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Bestrophin detected in the basal membrane of the retinal epithelium and drusen of monkeys with drusenoid maculopathy

P Gouras¹, K Braun², L Ivert³, M Neuringer⁴, and JA Mattison⁵

¹ Columbia University, Dept. of Ophthalmology, Stockholm, Sweden

² Dept. of Pathology, Stockholm, Sweden

³ St. Erik's Hospital, Stockholm, Sweden

⁴ Oregon National Primate Research Center

⁵ National Institute on Aging

Abstract

Purpose—To determine if bestrophin is present in the basal membrane of macular retinal pigment epithelium (RPE) and in drusen of rhesus monkeys with age related drusenoid maculopathy.

Methods—The macular region of three rhesus monkeys (*Macaca mulatta*), 23–24 years of age, with drusenoid maculopathy was dissected from eyes fixed with 4% paraformaldehyde. The macula was sectioned into rectangular pieces. The sclera was removed from each segment and the remainder separated into segments of neural retina with retinal epithelium or choroid with retinal epithelium. These segments were incubated with a goat polyclonal antibody to human bestrophin 1, reacted with gold-labeled rabbit antibody to goat IgG, silver enhanced, and processed for transmission electron microscopy.

Results—Bestrophin labeled gold particles were found in quasi-linear arrays on the basal surface of the macular RPE and also within drusen where bestrophin was found in segments of membranous-like material. The array density of the bestrophin linked gold particles on the basal membrane of the epithelium had a maximal value of about 100 bestrophin molecules/micron². Immuno-detection of bestrophin was most effective when examined in an RPE layer that remained attached to the neural retina, where the basal surface of the epithelium is more directly exposed to the antibodies.

Conclusion—Bestrophin is present on the basal membrane of macular RPE of rhesus monkeys with age related drusenoid maculopathy and also found in the membranous-like structures of drusen. The latter finding provides insight into the pathogenesis of drusen by indicating that segments of the basal membrane of RPE contribute to the material that accumulates within drusen.

Introduction

Bestrophin 1 is a protein located uniquely in the basal-lateral membrane of the retinal pigment epithelium (RPE) (6,8) and is considered to be an anionic conduction pathway (4). Members of this family of proteins are located in plasma and cytosolic membranes of many other cells in a wide variety of animals including arthropods (4). Mutations in the human gene (hBest 1), which expresses the unique RPE form of the protein, cause vitelliform macular dystrophy (7, 9). The precise functional role of RPE bestrophin is unclear although there is much evidence that it is a chloride channel (4). It is unclear why mutations in bestrophin protein lead to vitelliform macular degeneration. Recent immuno-histochemical studies using light microscopy found that bestrophin is less expressed in the macula than in the peripheral retina even though abnormalities produced by mutations in this protein localize mainly to the macula (8).

We have used immune-gold labeling to determine if bestrophin can be identified in the basal membrane of the RPE in the macula of rhesus monkeys with drusenoid maculopathy and whether it can also be found in drusen which are thought to arise partly by the budding and degeneration of segments of basal membrane of the RPE (1,2,5). The results reveal bestrophin's presence in the basal membrane of RPE and in membranous debris within drusen, providing more support for the hypothesis that some of the material accumulating within drusen comes from segments of RPE basal membrane.

Methods

The macular retinas of three female rhesus monkeys (*Macaca mulatta*), two 23 and one 24 years old, all with moderately severe drusenoid maculopathy (3), were examined for the presence of bestrophin, using immune-gold electron microscopy. After euthanasia, the eyes were fixed rapidly in 4% paraformaldehyde in phosphate buffered saline (PBS); the globes were pierced to facilitate diffusion of the fixative into the vitreous. After storage at 4°C in fixative for several weeks, the eyes were washed with PBS and dissected with the aid of a surgical microscope. The macula was identified and cut into a square approximately 15 × 15 mm² centered on the fovea. This segment was sectioned into multiple smaller rectangular pieces. The sclera and neural retina were removed from each piece. In some cases the RPE stayed with the neural retina and in others it remained with the choroid. One end of each piece was cut into 3 or 4 finger-like processes in order to facilitate diffusion of the antibodies into the tissues. Each piece was placed in a separate chamber of a 96 well plate and then immersed in a serial change of solutions. Each piece was first immersed in 0.05% glycine in phosphate buffer (PB) to inactivate aldehydes and then washed repeatedly with PB. The multi-welled plate was rotated continuously after a change of solution. Cell membranes were made more permeable using a 0.05% solution of triton X 100 in PB for 30 minutes and then washed repeatedly. The pieces were incubated in a blocking solution containing normal rabbit serum (Aurion Blocking solution, Wageningen, the Netherlands) for 1 hour at 4°C. The pieces were washed in PBS with 0.2% bovine serum albumin (BSA-cTM, Aurion) and then incubated with a polyclonal goat antibody to human bestrophin 1 (C14: sc-22027, Santa Cruz Biotechnology Inc, Santa Cruz, CA) diluted 1/10, 1/100, 1/200, 1/500 with PBS and 0.2% BSA-cTM. A negative control not exposed to the primary antibody was included. The 96-well plate was rotated in a cold room at 4°C overnight. The pieces were then washed with PBS with 0.2% BSA-cTM and incubated with the secondary antibody, rabbit anti-goat IgG labeled with 1 nm gold particles (Aurion), in PBS and 0.2% BSA-cTM and rotated overnight at 4°C. The pieces were washed with PBS and 0.2% BSA-cTM and post-fixed with 2.5 % glutaraldehyde in PB for 2 hours. After washing, the pieces were incubated in R-Gent SE-EM silver enhancement mixture (Aurion) for 90 minutes and washed with distilled H₂O. The pieces were immersed in 0.5% OsO₄ in PB for 15 minutes, dehydrated, epon embedded and sectioned for light and at selected sites for electron microscopy. Ultra-thin sections were examined by transmission electron microscopy using Zeiss 10C or Jeol 1200 EX2 instruments. Digital photographs were examined on Adobe Photoshop and measurements made of the spacing between silver/gold particles on the basal membrane of RPE cells.

All procedures were approved by the Institutional Animal Care and Use Committee of the ONPRC/Oregon Health & Science University and the Gerontology Research Center, NIA, and conformed to the Principles of Laboratory Animal Care (NIH publication No.85-23, revised 1985), the OPRR Public Health Service Policy on the Humane Care and Use of Laboratory Animals (revised 1986) and the U.S. Animal Welfare Act, as amended.

Results

Figures 1 and 2 illustrate silver enhanced gold particles along the basal surface of the RPE layer in the macula of both 23 year old rhesus monkeys with drusenoid maculopathy. There is a quasi-linear array of particles along the basal membrane of this RPE cell. An arrow points out such a particle in each figure. A few particles are seen in the cytoplasm. A magnified insert shows how close the particles are to the basal membrane of the RPE cell. No such particles were found along the basal membrane of the RPE in negative control specimens. Immuno-gold labeling was most extensive in specimens where the RPE layer had remained attached to the neural retina, thereby exposing it more to the antibodies.

We measured the spacing between silver/gold particles in samples containing the maximum density of particles (Figures 3 and 4). The particles counted are marked by arrows. Although some regions are distinct for the regularity of the spacing between particles, there is much variability. The majority of the particles are located on the basal membrane of the epithelial cells with very few in the cytoplasm. The spacing between particles had a mean of 224 nm and a median of only 176 nm. This gives an average spacing between particles of about 100 to 200 nm resulting in approximately 25–100 bestrophin channels/micron² of basal membrane.

Drusen were also found with silver/gold particles labeling bestrophin. Figure 5 shows an example of a drusen with membranous material (white rectangle) that contains typical silver/gold particles, better seen in a magnified view of this area (Figure 6). The particles are located on curled membranous-like structures that resemble the degenerating remains of plasma membrane. Figure 7 illustrates another example of a drusen containing bestrophin labeled silver/gold particle (white rectangle). Again the label is located on curled membranous-like material and in a quasi-linear array as observed along the base of the intact retinal epithelium (Figure 8). There was little to no label along the basal membrane of the retinal epithelial cell at the dome of these drusen. The drusenoid material may be blocking the antibodies accessibility to the basal membrane.

Discussion

These results confirm previous reports that used light microscopic immune-histochemistry to show localization of bestrophin to the basal membrane of the retinal epithelium (6,8). Immuno-gold labeling and electron microscopy detects single molecules of this protein and thus allows quantifying the density of bestrophin on the basal membrane. We estimated a density about 25–100 bestrophin channels/micron². However, this may be a low estimate because the antibody may not label every bestrophin molecule and variations in the accessibility of the antibodies to the protein may hinder such quantification. We found that removing RPE from Bruch's membrane led to greater staining presumably due to a greater accessibility of the antibody to the basal surface of the RPE cell. We observed less staining of the basal membrane of RPE cells at the dome of a drusen. Again, this difference could be due to less accessibility to the protein. In these current experiments, the tissue was exposed to the antibodies before ultra-thin sectioning. This process of post-embedding exposure of the antibodies to ultra-thin sections might reduce variability and give a more accurate measurement of bestrophin density. This process would be useful for elaborating on the observations made by light microscopy and biochemistry which indicate a lesser quantity of bestrophin on macular than on peripheral RPE (8) and also clarify whether the basal membrane of RPE over drusen express less bestrophin.

To our knowledge, this is the first evidence of the presence of bestrophin within drusen. Its location along segments of membranous material within the center of drusen further supports the hypothesis that some, perhaps much, of the material that accumulates within drusen is due

to the budding of segments of basal cytoplasm, including basal plasma membrane, from aging retinal epithelial cells (1,2,5).

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Figure 1.

Electron micrograph shows bestrophin linked silver/gold particles in a quasi-linear array along the basal membrane of an RPE cell of a 23 year old female rhesus monkey with age related drusenoid maculopathy. The arrows indicate the same silver-gold particle in the upper photo and the magnified inset. A calibration, below, indicates 100 nm for the upper photo.

Figure 2.

Electron micrograph shows bestrophin linked silver/gold particles in a quasi-linear array along the basal membrane of an RPE cell of the other 23 year old female rhesus monkey with age related drusenoid maculopathy. The arrows indicate the same silver-gold particle in the upper photo and the magnified inset. A calibration, below, indicates 100 nm for the upper photo.

Figure 3. shows the basal membrane of an RPE cell in which the spacing between the silver/gold particles was measured. The arrows show the particles considered for measurement. An asterisk marks the same structure in the upper photo and the magnified inset (lower right). A calibration, below, indicates 100 nm for the upper photo.

Figure 4. shows the basal membrane of another RPE cell in which the spacing between silver/gold particles was measured. An asterisk marks the same site in both the upper photo and the inset. A calibration, below, indicates 100 nm for the upper photo.

Figure 5.

Electron micrograph shows a druse in a 23 year old female rhesus monkey. BL refers to the basal lamina of the RPE. A white rectangle encloses an area with curled membranous material. Higher magnification of this area (Figure 6) reveals silver-gold particles indicating the presence of bestrophin. The calibration, below, indicates 100 nm.

Figure 6. shows a magnified view of the area enclosed by the rectangle in Figure 5. The arrows point out the silver-gold particles labeling bestrophin on the membranous material in this druse. Not all of the bestrophin linked particles are arrowed. A calibration, lower right, indicates 80 nm.

Figure 7. shows another druse in a 23 year old female monkey with drusenoid maculopathy. BL refers to the basal lamina of the RPE. Arrow heads mark silver/gold particles on the basal membrane. A white rectangle encloses an area with curled membranous material. Higher magnification of this area (Figure 8) reveals bestrophin linked silver-gold particles in this membranous material. The calibration, lower right, indicates 100 nm.

Figure 8. shows a magnified view of the area enclosed by the rectangle in Figure 7. The silver-gold particles labeling bestrophin are obvious. A calibration, lower right, indicates 80 nm.