,000).

of RPE cells containing lysosomes may be released into Bruch’s membrane. If such a process is indeed occurring, it appears that rupture of these bags of cytoplasm and dispersal of their contents is the source of the vesicles and lysosome-like bodies described above. Moreover, rupture of the bags must result in deactivation or masking of enzymes, since liberated lysosomes were all negative in old as well as young eyes.

ASB was used in this study in an attempt to enhance detection of possible vascular endothelial components in the Bruch’s membrane debris. The method failed in this regard, there being many fewer reactive sites in all cells compared to the AcPase method. Neither morphological nor cytochemical evidence were consistent with a contribution by choriocapillary cells (endothelium, pericytes) to the formation of Bruch’s membrane debris.

Sarks et al have presented histologic evidence from an 80-yr-old patient followed clinically for 14 yr that “softening” of drusen preceded neovascular invasion of Bruch’s membrane. Small, discrete drusen developed smudgy outlines and became larger and confluent prior to subretinal neovascularization. This interesting clinicopathologic observation suggests to us that a mechanism for dissolution of drusen in aged eyes may be the delivery by the RPE of lysosome-rich parcels of cytoplasm into drusen of Bruch’s membrane. The content of lysosomal enzymes of RPE cells is reportedly different from and more potent than that of other tissue such as retina and liver, particularly in protease activity. Although the direct ultrastructural localization of proteases is not feasible by current techniques, the positive AcPase reaction implies presence of proteases, as well as the entire complement of lysosomal enzymes capable of connective tissue degradation. Whether or not enzymes with pH optima of 5 have significant lytic activity at the presumably neutral pH of the extracellular space to damage the connective tissue barriers to neovascu-
Granular druse and adjacent RPE; ASB reaction. The enzyme reaction is less vigorous than for AcPase. None of the granules in the druse is positive (×10,000). Triangular area is enlarged and shown in lower right corner. Insets at left show reaction product in RPE lysosomes and lipofuscin granules. Lipofuscin granules (LF), when positive, show reaction only at periphery. 69-yr-old, 2 hr PM (×20,000). Lower right corner, negative reaction of druse granules (×20,000).

lar invasion of Bruch's membrane is an unanswered question.

The discovery in the late 1960's that fibroblasts secrete lysosomal enzymes in culture21 raised the possibility that all cells might normally secrete and subsequently recapture these enzymes, by receptor-mediated endocytosis.22 In the present study we carefully examined all surfaces of the cell, not just the basal region illustrated here, for evidence of extracellular enzyme. No evidence of extracellular AcPase or ASB activity was found. At the apical surface where secretion or leakage at the time of phagocytosis has been suggested,17,21,24 we were able to eliminate virtually all the cytochemical reaction (lead precipitate) on apical microvilli by addition of ouabain to the incubation medium. This suggests that an ATPase is responsible for this apical phosphatase reaction. Also, in our recently prepared human tissues, apically located extracellular manganese-dependent AcPase, as reported by Irons;25 was detected when Mn⁺⁺ was added to the incubation media (data not shown); most of the experiments reported here, however, were conducted without addition of manganese ion. Additionally, no lead reaction product was found along the RPE intercellular spaces, in contrast with results when these cytochemical methods are applied to epithelial cells that slough.26,27 Finally, at the basal Bruch's membrane surface, the primary interest of this study, no evidence of enzyme secretion was found. We conclude that RPE cells normally do not secrete or otherwise extrude lysosomal enzymes.

Key words: acid phosphatase, aging, apoptosis, aryl sulfate, Bruch's membrane
Acknowledgments

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References