Retinal Pigment Epithelial Cell Distribution in Central Retina of Rhesus Monkeys

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Purpose. To determine the cell density profile of the retinal pigment epithelium (RPE) in the central retina and relate it to the distribution of photoreceptors.

Methods. Wholemounts of rhesus monkey (*Macaca mulatta*) retinas with the choroid removed but the RPE attached were stained with the nuclear stain 4′,6-diamidine-2-phenylindole dihydrochloride (DAPI) and imaged with a fluorescence microscope. RPE cell nuclei were counted at the foveal center and at 0.4-mm intervals along the vertical meridian. The number of photoreceptors per RPE cell at each location was estimated by using previously published data on the distribution of rhesus photoreceptors.

Results. Data were collected from eight retinas. Mean RPE cell density increased from a relatively stable baseline of approximately 4000 RPE cells/mm^2 beyond 2 mm (10°) eccentricity to more than 7000 cells/mm^2 at the center of the fovea. The number of cones per RPE cell in the rhesus retina was approximately 20:1 in the foveal center (similar to the human retina) and only approximately 1.5:1 in the parafovea. However, when the rods were included, and the total number of photoreceptors per RPE cell were considered, the ratio of photoreceptors to RPE cells was lower in the fovea than in the remainder of the central retina.

Conclusions. In spite of the high cone density, there is a relatively low number of photoreceptors per RPE cell in the fovea. This may limit the metabolic demands on foveal RPE cells and help to preserve their functions in the face of aging and disease. (*Invest Ophthalmol Vis Sci.* 2002;43:2815–2818)

Photoreceptor density in the primate fovea changes rapidly with retinal eccentricity. In rhesus monkeys, the number of cones per unit area is maximal at the foveal center and declines rapidly to approximately 50% within 0.1 mm (0.5°) eccentricity[1,2] and approximately 20% at 0.5 mm (2.5°) eccentricity.[2] In parallel, the density of rods increases from zero at the foveal center to become equal to the density of cones at approximately 0.5 mm eccentricity. At greater eccentricities, the ratio of rods to cones increases until it reaches a maximum in the midperipheral retina. Similar topographic patterns have been documented in numerous other primates, including humans.[3]

The foveal photoreceptors, like those in the remainder of the retina, are dependent on the underlying retinal pigment epithelial (RPE) cells for metabolic support.[4,5] The RPE is an essential link in the vitamin A cycle that regenerates the visual pigments as well as being part of a system of breakdown and recycling of photoreceptor membrane components. However, rods and cones have different relationships with the RPE cells in at least two ways. First, they differ in spatial patterns of outer segment renewal.[4,5] In rods, proteins with similar "birthdays" form a compact band at the base of the outer segment that moves outward to be phagocytosed by the RPE as the discs in this band are shed. In cones, the newly manufactured proteins spread diffusely throughout the outer segment with the possibility that proteins with a wider range of birthdays and a greater range of oxidative damage are shed. Second, rods and cones differ anatomically in the depth of interdigitation between photoreceptor outer segments and RPE microvilli[6] (illustrated in Ref. 7). The implications of these and other rod–cone differences are still poorly understood, but it is reasonable to expect that they could lead to topographic differences in the effects of aging and disease,[8] as well as differences in the effects of such environmental factors as nutrition and light exposure.[7]

In recent studies of the topography of RPE cell density, the investigators have adopted two different approaches. One approach has focused on comparisons of the foveal center with other, fairly distant retinal regions[9] so that continuous topographic gradients are unknown. Others have measured continuous distributions, but they have used spatial sampling schemes that are too coarse for comparison with the steep gradients of foveal photoreceptor densities.[10,11]

The purpose of the present study is to report measurements of RPE cell density as a function of eccentricity along the vertical meridian in the central retina. These data allow us to estimate how many rods and cones the RPE cells at different eccentricities must support. We find that the fovea is favored by having the lowest number of photoreceptors per RPE cell in the central retina. These data can serve as a reference for studies of factors that may affect the relationships between RPE cells and photoreceptors in primate retinas.

Methods

Eyes were obtained from rhesus monkeys (*Macaca mulatta*), 7.6 to 33 years of age, that had been housed at the Oregon National Primate Research Center. Ocular tissues were obtained through the Center Tissue Distribution Program, from monkeys that were killed for other projects. All procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and to National Institutes of Health guidelines.

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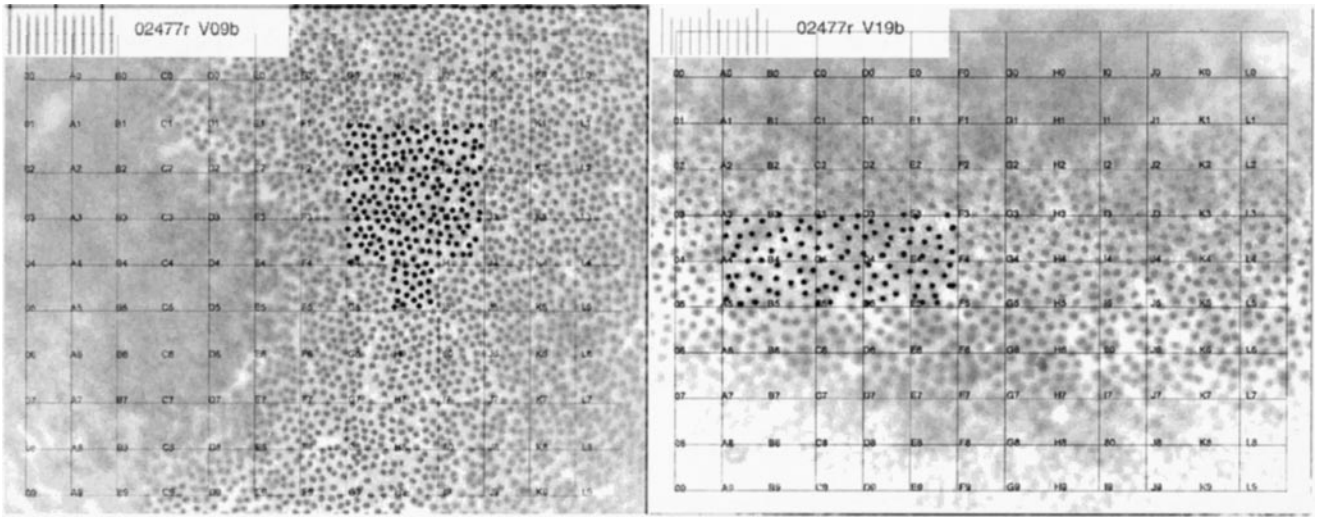

FIGURE 1. DAPI-labeled RPE cell nuclei in rhesus retinal wholemounts. *Left*: microscope field from a region 0.4 mm above the foveal center; *right*: a field 4.5 mm above the foveal center. The scale in the *upper left corner* of each image indicates 10 μm per division. A grid of *squares* 50 $\times$ 50 μm on a side was overlaid on the video image, and a sampling set was selected to include a region with a clear image near the center of the field. Well-focused nuclei were counted in 10 contiguous *squares* if they did not touch the bottom or right side of the *square* (unbiased counting). The nuclei that were counted are marked in *black* in the video frames shown here.

Monkeys were systemically perfused with 0.1 M phosphate buffer for 4 to 15 minutes to remove the blood followed by 4% paraformaldehyde in buffer for 12 to 60 minutes to preserve the tissue. The eyes were enucleated, hemisected at the equator, and postfixed in paraformaldehyde or a paraformaldehyde-glutaraldehyde mixture for durations ranging from several hours to overnight. The eyecups were stored at 4°C in 0.1 M phosphate buffer with 5% sucrose, 0.15 mM $CaCl_2$, and 0.04% sodium azide for an average of 1 year. A 10 $\times$ 10-mm square centered on the fovea was cut out of the eyecup, and the sclera was removed. With number 5 jewelers' forceps, the choroid was teased from the RPE, leaving the RPE attached to the retina. In 60% of the cases, the separation was patchy, with regions of choroid remaining attached to the RPE, or patches of RPE pulling loose from the retina. However, in 40% of the eyes examined it was possible to achieve a clean separation of the choroid from the RPE in the central retina.

Cell nuclei were labeled by incubating the tissue with the nuclear stain 4′6-diamidine-2-phenylindole dihydrochloride, (DAPI, 0.8% wt/vol in buffer; Roche Diagnostics, Indianapolis, IN) for 10 minutes. The piece of retina with the RPE attached was placed in a well 300 to 400 μm deep, constructed from a coverslip with a hole in it that was glued to a microscope slide with silicone rubber.[12] The well was filled with buffer, and a second coverslip was placed on top to form a closed chamber.

The slide was placed on the stage of an epifluorescence microscope (Eclipse E800; Nikon, Melville, NY) with the RPE side up. The center of the fovea was located by finding the thinnest point near the center of the tissue and the line between it, and the center of the optic disc was considered to be the approximate horizontal meridian. The location of the fovea was later confirmed by imaging the tissue on another microscope with a digital readout of stage position[13] and using retinal landmarks such as prominent vessels. Color images of microscope fields 0.5 by 0.65 μm at nominal 0.4-mm intervals along the vertical meridian were captured with a digital camera and software (Spot RT; Diagnostic Instruments, Inc., Sterling Heights, MI) and stored on disk. The retinal location was controlled by the stage manipulator, but because of the mechanical coarseness of the manipulator, the eccentric positions were sometimes slightly offset from the desired spacing. In all cases, the actual position was recorded from the vernier scale and the values corresponding to 0.4-mm spacing were estimated by interpolating between adjacent points.

Of 25 successfully dissected retinas, suitable images were obtained from 10 retinas from eight monkeys. Eight successfully dissected retinas were excluded from the study because of insufficient contrast between the RPE nuclei and the cell cytoplasm. The low contrast appeared to result from low levels of melanin that allowed fluorescence from nuclei in the retina to be transmitted through the RPE cell cytoplasm. The remaining seven retinas were used for other studies.

Calibrated image files with an embedded scale were imported into image analysis software (Photoshop; Adobe Systems, Mountain View, CA) converted to gray scale, and inverted (contrast reversed) so that the RPE cell nuclei appeared dark on a light background. For counting the nuclei, a grid with frames 50 $\times$ 50 μm square was superimposed on the images. The calibrated grid was generated with the aid of an image processing toolkit (Reindeer Games, Asheville, NC) that was added to the image analysis software (Photoshop; Adobe). Two example images with the counting grid superimposed are shown in Figure 1.

In each image, RPE cell nuclei were counted in 10 contiguous counting frames that were located in relatively central regions of the images where nuclei were clearly visible. Regions of the images that were out of focus or were damaged during the removal of the choroid were not used for counting. As a result, the frames that were used did not always form a perfect rectangle (Fig. 1), but they allowed sampling of the same amount of retinal area in each image. Although adjacent microscope fields overlapped slightly so that retinal position could be clearly assigned, the counting frames in each field were sufficiently close to the center of each microscope field that no nuclei were double counted. Within the frames, cell nuclei were counted in an unbiased manner by including only those that either did not touch the grid lines or touched only the top and left borders of each counting frame.[14] We assumed that the number of cell nuclei is a reasonable index of the number of cells, because only approximately 3% of rhesus RPE cells are thought to have two nuclei.[15]

RESULTS

In all 10 eyes studied, the number of RPE cells per unit area (cell density) was maximal near the foveal center and declined with retinal eccentricity. For the 2 animals in which both eyes were studied, the data from the two eyes were averaged to represent the best estimates for those individuals. Then averages and standard deviations were calculated across the eight animals (Table 1 and Fig. 2). The mean RPE cell density increased from a relatively stable baseline of approximately 4000 RPE cells/mm^2 beyond 2 mm eccentricity to more than 7000 cells/mm^2 at the center of the fovea. As indicated in the table,

TABLE 1. RPE Cell Density Along the Vertical Meridian of Rhesus Monkey Retinas

Vertical Eccentricity (mm)	Samples	RPE Cells/mm^2
−4	2	4160 ± 170
−3.6	4	3680 ± 219
−3.2	5	3976 ± 538
−2.8	7	3971 ± 492
−2.4	7	3997 ± 551
−2	6	4167 ± 611
−1.6	8	4210 ± 608
−1.2	8	4625 ± 759
−0.8	7	5174 ± 931
−0.4	8	6380 ± 998
0 (fovea)	8	7139 ± 1193
0.4	8	6373 ± 947
0.8	8	5513 ± 976
1.2	7	4600 ± 941
1.6	8	4000 ± 678
2	8	3905 ± 431
2.4	8	3983 ± 485
2.8	7	3837 ± 436
3.2	7	3886 ± 314
3.6	4	3750 ± 403
4	2	4050 ± 71

Data are the mean ± SD from eight monkeys. For macaques, 0.2 mm on the retina subtends about 1° of visual angle. Density was determined along the vertical meridian (inferior direction, negative eccentricity).

data were obtained from at least seven animals at 15 of the 21 measuring locations. Most of the missing data were from locations near the edges of the tissue where some specimens did not extend to the full distance. Because the profile of cell density is essentially flat outside the central ± 2 mm, the missing data would have little effect on the results.

The relatively large standard deviations indicated by the error bars are due to differences in RPE cell density from one animal to another, rather than to different shapes of the profiles. This fact is best illustrated by superimposing the profiles of individual animals, as is shown in Figure 3. Six of eight animals had the highest RPE cell density at the center of the fovea, and the other two had slightly higher density at one sampling location eccentric from the fovea. Sampling error or

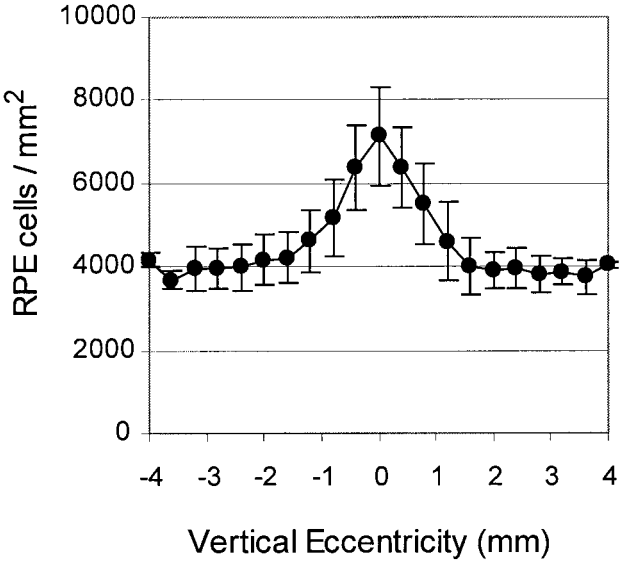

FIGURE 2. Average profile of RPE cell density along the vertical meridian of rhesus monkey retinas. The foveal center is designated as zero. Data are the mean ± SD (error bars) in eight animals.

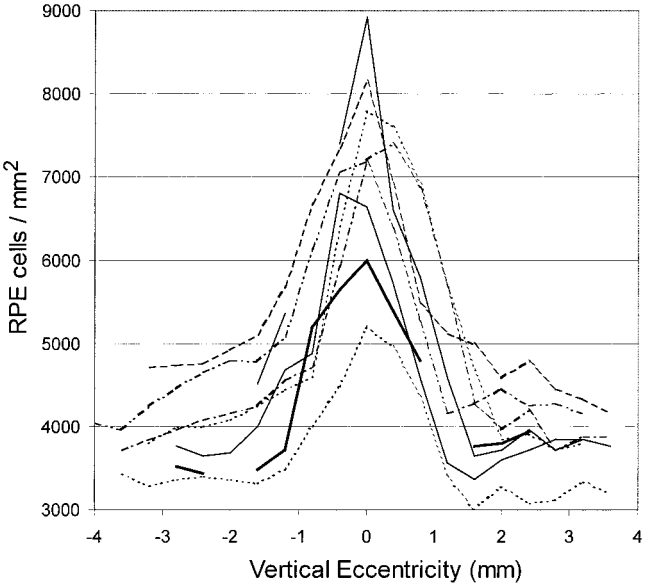

FIGURE 3. Profiles of RPE cell density along the vertical meridian in eight rhesus monkey retinas. All show a similar pattern with a peak density at or immediately adjacent to the foveal center, indicated as zero eccentricity. Characteristics of the monkeys (age; sex; eye—right, R; left, L; or average for the two eyes, Avg; and ID number), ordered by their RPE peak densities from highest to lowest were: 22.3 years F, R (13313); 33 years M, Avg (02477); 20.5 years F, L (11133); 14 years F, R (20078); 31 years F, Avg (03353); 16 years M, R (12697); 7.6 years F, R (16689); 7.8 years F, L (16537).

slight errors in locating the center of the fovea are probably sufficient to account for the two profiles that did not peak at the center. The two animals represented by average data from both eyes—which should reduce sampling error— had profiles that peaked at the center.

The ages and sexes of the animals are listed in the legend of Figure 3. There was a moderate increase of peak RPE cell density with age ($r = 0.56$) that was not statistically significant with this small sample ($P = 0.15$). Additional data are being collected from sections of a separate group of monkey retinas for comparison with data in studies of the effects of aging on human RPE cell density.[9–11]

The RPE cell density profile is relatively wide compared with the foveal depression. The average curve of Figure 2 is replotted in Figure 4 and superimposed on a graph of the retinal thickness along the vertical meridian of a representative rhesus retina. These preliminary thickness data were collected by focusing through a retinal wholemount at specified eccen-

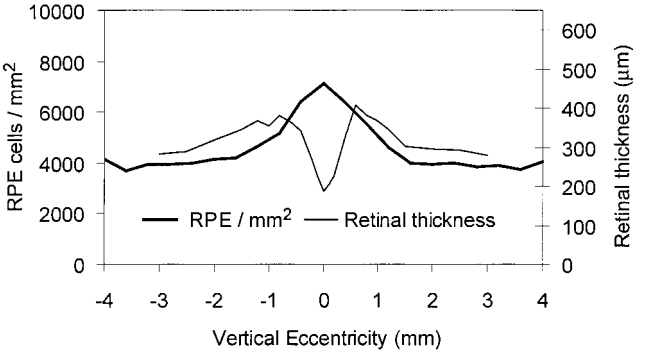

FIGURE 4. Relationship of the profile of RPE cell density (*thick line*) to the profile of retinal thickness (*thin line*). The increased density of RPE cells goes well beyond the foveal depression. The density declines to a steady level beyond approximately 2 mm eccentricity.

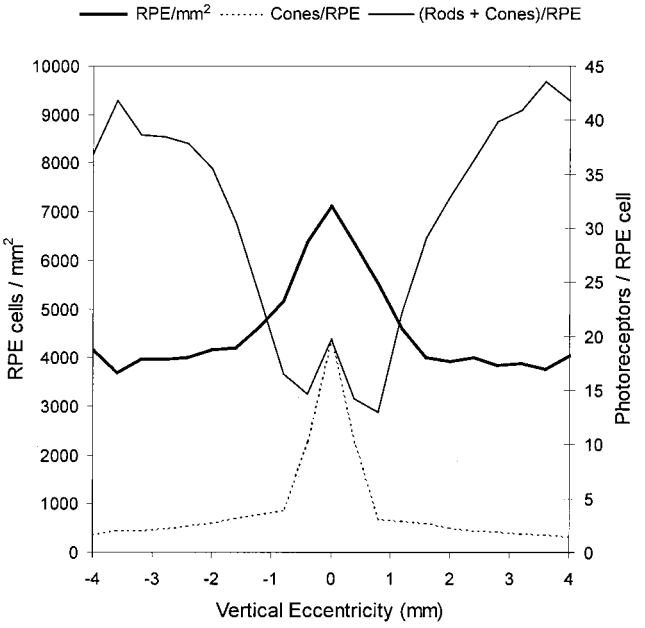

FIGURE 5. Relationship of RPE cells to photoreceptor cells in rhesus retinas. *Thick solid line*: mean RPE cell density from Figure 2. *Dashed line*: mean number of cones per RPE cell. Cone densities were estimated as means of data extracted from Figure 3B in Wikler et al.[2] and Figure 3B in Perry and Cowey.[1] *Thin solid line*: mean number of photoreceptors (rods plus cones) per RPE cell. Rod densities were extracted from Figure 3B in Wikler et al.[2] and added to the cone densities described above.

tricities, as described previously.[13] A more complete description will be published later.

DISCUSSION

By referring to published data on the density profiles of cones and rods in the foveal region of rhesus retinas,[1,2] we estimated the number of photoreceptors that each RPE cell must support. Curves describing these relationships are presented in Figure 5.

The density of cone photoreceptors peaks in the fovea and then declines with eccentricity to very low density. Consequently, the calculated number of cones per RPE cell was approximately 20:1 in the center and only approximately 1.5:1 in the parafovea. However, when the rods were included and the total number of photoreceptors per RPE cell was considered, the ratio of photoreceptors to RPE cells increased rapidly in the parafovea. As a result, except for the very center, the foveal region actually had the lowest number of photoreceptors per RPE cell in the central retina. Furthermore, inspection of the wholemounts suggested that although there is a sharp maximum of cone density at the very center of the fovea, the RPE cell distribution is less peaked. This implies that the foveolar area containing the local maximum of cones to RPE cells is even more limited than implied by our graph (Fig. 5), because of the relatively large sampling intervals.

Although it has been reported that RPE cell density is maximal at the center of the human fovea,[9] apparently no one has established a complete profile of RPE cell density in the foveal region of human eyes. However, the mean cone-to-RPE ratio has been determined for the foveal center (23:1 in the second to fourth decades[9]), and it is quite similar to the ratio we found for the foveal center of rhesus retinas (20:1). This similarity between the human and primate data argues for the utility of rhesus monkeys as models for humans in the search for preventive approaches to macular disease.

Each RPE cell nucleus must support the synthesis of enzymes that participate in the vitamin A cycle, as well as enzymes that degrade the proteins and lipids shed by the photoreceptors. It would be valuable to estimate as a function of eccentricity how much vitamin A and photoreceptor membrane RPE cells must metabolize per day, but available data are not sufficient to derive a realistic estimate; the photoreceptor-to-RPE cell ratio is our best current surrogate. Presumably, the smaller number of photoreceptors overlying foveal RPE cells should produce less total material requiring recycling and hence should place less metabolic demand on the RPE cells. Consequently, the relatively low number of photoreceptors per RPE cell in the fovea may be beneficial for foveal RPE cells and photoreceptors and help to preserve their function in the face of aging and disease. This advantage may contribute to the relative sparing of foveal photoreceptors in aging[8] and in some diseases of the retina,[16,17] including varieties of retinitis pigmentosa that involve genetic abnormalities of the RPE.[18]

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