June 1992 Vol. 33/7
Investigative Ophthalmology
& Visual Science

Articles

Autoradiographic Studies of Aged Primate Macular Retinal Pigment Epithelium

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The authors used ^{35}S-sulfate and ^{3}H-proline to trace labeled molecules in autoradiograms of aged monkey and human macular retina to detect the synthesis of extracellular matrix (ECM) components by retinal pigment epithelial (RPE) cells. Quantitative analysis of silver grains 6 hr and 3 d after intravitreal injection of ^{35}S-sulfate in the monkey showed that secretion from the basal pole of the RPE occurs at a slower rate than from the apical pole. In vitro incubation of human maculas produced poor autoradiographs with ^{35}S-sulfate. Good autoradiographs were obtained using ^{3}H-proline. Human macular RPE showed uneven labeling, but densely labeled cells did not correlate with sites of basal linear deposits, ECM thought to be basement membrane material, and a hallmark of age-related maculopathy. The time course of labeling in adult primate tissue showed a fairly high turnover rate for these molecules. Scant labeling of ECM at drusen sites and no labeling in basal linear deposits suggested that either (1) these structures have a slow turnover or (2) their components contain scant sulfate and proline. Alternatively, faulty degradative processes rather than enhanced synthesis may account for the accumulation of abnormal ECM at the RPE–Bruch's membrane interface in aged maculas. Invest Ophthalmol Vis Sci 33:2079–2090, 1992

Age-related maculopathy is an advanced stage of ill-defined deteriorative processes that occur in aged human eyes. The degeneration of photoreceptors responsible for loss of central vision seems to be a secondary result of complex changes in retinal pigment epithelial (RPE) cells and Bruch's membrane.[1–3] We were interested particularly in two structural features that appear during the early stages of disease, drusen[4] and basal linear deposits (BLD),[5] both of which are located at the base of RPE cells but seem to be formed by different mechanisms. Whereas BLD appear to be a secretory product that lies internal to the RPE basement membrane,[5–7] drusen lie external to the RPE basement membrane, beginning as focal accumulations of membranous and vesicular debris to which is added varying amounts of material apparently secreted by RPE cells.[5–10]

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Supported by research grant EY-03274 (LFB) from the National Eye Institute, National Institutes of Health, Bethesda, Maryland, and by grants (Departmental and Senior Scientific Investigator) from Research to Prevent Blindness, Inc., New York, New York.

Submitted for publication: November 20, 1990; accepted December 13, 1991.

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The first clinical signs of age-related maculopathy, pigmentary mottling, and/or development of drusen in the macular area may involve only one or a few RPE cells. Therefore, an experimental probe of cell pathologic disorders must reveal tissue morphology before functional information can be interpreted. Autoradiography is a valuable technique for investigating cell pathology because it concurrently reveals the functional capacity of a cell and visualizes its morphologic features.

To investigate the possible contribution of RPE cells to the formation of drusen and BLD, we did radiolabeling experiments on macular tissue of monkeys and humans to detect (by autoradiography) the synthetic activity of these cells. Because there is no readily available animal model of age-related maculopathy, we wanted to explore the possibility of using aged human donor tissue for laboratory studies. Of particular interest to us were the basement membrane components heparan sulfate proteoglycan and type IV collagen because they are normal constituents of the basal RPE–Bruch's membrane complex,[11–21] have been studied well in other organs,[22,23] and are likely candidates for age-related molecular alteration during drusen and/or BLD formation. Using ^{35}S-sulfate and ^{3}H-proline as precursors of these ECM molecules, we found autoradiographic evidence that basal secretion of sulfate-labeled molecules occurs in adult tissue.

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However, we did not find extracellular accumulation of labeled molecules in human or monkey BLD or in human drusen.

Materials and Methods

Materials

Two rhesus monkeys (both aged 23 yr) were selected by Dr. Michael Klein because they had bilateral macular drusen (scattered bright spots in the fundus). They were obtained from the Oregon Regional Primate Research Center (Beaverton, OR). A 23-yr-old monkey is equivalent to a 70-yr-old human. One animal was used for sulfate labeling experiments during two periods, and the other one was used to compare in vivo and in vitro distribution of proline labeling (Table 1). Maintenance and care of monkeys was in accordance with the National Institutes of Health Guide for the care and use of laboratory animals. These experiments were conducted according to the ARVO Resolution on the Use of Animals in Research.

Human eyes from donors older than 75 yr of age (n = 29) were obtained from the Missouri Lions Eye Tissue Bank (Columbia, MO; Table 1).

Radiochemicals

We used ^{35}S-sulfate ($Na_2{}^{35}SO_4$:carrier free, concentration 10 mCi/ml; DuPont, Wilmington, DE) and L-[3,4-^{3}H(N)]-proline (specific activity 50 Ci/mM: American Radiolabeled Chemicals, St. Louis, MO).

Sample Labeling

The rhesus monkeys were anesthetized initially by intramuscular injection of ketamine hydrochloride (12 mg/kg; Ketalar, Parke-Davis, Morris Plains, NJ), followed by intubation and administration of Halothane (Abbott, Abbott Park, IL). The ocular surface was anesthetized with proparacaine (0.5%; Alcaine, Alcon, Fort Worth, TX) and kept moist with artificial tears throughout the procedure. The radiochemicals were evaporated first to dryness, resuspended in saline solutions at a concentration of 10 mCi/ml, and then injected slowly into the vitreous cavity through a 25-gauge needle inserted at the sclera approximately 3 mm posterior from the limbus. We used a continuous infusion pump (Motomatic model E-550-BV; Electro-Craft, Hopkins, MN) for injection at a rate of 5 μl/min to avoid abrupt increases in intraocular pressure. The total volume injected into each eye was 100 μl (1 mCi). At 6 hr and 3 d after the intravitreous injection of the radiochemicals, the eyes were enucleated. The animals were killed after we removed their fellow eyes. The anterior segment was removed, and the posterior hemisphere was dissected free of the vitreous. The macular area of the full-thickness eye wall was excised and used in our study.

Table 1. Specimens used in this study

No.	Age (yr)	PM-Enuc (hr)	T (4°C)	Pulse (hr)	Chase (hr)
			^{35}S-sulfate		
Monkey					
PC2569	23	In vivo		6	NA
		In vivo		3	NA
Human					
1261	80	3	5	1	0.5
1262				3.5	0.5
1263	87	3	6	2	0.5
1264				6	0.5
1265	72	3	9	21	4
1266				21	1
			^{3}H-proline		
Monkey					
PC2224	23	In vivo		3	NA
		In vitro		2	1
Human					
89615	12	5	4	0.5–4*	0.1
89616			5	0.5–4*	0.1
89335	19	4	7	0.5–4*	0.1
89336			8	0.5–4*	0.1
1330	79	1.5	5.5	2	0.5–1*
1331				2	0.25–0.75*
1314	80	4	1.5	24	0.1
1315			2	48	0.1
1367	80	3	6	3	1
1368				2	1
1271	83	1	9	2	1
1272				4	0.5
1310	84	2	9	2	0–1*
1311			10	4	1
1371	84	2	5	1.5	0.5
1372				1.5	0.5
1391	85	3	6	1	0.5
1392				2	0.5
1345	89	2.5	5	4	0.5
1346				8	0.5
1359	90	2	7	0.5–4*	0.1
1360				0.5–4*	0.1
1343	93	3	7	2	0.25–1*
1344				2	0–0.75*
1281	95	1	4	1	0.5
1282				2	0.5

Abbreviations: NA, not applicable; PC, Oregon Regional Primate Research Center; PM-Enuc, time between death and enucleation; T, time (hr) between enucleation and start of experiment.
* Maculas were bisected or trisected.

The macular area (approximately, 7 × 7 mm) of the eye (Table 1) was excised and pulse incubated in a Petri dish containing one specimen per dish in a final volume of 1 ml of incubation medium. The pulse medium used was Eagles' minimal essential medium with antibiotics containing 800 μCi/ml of $Na_2{}^{35}SO_4$ for the sulfate-labeling experiments and 250 μCi/ml of ^{3}H-proline for proline-labeling experiments. Pulse labeling was done first for 10 min at 4°C to permit equilibration of medium components with the tissue

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and then at 37°C for the indicated intervals (total, 30 min–6 hr) under 95% air and 5% carbon dioxide with rotary agitation in an incubator. The tissues then were incubated in two changes of warm aerated medium containing two- to tenfold concentrations of nonradioactive substrate (chase media) for various periods. Radioactivity changes at each step were monitored using a liquid scintillation counter.

Characteristics of Pulse Labeling

To assess the viability of the human maculas we used and to characterize the efficiency of pulse labeling, the kinetics of isotope incorporation into trichloroacetic acid (TCA)-precipitable and TCA-nonprecipitable materials were investigated. For this analysis, tissues incubated with ^{3}H-proline or ^{3}H-leucine (L-[4,5-^{3}H(n)]-leucine, specific activity, 60 Ci/mM; American Radiolabeled Chemicals)[24] were rinsed as described, homogenized, and placed in a microcentrifuge tube containing 1 ml of cold TCA 10%. They were allowed to stand for 2 hr on ice. The TCA-precipitable material was collected by centrifugation (15 min, 13,000 rpm, Eppendorf model 5414 microcentrifuge; Brinkman Instruments, Westbury, NY). The pellet was washed three times with 1 ml of cold TCA 5% and solubilized in 1 ml of 1 N NaOH. The specific activity of both the TCA-precipitable and TCA-nonprecipitable material was determined by assaying aliquots for radioactivity and protein concentrations using the bicinchoninic acid (Pierce, Rockville, IL) protein assay.

Preparation of Samples for Autoradiography

The samples were fixed at 4°C with glutaraldehyde 2% and paraformaldehyde 1% in 0.06 mol/l sodium cacodylate buffer (pH 7.4) containing 0.02 mol/l $CaCl_2$, 0.007 mol/l KCl, and sucrose 1%. They were rinsed in 0.1 mol/l sodium cacodylate buffer (pH 7.4) containing sucrose 7%. The samples were postfixed for 1 hr at room temperature with osmium tetroxide 2% in 0.1 mol/l sodium cacodylate buffer, rinsed, dehydrated in a graded acetone series, and embedded in epoxy resin.

Preparation of Light Microscopic Autoradiographs

Sections (0.5-μm thick) were cut and placed on a glass microscope slide. The slides were coated by dipping with Ilford K5 (Ilford Ltd., Ilford, England) nuclear emulsion under darkroom conditions and exposed in light-tight boxes at 4°C. After exposure for 1–5 weeks, the autoradiograms were developed with D-19 for 2 min at 20°C and fixed with Kodak rapid fixer for 5 min (Eastman Kodak, Rochester, NY). The slides were examined by light microscopy.

Preparation of Electron Microscopic Autoradiographs

Thin sections (100–250-nm thick) of radiolabeled tissue were cut and transferred onto formvar-coated grids, stained with uranyl acetate 2% in 0.1 M sodium maleate buffer (pH 6.0) for 30–60 min, and then carbon coated. Sections of unlabeled retina–choroid from the same eye were placed on grids to serve as control specimens for background grain counts. The grids were attached to a glass microscope slide with double-sided tape and covered with a thin layer of Ilford L4 nuclear emulsion using the loop technique.[25] After exposure in light-tight boxes at 4°C with desiccant for 3–8 weeks, the grids were developed with Microdol-X (Kodak) for 5 min at 20°C and fixed. The grids were examined with a JEOL 1200 EX electron microscope by scanning transmission electron microscopy.

Quantitative Analysis of the Autoradiographs

Our quantitative analysis procedure was adapted from methods developed earlier.[26] Electron micrographs were taken in an unbiased manner and printed at the same magnification (×7500). Individual grains were assigned to five compartments: (1) photoreceptors, (2) apical cytoplasm of the RPE, (3) nucleus of the RPE, (4) basal cytoplasm of the RPE, and (5) Bruch's membrane. The assignment was according to the position of a circumscribed 50% probability circle.[26] The background level of silver grains, ie, grain density in tissue-free areas of the same thin section, was subtracted from the mean grain counts of each compartment. After the grain distribution was determined for a set of micrographs, a "circle" analysis[26] was done on the same micrographs to estimate the proportional areas occupied by each compartment. Grain densities over specific compartments then were calculated for analysis. Comparisons of these data were made after analyzing at least eight such compartments from each section. We used a student t-test for statistical analysis.

Results

The 23-yr-old monkeys used in this study were chosen from a large pool of animals because they had bilateral macular yellowish-white spots interpreted as drusen (Fig. 1). When histologic examination was done after the autoradiographic procedures were completed, the fundus spots were found to be vacuolated RPE cells, not drusen, a result found in previous clinicopathologic studies of monkey[27–30] and human[31] maculas. Therefore, data on "real" drusen, ie, clinically visible (> 25 μm) mounds in the inner collag-

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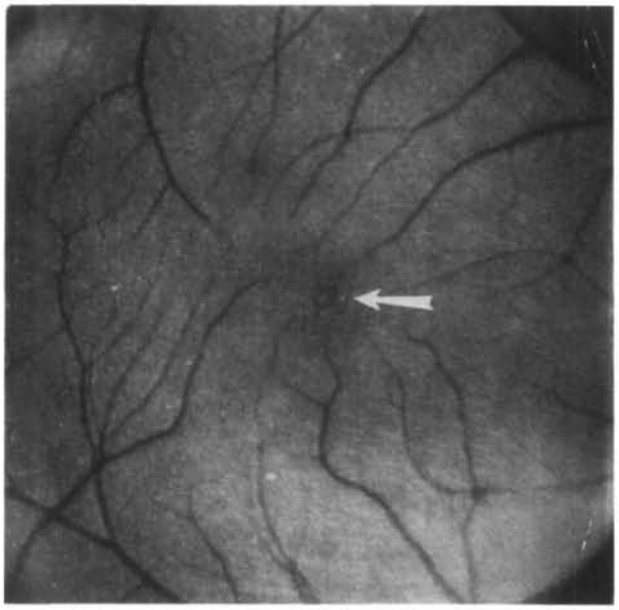

Fig. 1. Fundus photograph of 23-year-old rhesus monkey showing cluster of bright spots (arrow) in the macula. Although the term "drusen" is useful to describe these spots as seen clinically, their histologic basis in this instance was vacuolated RPE cells, not mounds in Bruch's membrane.

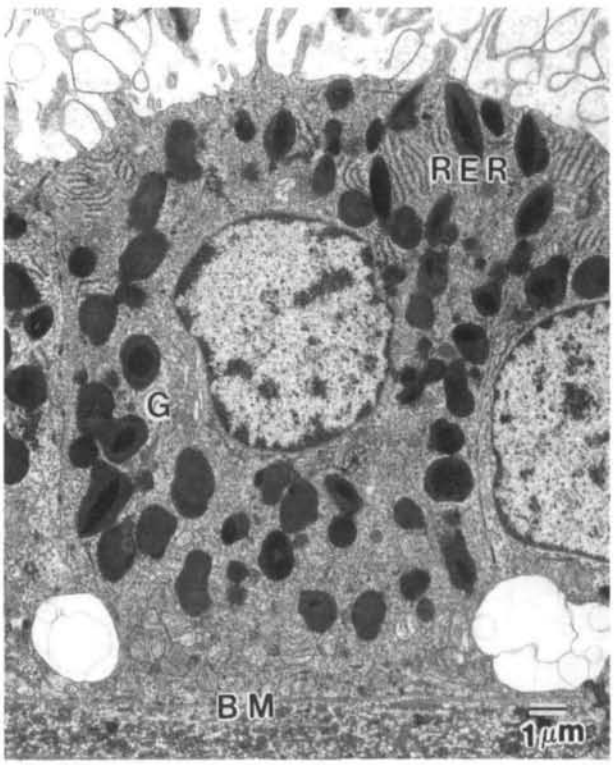

Fig. 2. Electron micrograph of 65-year-old human macular RPE showing normal polarized structural features. RER, rough endoplasmic reticulum; G, Golgi apparatus; BM, basement membrane.

enous zone of Bruch's membrane, were not obtained in our monkey experiments.

The ultrastructure of aged macular RPE is shown in Figure 2. The basal portion of the RPE rests on a basement membrane attached to Bruch's membrane; the apical portion of the cell interacts with the photoreceptors. Laterally, the cell associates with its neighbors through apicolateral junctional complexes that form part of the blood–retinal barrier. In the cell, organelles and inclusions are polarized; melanin granules and rough endoplasmic reticulum lie apically with lipofuscin granules and mitochondria placed more basally. Products synthesized in polarized epithelial cells such as RPE are known to undergo a sorting process in the Golgi apparatus[32] located in the paranuclear region (Fig. 2). Some molecules are targeted apically and others basolaterally. In aged human maculas, extracellular material (BLD) often is found along the basolateral plasma membrane of scattered single cells, groups of cells, or the entire RPE of a given sample (Fig. 3). Our study was designed to examine this putative synthetic product of the RPE.

^{35}S-Sulfate Autoradiography

In sulfate-labeled autoradiographs of monkey maculas, although grains were present over all retinal strata, the intensely labeled compartments were: inner plexiform layer, outer plexiform layer, photoreceptor inner and outer segments, and the RPE layer

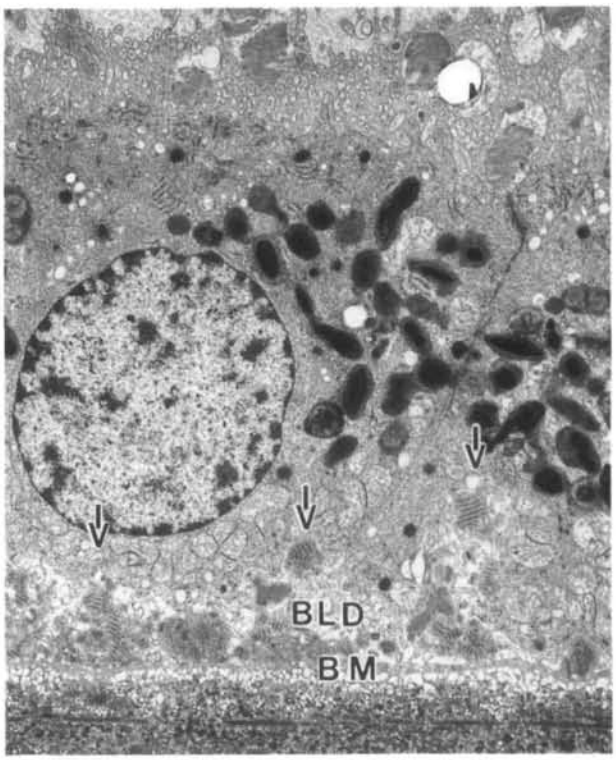

Fig. 3. RPE of 79-year-old human macula that had basal linear deposits (BLD) along the basolateral cell surfaces (arrows) internal to the normal RPE basement membrane (BM).

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(Fig. 4). Electron microscopic autoradiographs revealed that, after 6 hr of labeling, the apical side of the RPE was labeled markedly with grains and the basal side of the RPE and Bruch's membrane were well labeled (Fig. 5). After 3 d of labeling, the grains over the basal side of the RPE and in Bruch's membrane were enhanced (Fig. 6).

The quantitative grain density distribution taken from electron microscopic autoradiographs is shown graphically in Figure 7. At the apical side of the RPE, the grain density was almost stable (or slightly decreased) from 6 hr to 3 d. However, at the basal side of the RPE, it increased ($P < 0.05$), suggesting that radioactivity at the apical side of the RPE already had peaked by 6 hr but that of the basal side still was accumulating.

In Vivo Versus In Vitro ^{35}S-Sulfate

By contrast with the monkey autoradiographs, human macular tissues incubated with ^{35}S-sulfate in vitro produced poor autoradiograms. Weak labeling was detected in the sensory retina, but no label was found over Bruch's membrane or the RPE. Using a precipitating agent for sulfate, cetylpyridium chloride (Sigma, St. Louis, MO), in the fixative did not improve the results.[22,33]

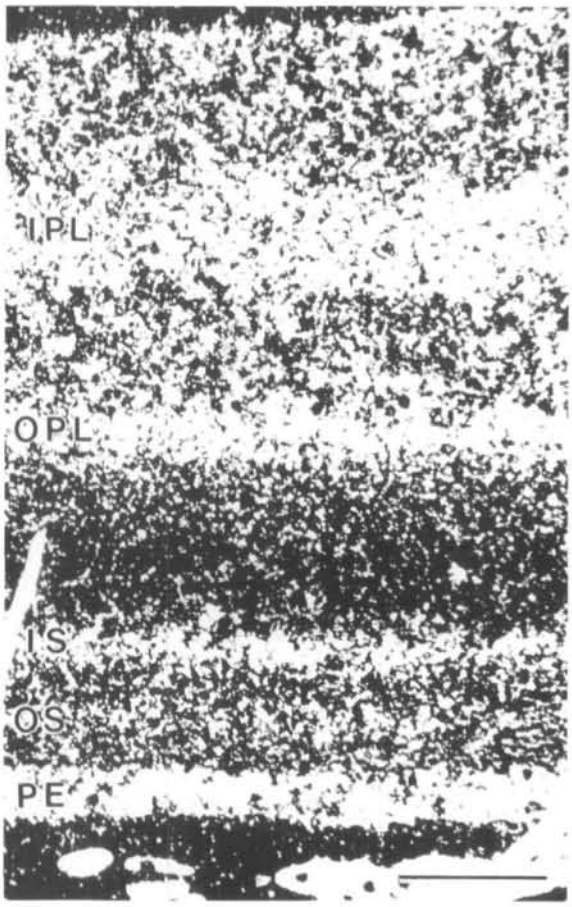

Fig. 4. Light microscope autoradiograph, dark-field illumination, of a monkey macular retina 6 hr after intravitreal ^{35}S-sulfate injection (exposed for 3 weeks). Note the heavy grain accumulation over the inner plexiform layer, outer plexiform layer (OPL), inner segments (IS) and outer segments (OS) of the photoreceptors, and pigment epithelial (PE) cells. Bar = 50 μm.

Test of In Vitro Viability

To discover whether in vitro pulse labeling in human tissue was achieved properly and, if so, to determine its characteristics, we did a series of experiments with radiolabeled amino acids of the type illustrated in Figure 8. Our results showed stable activity in TCA-precipitable materials (proteins, glycoproteins, and proteoglycans) and an abrupt decrease in radioactivity in TCA-nonprecipitable materials (free amino acids) after 15 min of chase incubation. Therefore, true pulse labeling was confirmed for both leucine and proline, and wash out of unincorporated activity was done efficiently. In addition, to confirm the viability of the tissue preparation during the incubation period, the specific activity of ^{3}H-proline-labeled TCA-precipitable materials (Fig. 9) and the density of silver grains in autoradiographs were analyzed. They increased linearly with increasing incubation time.

^{3}H-Proline Autoradiography

In proline-labeled autoradiographs of monkey specimens, the grains did not chase to the basal side of the RPE after a 2-hr pulse and a 1-hr chase incubation. Vacuolated RPE cells had label in the cytoplasm, but there was no specific correlation with any organelles (Fig. 10).

Experiments with human maculas incubated with ^{3}H-proline produced good autoradiograms. The RPE was labeled well after a 2-hr pulse incubation (Fig. 11). In several specimens, single scattered RPE cells, about 20–30% in a given section, had above-average silver grains (Figs. 11B–C). These cells stained somewhat more intensely with toluidine blue and had a greater electron density caused by an abundance of polyribosomes and denser cytosol (Fig. 11D). A "dark" proline-labeled cell appeared to extend basal processes under neighboring cells (Fig. 11B).

Using autoradiographs of human macular tissue incubated with ^{3}H-proline for various periods, the fate of labeled macromolecules was analyzed (Fig. 12). Basal labeling occurred at almost the same rate as the apical labeling but seemed to peak later. The rapid labeling and transport of the synthesized products indicated that the turnover rate for these RPE-associated proline-labeled molecules was fairly high.

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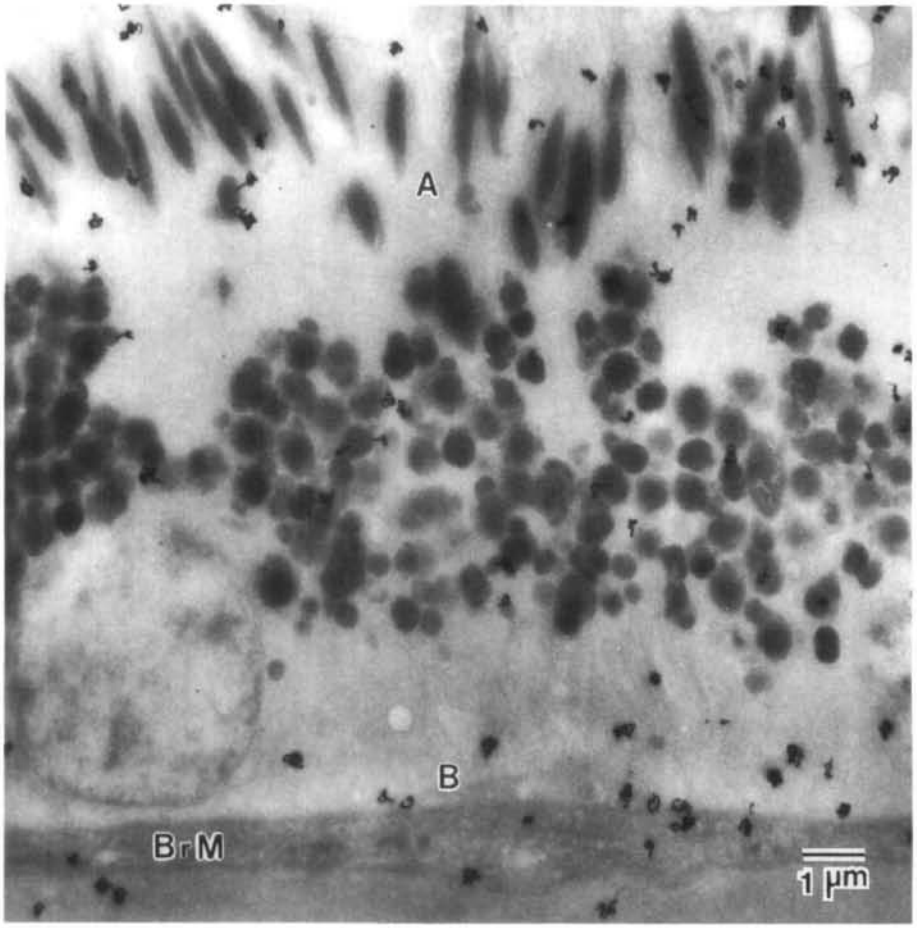

Fig. 5. Electron microscope (STEM) autoradiograph of a monkey retina 6 hr after intravitreal ^{35}S-sulfate injection. Note the grains around outer segments of photoreceptors and at the apical (A) and basal (B) side of the RPE. Bruch's membrane (BrM) is also well labeled.

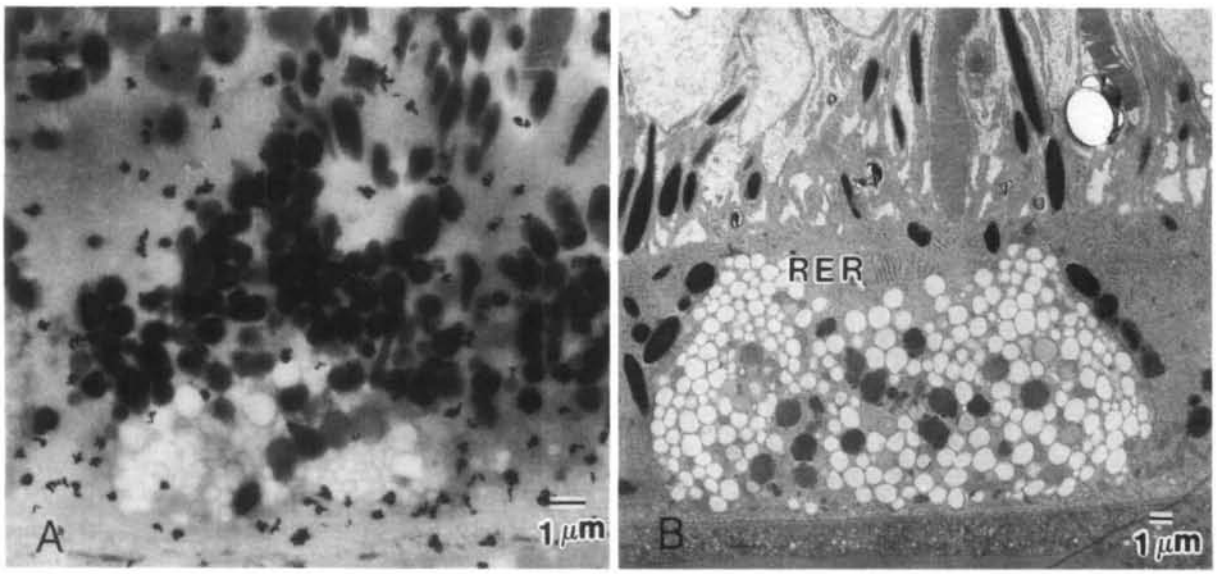

Fig. 6. (**A**) Monkey retina 3 days after ^{35}S-sulfate injection (STEM micrograph). The basal side of the RPE and Bruch's membrane are labeled. The vacuolated RPE cell, equivalent to the drusen seen clinically, is not notably labeled. (**B**) TEM of similar vacuolated RPE from another animal. Note normal cellular polarity and lateral attachments are maintained as cell undergoes lipoidal degeneration.

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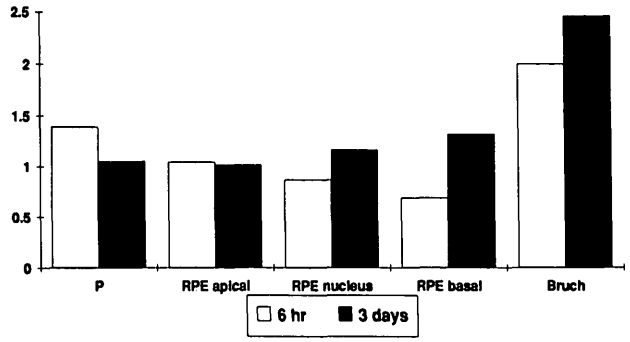

Fig. 7. Histograms showing the mean silver grain density taken from the indicated compartments of the monkey 6 hr (unshaded) and 3 days (shaded) after ^{35}S-sulfate injection. Counts were taken from ~100 cells in electron microscopic autoradiographs printed at ×7500 magnification. Note the increasing label at the basal RPE compared with stable apical RPE label. P, photoreceptor layer.

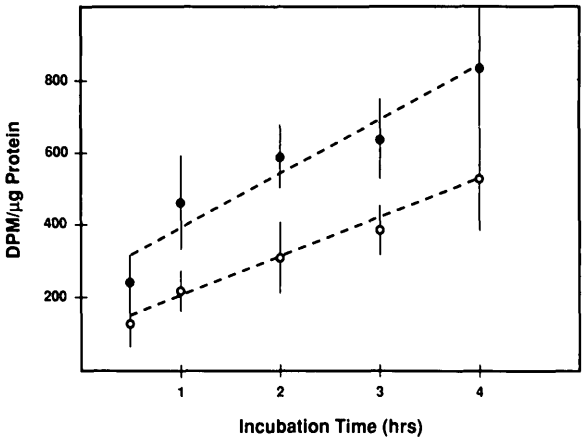

Fig. 9. Incorporation of ^{3}H-proline into TCA-precipitable materials in old and young human specimens. Note the progressive increase in ^{3}H-proline incorporation during the period examined. Determinations of radioactivity and protein at each time point performed on three different tissues. Open symbols, 90-year-old; filled symbols, 19-year-old.

Drusen

Drusen (ie, mounds in the inner collagenous zone of Bruch's membrane) were found in some human specimens that had been incubated with ^{3}H-proline (Fig. 13). Ultrastructurally, these were granular "hard"[10,34] drusen (Fig. 13B). Labeling of the drusen was poor and showed no consistent pattern. The largest number of silver grains was found over the inner surface of drusen where the RPE appeared morphologically to be releasing material from basal pockets that merged with the basement membrane (Fig. 13A).

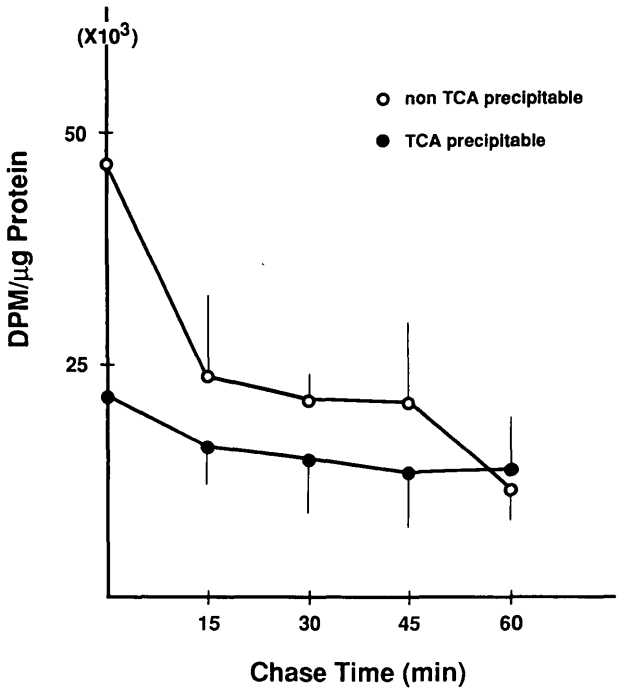

Fig. 8. Kinetics of pulse-labeling (2 hr) of aged human macular tissue. Note the stable activity of ^{3}H-proline in TCA-precipitable materials, and the abrupt decrease in radioactivity in non-TCA-precipitable materials. Each time point is the average of radioactivity and protein determination on macular specimens from two old donors.

Discussion

Efforts to investigate biologic processes that cause the formation of macular drusen and BLD are limited severely by the availability of suitable experimental tissue. Although drusenoid maculas are common among human eyes from donors older than 70 yr of age, detection of their presence in the eyecup preparation often is obscured by the opacified postmortem neural retina. If the neurosensory retina is removed to visualize sub-RPE pathologic findings, damage occurs to the apical surface of the RPE. In addition, this procedure disturbs the normal relationships between the apices of the cells and the apical extracellular matrix (ECM). Another limitation that precedes the diagnosis of macular disease is the difficulty of acquiring donor tissue rapidly, a procedure that is essential if dynamic experiments are being done on viable cells. Although we did not test all maculas for viability because of the scarcity of our specimens, our experience shows that eyes that are more than 10 hr postmortem do not function well in organ culture. Also, our attempts to do labeling experiments for longer than 6 hr in vitro were unsuccessful. In summary, many technical and logistic problems complicate the use of human donor maculas for research on age-related maculopathy. Nonetheless, these pilot experiments show the potential for detecting hitherto obscure biologic

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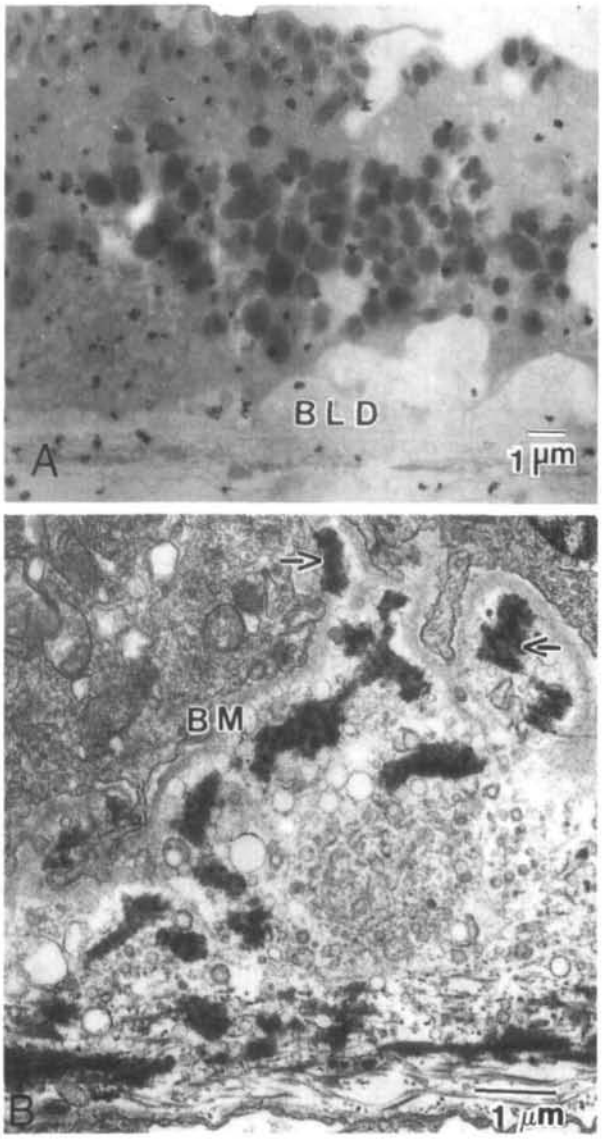

Fig. 10. (**A**) Monkey retina pulse-labeled 2 hr in vitro with ^{3}H-proline, chase incubation 1 hr. Macular RPE overlying a site where the basal aspect of the cell is elevated by a drusen-like mound that contains material thought to be the monkey equivalent of human basal linear deposits (BLD). Silver grains did not chase to the basal side of the RPE, nor did they label the BLD-like deposits. STEM; 250-nm section. (**B**) TEM of comparable site shown in (**A**). Material similar to human BLD (arrows) seems to be secreted from the basal surface of RPE, but, unlike human BLD, this faintly banded material passed through the BM rather than accumulating internal to it; compare location of BLD in Figure 3. BM, basement membrane of RPE.

processes involved in the development and progression of this disease.

Although duplication of human experiments in animal maculas is desirable for in vivo verification and cross-checking of biologic phenomena found in vitro, drusen occur rarely in maculas of nonhuman primates. (An exception may be the rhesus colony on Cayo Santiago Island.[35]) Moreover, monkeys diagnosed clinically as having macular drusen instead often have scattered vacuolated RPE cells as the basis for the white spots in their fundi.[27-30] Because the term "drusen" is used so widely in clinical ophthalmology, the fact that these fundus spots vary in their histologic morphology and even in the anatomic position of the defect does not warrant creation of new terminology.[36] Vacuolated RPE apparently have optical properties that mimic thinned RPE over drusen, and their transparency produces window defects in fluorescein angiograms that also resemble drusen. Although "real" drusen occur in some monkey maculas,[35,36] the 23-yr-old monkeys we used unfortunately had vacuolated RPE instead of drusen. Therefore, our attempt to label drusen isotopically in long-term in vivo experiments for comparison with short-term in vitro human experiments was thwarted.

We obtained important pilot information concerning the usefulness of in vivo labeling of the macula despite the disappointing outcome for the drusen inquiry per se. There was excellent sulfate labeling of the RPE in vivo, indicating that sulfated macromolecules are being produced in aged adult primate cells at a fairly rapid rate. Movement of labeled molecules from intracellular to more peripheral, or extracellular, sites also was detected. Movement of sulfated macromolecules toward the basal pole of RPE cells and the presence of label in Bruch's membrane provided dynamic evidence of synthesis and polarized secretion of sulfated substances by these cells. This confirms and extends histologic and biochemical findings in animals,[13,14,16,19] in human tissue in situ,[11,12] and in tissue cultured RPE.[15,17,19,20]

We assume that heparan sulfate is the major product visualized at the basal RPE–basement membrane zone in our autoradiograms. Studies of glomerular basement membrane show 80% of ^{35}S-sulfate label in the heparan sulfate. In addition, it has a rapid biphasic (20 hr and 60 hr) turnover.[22] Heparan sulfate proteoglycans in cell membranes and in the ECM are receptors for basic fibroblast growth factor,[37] a potent cytokine postulated to be involved in the transformation of RPE and vascular wall cells that lead to proliferative vitreoretinopathy[38] and the subretinal angiogenesis of age-related maculopathy.[17] Therefore, changes in sulfated ECM molecules may be a factor in these diseases. Whether sulfated macromolecules are components of BLD remains to be determined.

Several factors other than the relatively short exposure to isotope probably contributed to the poor labeling result with sulfate in human compared with monkey maculas. Breakdown of labeled macromolecules by either hydrolytic enzymes that leak from dying or

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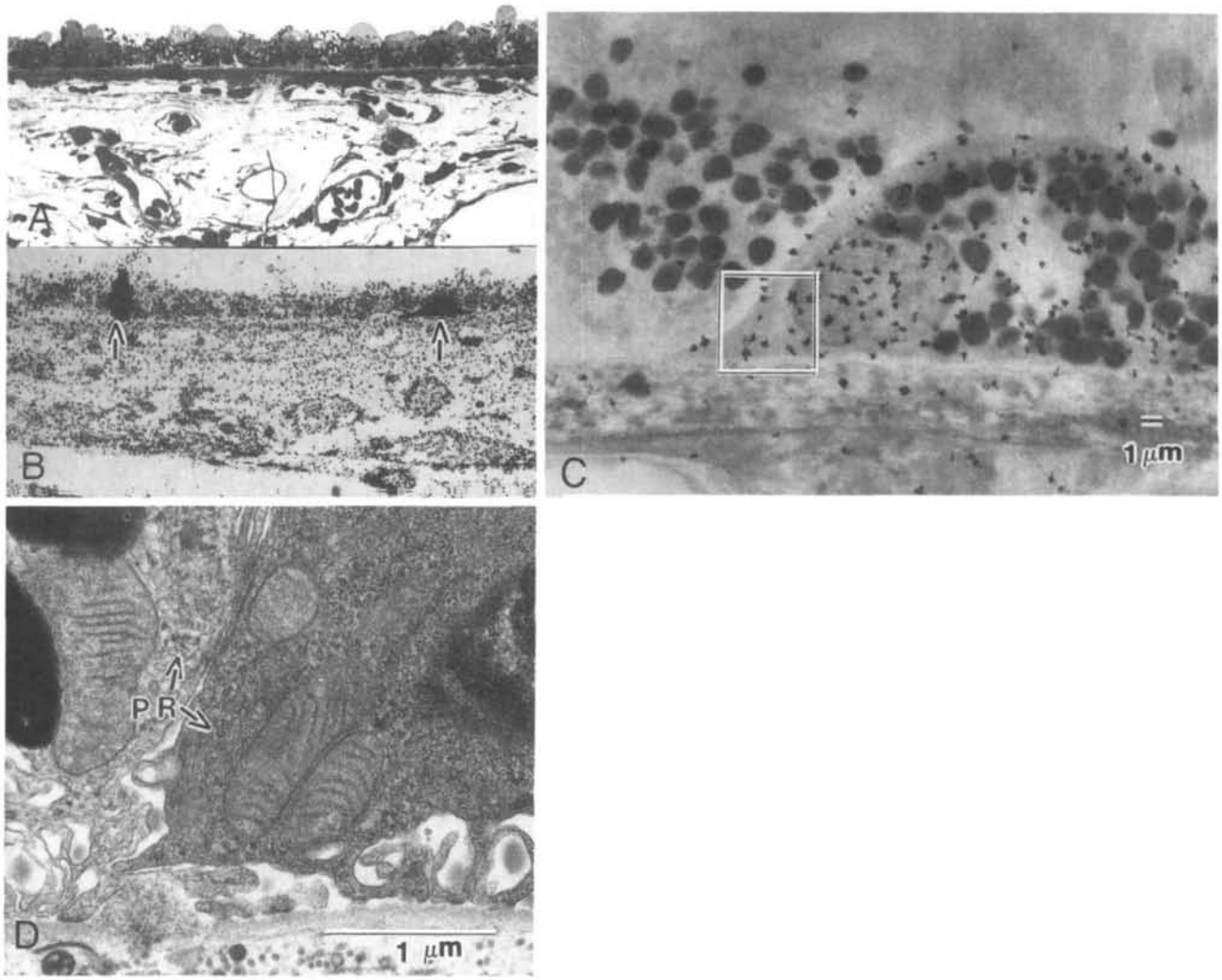

Fig. 11. Human macular RPE (83-year-old, 10 hr PM) **(A)** Light micrograph of 0.5-μm thick section stained with toluidine blue. **(B)** ^{3}H-proline autoradiograph of next section showing two RPE cells that are more densely labeled than the others (arrows); 2-hr pulse, 1-hr chase. **(C)** Electron microscope (STEM) autoradiograph of 250-nm thick section of one of the cells shown in **(B)**; this well labeled RPE cell has darker cytoplasm. The boxed area is shown by TEM in **(D)**. **(D)** TEM of a dark RPE cell. The dense cytoplasm of the proline-labeled cells is due to the presence of numerous polyribosomes (PR) and denser cytosol.

edge-damaged cells or by tissue proteinases activated by the incubation conditions may have occurred. Proteinase inhibitors added to the medium might be effective in lessening the postulated loss of newly synthesized glycosaminoglycans.

Our findings in the proline-labeling experiments are intriguing and require additional study. Proline is the customary tracer for type IV (basement membrane) collagen biosynthesis,[23] and we wanted to see whether or not RPE cells with abundant BLD at their bases and morphologic signs of secretory activity would show evidence of synthesis and secretion of labeled material. Although collagen is known to have a slow turnover ($t_{1/2}$ = 100 d[22]), numerous studies using ^{3}H-proline[23,39] show that de novo synthesis and secretion occurs within 2 hr. Therefore, our incubation protocols were thought to be sufficient to detect labeled BLD if it were made of collagen. Although no labeled BLD was found in the maculas examined, we still anticipate finding an optimal specimen for demonstrating the secretory phase of this basal deposit. These results, instead, suggest that the accumulated BLD may result more from failure of removal (degradation) than enhancement of deposition. Posttranslational modifications of procollagen by both intra- and extracellular proteolytic enzymes are important processes in normal collagen secretion.[40] New information regarding normal and pathologic degradative mechanisms in basement membranes[41] may shed additional light on collagen turnover, the physiologic

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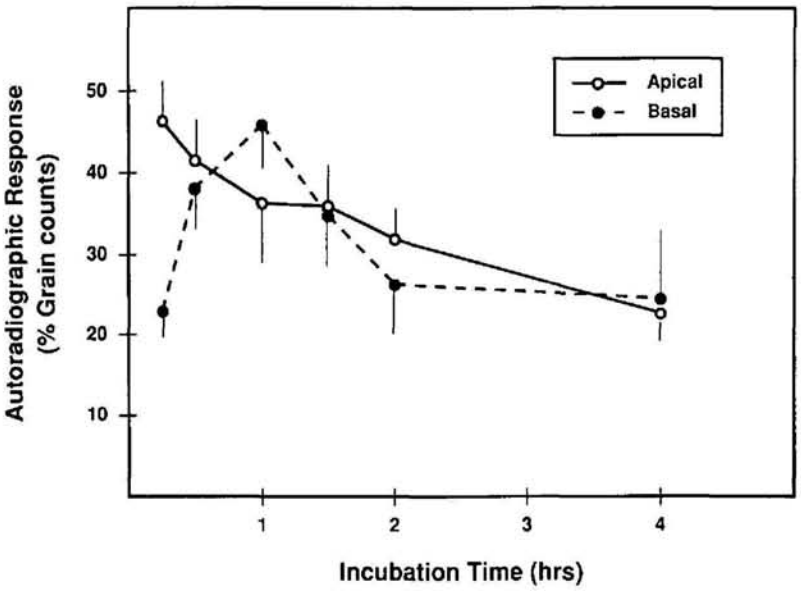

Fig. 12. ^{3}H-proline grain counts per unit area is plotted as autoradiographic response (ordinate). The results are from human macular tissues pulse-incubated for the indicated time (abcissa). Note that basal accumulation peaks later but occurs at almost the same rate as apical labeling.

impact of basement membrane "clogging," and mechanisms of neovascular invasion, all important considerations in understanding age-related maculopathy.

The proline labeling was stable in the RPE cells for up to 4 hr incubation without apparent secretion of labeled molecules. Therefore, other biologic processes must be considered to understand the heavy proline labeling of scattered macular RPE cells, eg, synthesis of cytoskeletal proteins, perhaps required for cell migration over basement membrane denuded by cell death; preparation of the nucleus for mitosis; or synthesis of stress proteins[42] that may help them survive postmortem shock or prepare for apoptotic cell death.[43] Additional studies with proline and other precursor isotopes may elucidate the activities of human RPE cells, particularly during the early postmortem period when cells are harvested for tissue culture experiments.

To our knowledge, autoradiography has not been used to study the maculas of aged humans. Our results with human tissue varied from one specimen to another, despite the similarity in age of the donors and postmortem delay times. During incubation, the neural retina came off most specimens. Also, elevation of some or all of the RPE from Bruch's membrane was common. In some instances, RPE detachment correlated with extensive amounts of BLD, also observed in patients,[44] and/or the presence of large

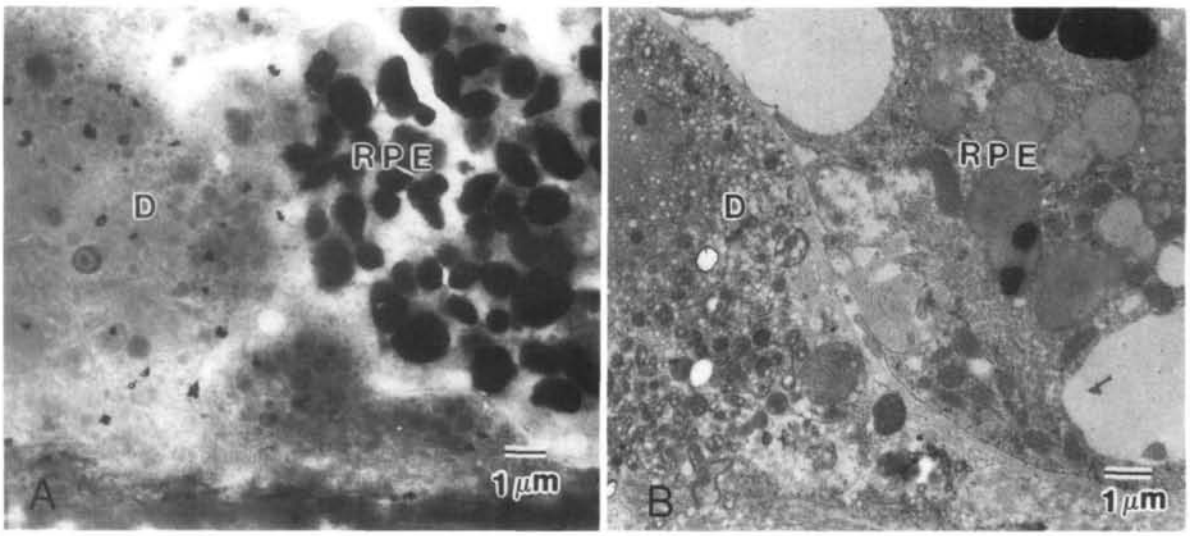

Fig. 13. (A) Human macular specimen pulse-labeled for 4 hr with ^{3}H-proline. Druse (D) has a few more silver grains than the RPE cell. (B) TEM of comparable "hard" druse composed of heterogeneous granules and vesicles beneath the basement membrane of an RPE cell, ie, in Bruch's membrane.

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"soft"[34] drusen. Nonetheless, a sufficient number of specimens responded well to the autoradiography protocol, and this encouraged us to consider additional studies of macular RPE biology using these methods.

The repeated experience of having clinically diagnosed drusen in monkeys that were found to be vacuolated RPE cells probably indicates that some cases of human macular drusen, particularly tiny shining yellow ones, may be vacuolated RPE cells. A relationship between vacuolated RPE and subsequent development of "real" drusen in monkeys was not found in a previous 14-yr study of monkeys with window defects from vacuolated RPE.[30] In humans, however, others[31] found a correlation between vacuolated RPE and the presence of soft[34] drusen, a subset of lipoidal drusen followed by adverse macular consequences.[10,45]

In summary, autoradiography is a powerful tool for investigating questions concerning normal and abnormal synthesis and secretion by cells during the development of age-related maculopathy.

Key words: extracellular matrix, retinal pigment epithelium, macula, age-related maculopathy, autoradiography

Acknowledgments

The authors thank Dr. Michael Klein, University of Oregon Health Sciences Center, for the clinical workup on the two monkeys; Dr. Martha Neuringer, Oregon Regional Primate Research Center, for arranging their transfer to Missouri; Mr. John Sears, Missouri Lions Eye Tissue Bank, for securing the human donor eyes; and Dr. Chun-Lan Gao, Mr. Michael Norberg, and Mr. Michael Carter for their technical assistance.

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