Graefe's Arch Clin Exp Ophthalmol
(2005) 243:250–256

LABORATORY INVESTIGATION

DOI 10.1007/s00417-004-0967-y

Lena Ivert
Hild Keldbye
Peter Gouras

Age-related changes in the basement membrane of the retinal pigment epithelium of Rpe65 –/– and wild-type mice

Received: 24 February 2004
Revised: 21 May 2004
Accepted: 11 June 2004
Published online: 17 September 2004

L. Ivert (✉)
Department of Ophthalmology,
St Erik's Eye Hospital, Karolinska Institute,
Polhemsgatan 50, 112 82 Stockholm,
Sweden
e-mail: lena.ivert@sankterik.se

H. Keldbye · P. Gouras
Department of Ophthalmology,
Columbia University,
New York City, New York, USA

Abstract *Purpose:* To investigate unusual changes in the basal surface of the retinal pigment epithelium (RPE) cell layer in aging Rpe65 –/– and wild-type mice. *Methods:* The retinas of Rpe65 –/– and wild-type mice of different ages—6 weeks and 3, 6, 12–13 and 16 months—were examined by electron microscopy. *Results:* There was an age-related increase in the width of the basement membrane of both Rpe65 –/– and wild-type mice which was associated with loss of basal infoldings of the plasma membrane of the RPE cells and protrusions of basement membrane material deep into the cytoplasm of these cells. These changes were evident at 6 months of age in RPE65 –/– mice and became extensive at 1 year of age. Similar changes occurred in wild-type mice but were less extensive and were only evident after 1 year of age. *Conclusions:* There is an age-dependent abnormality that develops at the basal surface of murine RPE cells, which resembles some of the changes observed in human age-related macular degeneration. These changes occur earlier in life and are more extensive in Rpe65 mutant mice.

Introduction

Senescent changes in the retinal pigment epithelium (RPE) and Bruch's membrane are suspected causal factors for age-related macular degeneration [6, 7, 16, 23–25, 35]. Our understanding of the relationship of these changes to the pathogenesis of human macular degeneration is limited by the dearth of animal models. There is evidence that similar changes occur in aging non-human primates [2, 10, 11, 14, 36, 38, 40], but the slow development of this process in primates handicaps experimentation. It would be valuable to have models for these changes in the RPE and Bruch's membrane in small animals, such as rodents, which occur over a more rapid time course, providing a greater range of possible experimentation. There have been several reports of age-related changes in the RPE and/or Bruch's membrane of rats [20] and mice [4, 5, 8, 15, 22, 27, 29–32, 42]. Some of these reports describe unusual changes in the basal infoldings and basement membrane of rodents that develop between 1 and 2 years of age [20, 30, 31]. We find a similar pattern of age-related changes in the Rpe65 –/– mouse, but the alterations occur earlier and more extensively than in wild-type mice. This is interesting because the Rpe65 mutant evinces a drastic reduction of lipofuscin fluorophores and excessive accumulation of all-*trans* retinyl palmitate in the RPE [19] that may influence this process.

Methods

Two strains of mice were studied, the Rpe65 mutant and a wild-type strain. The Rpe65 –/– mice were established in a C57Bl/6 strain and obtained from breeding pairs provided by Michael Redmond (National Institutes of Health, Bethesda, MD). Wild-type C57Bl/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and served as normal controls. Both eyes of eight Rpe65 –/– and nine wild-type mice were studied. Two mice from each strain were examined at 3, 6, 12–13 and 16 months of age; one wild-type mouse was examined at 6 weeks of age. Mice were killed with an intraperitoneal injection of Euthasol (40 mg/kg). The eyes were rapidly removed and punctured with a 30-gauge needle at the temporal limbus for orientation and placed in 3% glutaraldehyde in

phosphate-buffered saline, pH 7.4. They were kept at 4°C for 3–20 days before being washed and dissected. The anterior segment and the lens were removed and the remaining eye cup sectioned into two pieces with a razor blade along the vertical meridian through the optic nerve head. These segments were osmicated, dehydrated using ethanol and propylene oxide and embedded in epoxy resin. Blocks were sectioned semi-serially at 1–2 μm in thickness, stained with 2% toluidine blue and examined by light microscopy. At selected points, mainly at the posterior pole, the block was trimmed and ultra-thin sections were cut, stained with uranyl acetate and lead citrate and examined using an electron microscope (Zeiss 100B). In two eyes from Rpe65 –/– mice, aged 13 and 16 months of age, blocks were trimmed and sectioned in the inferior peripheral retina to compare this area with changes seen at the posterior pole. All sections were viewed and selected areas photographed at magnifications of 5,000–27,000×.

Results

No abnormalities were detected in wild-type mice up to 1 year of age. Figure 1 shows an example of the normal-appearing basal surface of the RPE of a 6-week-old and a 1-year-old wild-type mouse. There are numerous, slender basal infoldings of the plasma membrane of the RPE cell adjacent to Bruch's membrane without significant thickening of the basement membrane. Above the basal infoldings are numerous mitochondria and pigment granules adjacent to the nucleus of the RPE cells.

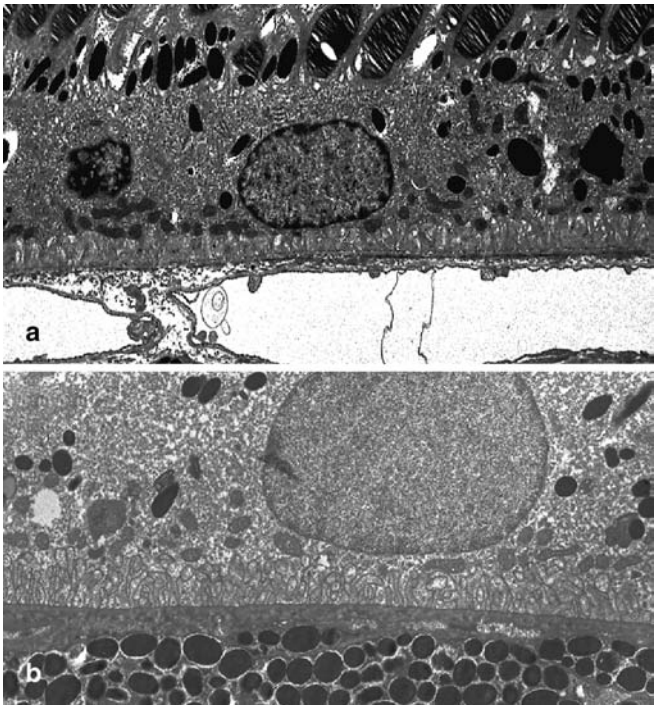

Fig. 1 Electron micrographs of **a** 6-week-old and **b** 1-year-old wild-type mice show typical infoldings of the basal plasma membrane of RPE cells adjacent to Bruch's membrane. There is no significant thickening of the basement membrane (magnification **a** 4,000×; **b** 8,000×)

After 13 months of age changes could be observed at the basal surface of the RPE layer of wild-type mice; these are illustrated in Fig. 2. The most frequent change was a thickening of the basement membrane with small protrusions into the infoldings of the plasma membrane of the RPE cell (Fig. 2a). In some cases there were areas where the basal infoldings were completely absent (Fig. 2a,b). In some locations there was a prolongation of these protrusions so that they extended deeply into the basal surface of the RPE cell (Fig. 2c,d); a similar protrusion is also visible on the right side of Fig. 2b. The mitochondria, melanin and melano-lysosomal bodies as well as the apical surface of the RPE appeared to be normal.

In Rpe65 –/– mice, similar changes were apparent at the basal surface of the RPE by 6 months of age (Fig. 3) but not at 3 months of age (data not shown). In Fig. 3a there is an area at the basal surface that appears normal with characteristic basal infoldings (short arrow on the left). But on the right side of this arrow, there are abnormal protrusions of the basal lamina into the plasma membrane (longer arrow) and an absence of basal infoldings. This change is even more striking in Fig. 3b–d. At higher magnification (Fig. 3d) one sees an amorphous, granular appearance to these protrusions or deposits extending from the basement membrane into the basal plasma membrane of the RPE cell. There are a number of vacuoles, presumably containing lipid, in the cytoplasm of these cells seen as gray homogeneous round structures, which are considered to contain retinyl esters.

At 16 months of age, these changes at the basement membrane the RPE of Rpe65 –/– mice have become most extraordinary, with long fingers of amorphous material penetrating deeply into the basal surface of the RPE cell (Fig. 4a,b). In some places in the section, islands of this material extend deeply into the cytoplasm of the RPE cell. In other areas these processes appear to completely encircle and isolate parts of the cytoplasm from the rest of the RPE cell (Fig. 4c,d). A slight suggestion of this can also be seen in the aged normal retina (Fig. 2d). Basal infoldings of the basement membrane are completely absent. This appearance was characteristic of many areas of the basal surface of the RPE layer of aged Rpe65 –/– retinas but seemed to be more prominent in the central area than the periphery of the retina.

Discussion

Age produces characteristic changes in the basal surface of RPE of wild-type mice [30, 31] and rats [20]. Mishima and Kondo [31] considered that these changes were due to a fusion of the plasma membranes of the basal infoldings of the RPE cell. Katz and Robison [20] noted a thickening of the basement membrane of RPE as well as an enlargement and prolongation of the basal infoldings. They

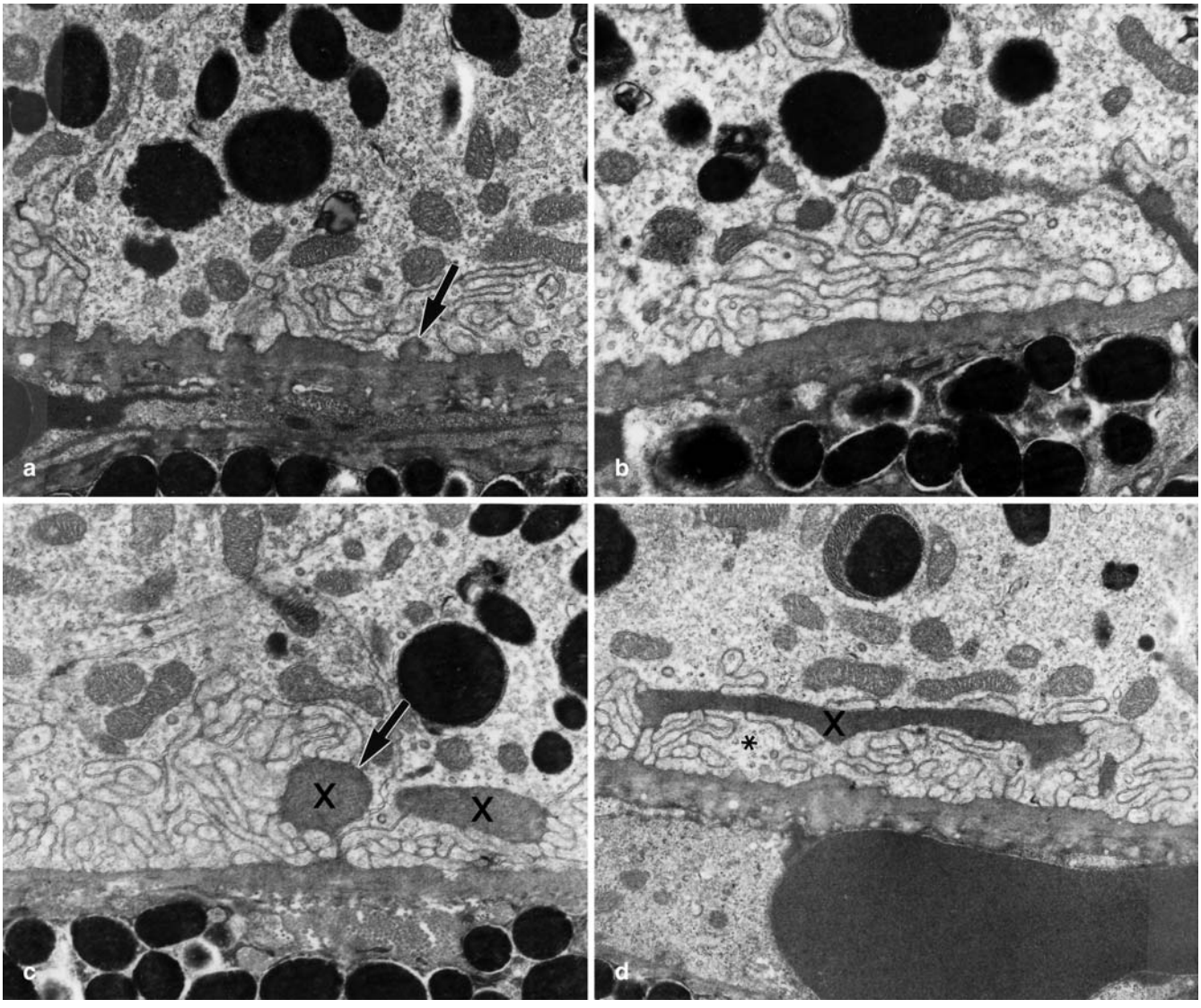

Fig. 2 Electron micrographs show the basal surface of RPE cells of a 13-month-old wild-type mouse. **a** Protuberances of the basement membrane into plasma membrane of the cell (*arrow*) and local loss of basal infoldings. **b** Thickening of the basement membrane with irregularities and some loss of the basal infolding. **c**, **d** Abnormal prolongations (*arrow* in **c**) of the basement membrane deep into the cytoplasm of the cell. These prolongations of basal laminar-like material are marked with an *x*. In both **c** and **d**, a thin stalk connects this large protuberance with the rest of the basement membrane. A *small asterisk* indicates an area of basal laminar infoldings that is being isolated from the remainder of the cell by the prolongations of the basal lamina (magnification 12,500×)

also noted areas of loss of basal infoldings. In both studies [20, 31] these changes appeared only in animals that were more than 1 year old. We have found similar changes in the Rpe65 –/– mutant which appear much earlier, being detectable at 6 months of age. In these mice the thickening appears to be due to an excessive formation of basement membrane material, which extends into the basal plasma membrane of the RPE and is associated with a loss of basal infoldings. In extreme cases these extensions surround areas of cytoplasm of the RPE cell, separating these areas from the rest of the cell, which could lead to local areas of degeneration.

Any correlation with the pathology of human age-related macula degeneration is difficult because the human changes are much more extensive, involving most of Bruch's membrane [6, 7, 16, 23–25, 35]. They characteristically involve the formation of drusen which are located external rather than internal to the basement membrane of the RPE and have not been reported in rodent eyes. There are, however, changes in the aging human retina that occur between the basement membrane and the basal plasma membrane of the RPE cell which extend over large areas, often being more extensive than drusen. In their late stages these are characterized by a

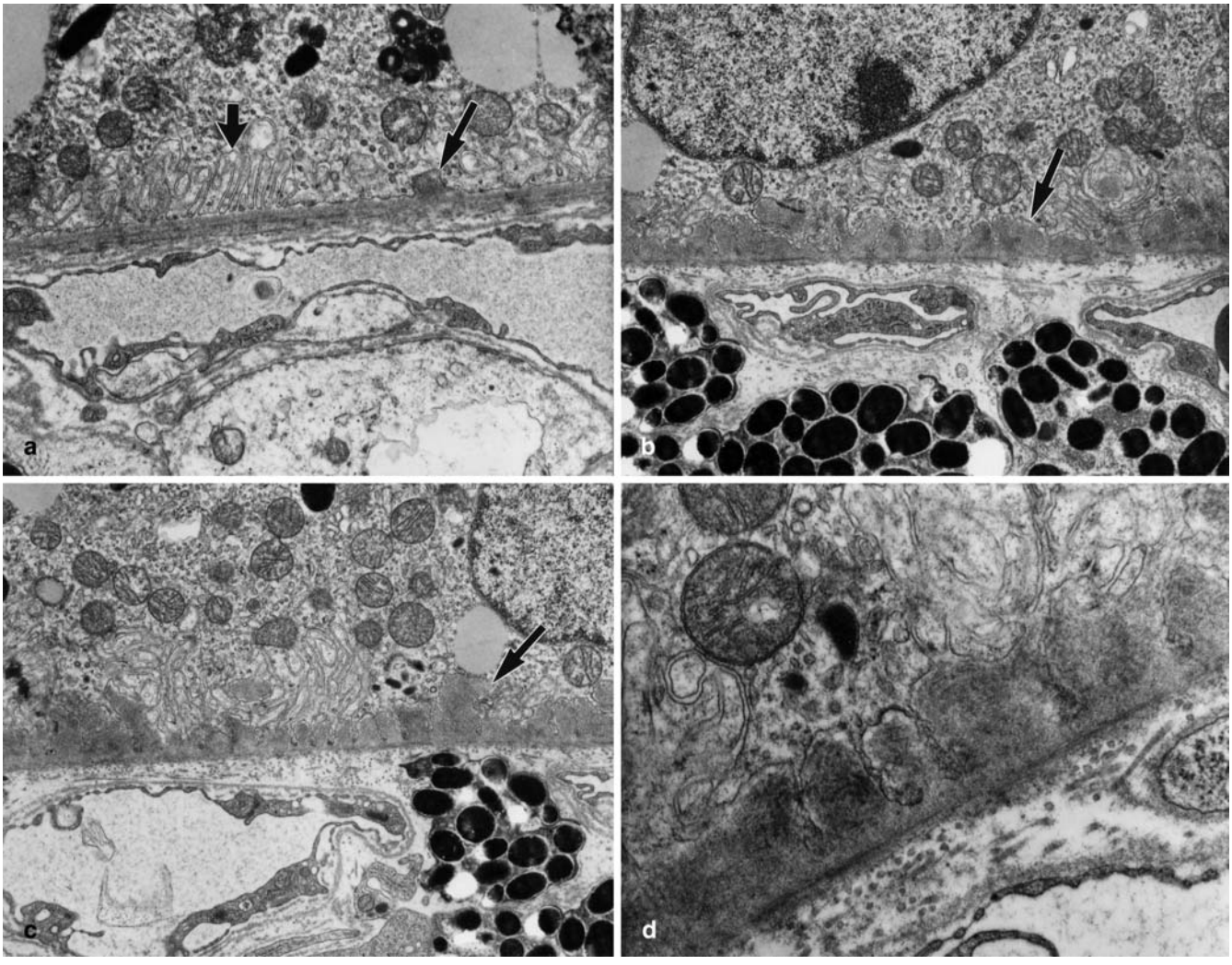

Fig. 3 Electron micrographs show the basal surface of an RPE cell of a 6-month-old Rpe65 –/– mouse. **a** An area of normal basal infoldings (*short arrow*) adjacent to an area where they are absent. At this point there is an abnormal protuberance of basement membrane into the plasma membrane of the cell (*long arrow*) (magnification 10,000×). **b** An area where basal infoldings are absent and the basement membrane is thickened by the accumulation of amorphous material (*arrow*) (magnification 8,000×). **c** A prolongation of such a basement membrane protuberance penetrating the cytoplasm and contacting a lipid droplet (*arrow*) (magnification 8,000×). **d** A more extreme example of the protuberances of the basement membrane contacting the plasma membrane of the cell where basal infoldings are absent (Magnification 25,000×)

linear palisade-like appearance but in earlier stages as excrescences on the basement membrane of the RPE, just as we and others [20, 30, 31] have described in murine retina. In humans, these changes in the basement membrane lead to the formation of putative long-spacing collagen [6, 24, 41], which is also found in aging basement membrane in other parts of the body. We have not observed such long-spacing collagen in our mice, but Katz and Robison [20] have found an example in rats. In man the increase in the basement membrane deposits that accumulate under the plasma membrane of the RPE can lead to detachment of the RPE cell and have been associated with choroidal neovascularization [16, 34, 37], although choroidal neovascularization in humans seems to occur in the plane of basal linear deposits [6]. This excessive deposition of basement membrane material observed in these aging rodent eyes could be related to the pathogenesis of human age-related macular degeneration.

This tendency towards a thickening of the basement membrane of the RPE is consistent with most other rodent models of possible age-related macular degeneration [4, 5, 8, 15, 17, 20, 22, 27, 29–32]. Why this occurs with age is difficult to explain. It seems that this amorphous material is an overexpression of basement membrane. The basement membrane is far more complex than standard electron microscopy has traditionally shown. All base-

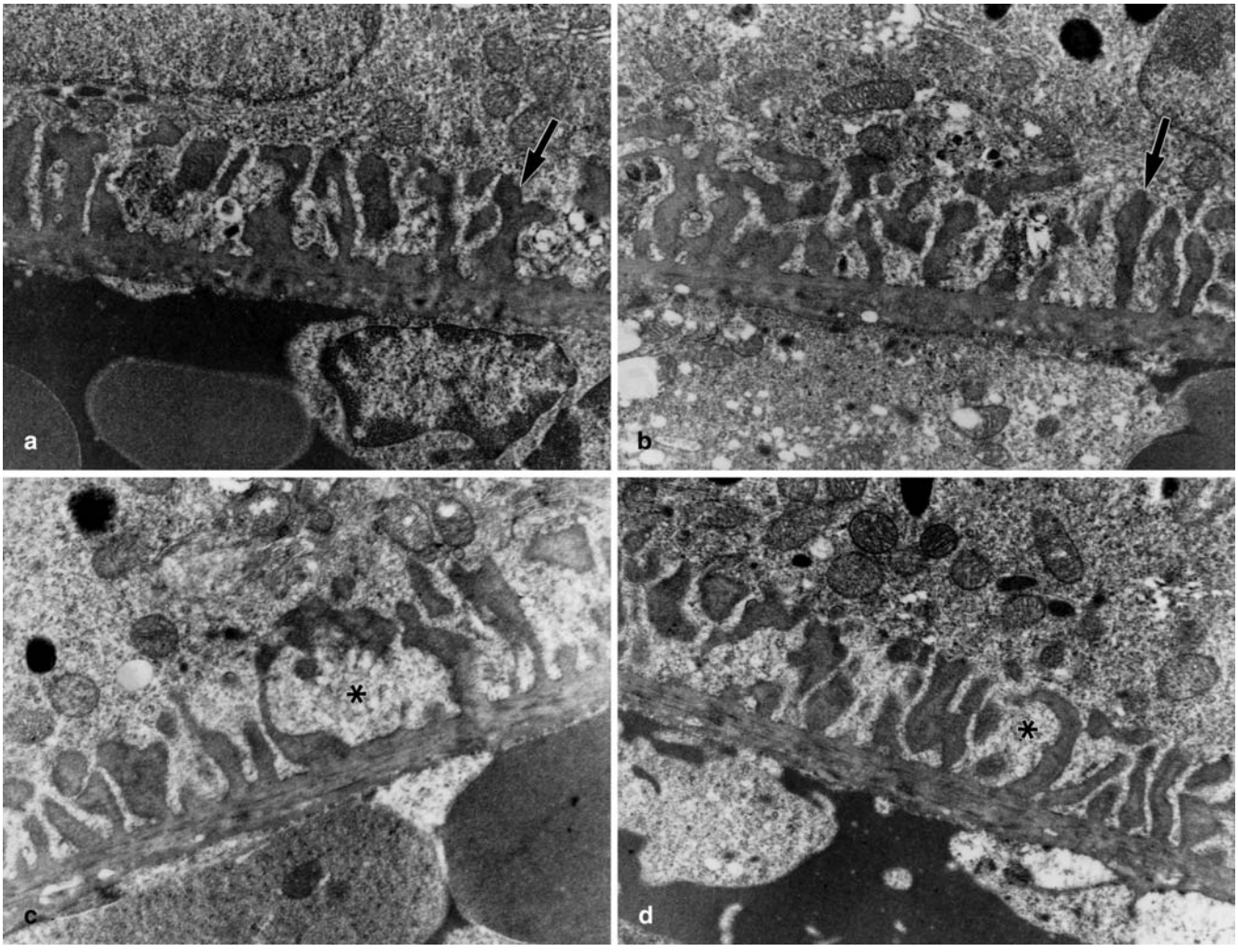

Fig. 4 Electron micrographs show the basal surface of RPE cells of a 16-month-old Rpe65 –/– mouse. **a**, **b** Enormous prolongations of the basement membrane into the plasma membrane of the cell (*arrows*) with total absence of basal infoldings (magnification 10,000×). **c**, **d** These prolongations of the basement membrane tend to isolate whole segments of the cytoplasm (*asterisks*) from the remainder of the cell (magnification 12,500× and 10,000×, respectively)

ment membranes contain laminins, entactin-1/nidogen-1, type IV collagen, heparan sulfate proteoglycans including type XVIII collagen, agrin, perlecan and various growth factors and proteases [12, 13]. Many growth factors and cytokines are stored in the basement membrane matrix and are released and activated only on dissolution. Little is known about the specific constituents of RPE basement membrane and the turnover of these constituents. Therefore it is difficult to hypothesize why this material seems to increase with age. Further studies with immunohistochemical methods, especially at the electron-microscopic level, will be necessary to elucidate the evolution of these changes in murine retina and their possible relevance to human age-related macular degeneration.

Perhaps the excessive accumulation of this material detected in the Rpe65 mutant provides an insight into the factors responsible. Katz and Robison [20] noted that there was an increase in the ratio of retinyl palmitate to stearate with age, which could reflect RPE cell senescence. This is interesting with regard to the Rpe65 –/– mutant, where there is a considerable increase in the amount of retinyl palmitate in the RPE due to a failure of isomerization of all-*trans* to 11-*cis* retinol [33]. Perhaps this is a sign of premature senescence in the mutant RPE. Human subjects with defects in the Rpe65 gene have a retinal degeneration with early signs of macular degeneration [18], but there is no evidence that they have thickened basal laminar deposits.

It is also interesting that the Rpe65 –/– strain has smaller amounts of lipofuscin accumulation in the RPE than wild-type mice, presumably because of the reduced turnover of rhodopsin [19]. Excessive lipofuscin accu-

mulation is characteristic of autosomal recessive Stargardt's dystrophy [3, 9, 21, 26, 28, 39], which is usually manifested as a severe macular degeneration with possible genetic relevance to age-related macular degeneration [1]. In the Rpe65 mutant we have an example of excessive basement membrane deposition, a possible causal factor in age-related macular degeneration, with minimum lipofuscin accumulation [19].

Acknowledgements We are very grateful to Christine Curcio for helpful discussions on the classification of membranous structures and their position relative to the basal lamina of the RPE. We thank Research to Prevent Blindness, Inc. for support.

References

1. Allikmets R, Shroyer NF, Singh N, Seddon JM, Lewis RA, Bernstein PS, Peiffer A, Zabriskie NA, Li Y, Hutchinson A, Dean M, Lupski JR, Leppert M (1997) Mutation of the Stargardt disease gene (ABCR) in age-related macular degeneration. Science 277:1805–1807
2. Barnett KC, Heywood R, Hague PH (1972) Colloid degeneration of the retina in a baboon. J Comp Path 82:117–118
3. Birnbach CD, Jarvelainen M, Possin DE, Milam AH (1994) Histopathology and immunocytochemistry of the neurosensory retina in fundus flavimaculatus. Ophthalmology 101:1211–1219
4. Cousins SW, Espinosa-Heidmann DG, Alexandridou A, Sall J, Dubovy S, Csaky K (2002) The role of aging, high fat diet and blue light exposure in an experimental mouse model for basal laminar deposit formation. Exp Eye Res 75:543–553
5. Cousins SW, Marin-Castano ME, Espinosa-Heidmann DG, Alexandridou A, Striker L, Elliot S (2003) Female gender, estrogen loss, and sub-RPE deposit formation in aged mice. Invest Ophthalmol Vis Sci 44:1221–1229
6. Curcio CA, Millican CL (1999) Basal linear deposit and large drusen are specific for early age-related maculopathy. Arch Ophthalmol 117:329–339
7. Curcio CA, Millican CL, Bailey T, Kruth HS (2001) Accumulation of cholesterol with age in human Bruch's membrane. Invest Ophthalmol Vis Sci 42:265–274
8. Dithmar S, Sharara NA, Curcio CA, Le NA, Zhang Y, Brown S, Grossniklaus HE (2001) Murine high-fat diet and laser photochemical model of basal deposits in Bruch membrane. Arch Ophthalmol 119:1643–1649
9. Eagle RC Jr, Lucier AC, Bernadino VB Jr, Yanoff M (1980) Retinal pigment epithelial abnormalities in fundus flavimaculatus: a light and electron microscopic study. Ophthalmology 87:1189–1200
10. El-Mofty AAM, Gouras P, Eisner G, Balasz EA (1978) Macular degeneration in rhesus monkey (*Macaca mulatta*). Exp Eye Res 27:499–502
11. El-Mofty AAM, Eisner G, Balasz EA, Denlinger JL, Gouras P (1980) Retinal degeneration in Rhesus monkeys, *Macaca mulatta*. Survey of three seminatural free-breeding colonies. Exp Eye Res 31:147–166
12. Engbring JA, Kleinman HK (2003) The basement membrane matrix in malignancy. J Pathol 200:465–470
13. Erickson AC, Couchman JR (2000) Still more complexity in mammalian basement membranes. J Histochem Cytochem 48:1291–1306
14. Fine BS, Kwapian RP (1978) Pigment epithelial windows and drusen: an animal model. Invest Ophthalmol Vis Sci 17:1059–1068
15. Gottsch JD, Bynoe LA, Harlan JB, Rencs EV, Green WR (1993) Light-induced deposits in Bruch's membrane of protoporphyric mice. Arch Ophthalmol 111:126–129
16. Green WR, Enger C (1993) Age-related macular degeneration histopathologic studies. The 1992 Lorenz E. Zimmerman Lecture. Ophthalmology 100:1519–1535
17. Grimes PA, Laties AM (1980) Early morphological alteration of the pigment epithelium in streptozotocin-induced diabetes: increased surface area of the basal cell membrane. Exp Eye Res 30:631–639
18. Hamel CP, Griffoin JM, Lasquellec L, Bazalgette C, Arnaud B (2001) Retinal dystrophies caused by mutations in RPE65: assessment of visual functions. Br J Ophthalmol 85:424–427
19. Katz ML, Redmond TM (2001) Effect of Rpe65 knockout on accumulation of lipofuscin fluorophores in the retinal pigment epithelium. Invest Ophthalmol Vis Sci 42:3023–3030
20. Katz ML, Robison WG (1984) Age-related changes in the retinal pigment epithelium of pigmented rats. Exp Eye Res 38:137–151
21. Klein BA, Krill AE (1967) Fundus flavimaculatus: clinical, functional, and histopathologic observations. Am J Ophthalmol 64:3–23
22. Kliffen M, Lutgens E, Daemen MJAP, de Muinck ED, Mooy CM, de Jong PTVM (2000) The APO*E3-Leiden mouse as an animal model for basal laminar deposit. Br J Ophthalmol 84:1415–1419
23. Loeffler KU, Lee WR (1986) Basal linear deposit in the human macula. Graefes Arch Clin Exp Ophthalmol 224:493–501
24. Loeffler KU, Lee WR (1992) Is basal laminar deposit unique for age-related macular degeneration? Arch Ophthalmol 110:15–16 (letter)
25. Loeffler KU, Lee WR (1998) Terminology of sub-RPE deposits: do we all speak the same language? Br J Ophthalmol 82:1104–1105
26. Lopez PF, Maumenee IH, de la Cruz Z, Green WR (1990) Autosomal-dominant fundus flavimaculatus: clinicopathologic correlation. Ophthalmology 97:798–809
27. Majji AB, Cao J, Chang KY, Hayashi A, Aggarwal S, Grebe RR, de Juan Jr E (2000) Age-related retinal pigment epithelium and Bruch's membrane degeneration in senescence-accelerated mouse. Invest Ophthalmol Vis Sci 41:3936–3942
28. McDonnell PJ, Kivlin JD, Maumenee IH, Green WR (1986) Fundus flavimaculatus without maculopathy: a clinicopathologic study. Ophthalmology 93:116–119
29. Miceli MV, Newsome DA, Tate Jr DJ, Sarphie TG (2000) Pathological changes in the retinal pigment epithelium and Bruch's membrane of fat-fed atherogenic mice. Curr Eye Res 20:8–16
30. Mishima H, Hasebe H (1978) Some observations in the fine structure of age changes of the mouse retinal pigment epithelium. Graefes Arch Clin Exp Ophthalmol 209:1–9
31. Mishima H, Kondo K (1981) Ultrastructure of age changes in the basal infoldings of aged mouse retinal pigment epithelium. Exp Eye Res 33:75–84

32. Rakoczy PE, Zhang D, Robertson T, Barnett NL, Papadimitriou J, Constable IJ, Lai C-M (2002) Progressive age-related changes similar to age-related macular degeneration in a transgenic mouse model. Am J Pathol 161:1515–1524
33. Redmond TM, Yu S, Lee E, Bok D, Hamasaki D, Chen N, Goletz P, Ma JX, Crouch RK, Pfeifer K (1998) Rpe65 is necessary for production of 11-cis-vitamin A in the retinal visual cycle. Nat Genet 20:344–351
34. Sarks SH (1973) New vessel formation beneath the retinal pigment epithelium in senile eyes. Br J Ophthalmol 57:951–965
35. Sarks S, Arnold J, Killigsworth M, Sarks J (1999) Early drusen formation in the normal and aging eye and their relation to age-related maculopathy: a clinicopathological study. Br J Ophthalmol 83:358–368
36. Schmidt RE (1971) Ophthalmic lesions in nonhuman primates. Vet Pathol 8:28–36
37. Spraul CW, Lang GE, Grossniklaus HE (1996) Morphometric analysis of the choroid, Bruch's membrane, and retinal pigment epithelium in eyes with age-related macular degeneration. Invest Ophthalmol Vis Sci 37:2724–2735
38. Stafford TJ (1974) Maculopathy in an elderly subhuman primate. In: Strieff EB (ed) Limitations and prospects for retinal surgery, Miami, 1972 (Modern problems in ophthalmology, vol 12). Karger, Basel, pp 214–219
39. Steinmetz RL, Garner A, Maguire JI, Bird AC (1991) Histopathology of incipient fundus flavimaculatus. Ophthalmology 98:953–956
40. Vainisi SJ, Beck BB, Apple DJ (1971) Retinal degeneration in baboon. Am J Ophthalmol 78:279–284
41. Van der Schaft TL, de Bruijn WC, Mooy CM, Ketelaars DAM, de Jong PTVM (1991) Is basal laminar deposit unique for age-related macular degeneration? Arch Ophthalmol 109:420–424
42. Zhang D, Lai MC, Constable IJ, Rakoczy PE (2002) A model for a blinding eye disease of the aged. Biogerontology 3:61–66