Morphometric Analysis of the Extramacular Retina from Postmortem Eyes with Retinitis Pigmentosa

Mark S. Humayun,1 Maria Prince,1 Eugene de Juan, Jr,1 Yolanda Barron,1 Mosheh Moskowitz,1 Ingrid B. Klock,2 and Ann H. Milam2

Purpose. To evaluate the degree of inner retinal preservation in the extramacular regions of postmortem retinitis pigmentosa (RP) eyes.

Methods. Eighteen RP retinas and 11 age-matched healthy retinas were sectioned for morphometric analysis by light microscopy. The 18 RP retinas were classified by disease severity and mode of inheritance. Cell nuclei in the outer nuclear layer (ONL), inner nuclear layer (INL), and ganglion cell layer (GCL) were counted in adjacent 125-μm segments from an area spanning the region between 4 mm and 10 mm from the fovea.

Results. A mixed-effects model showed a decrease in mean cell counts for each of the cell layers when the severity groups and inheritance types compared with those of control retinas. There was no statistically significant difference in the number of nuclei preserved in the INL and GCL in the moderate group compared with the severe group. Results from the INL counts for the different inheritance types of RP showed a higher overall mean percentage of cells was preserved for the autosomal dominant RP (ADRP) group when compared with the X-linked (XLRP) and simplex RP groups. Analysis of the GCL counts revealed significantly more counts only in the ADRP group compared with the XLRP group; the other group comparisons were not significant.

Conclusions. Retinitis pigmentosa results in cell loss in all retinal layers, with the most profound loss in the ONL, followed by the GCL and then the INL. The preservation of the INL and GCL in the extramacular region is less than that previously reported for the macular region of the same retinas.

(Retina 1999; 19:143–148)

Retinitis pigmentosa (RP) is the most common cause of inherited blindness, with a prevalence of 1 in 4000. RP results in blindness due primarily to photoreceptor degeneration and retinal pigment epithelium damage.2,3 Because no effective therapy exists for RP, the impact of RP on the patients, their families, and society is devastating both psychologically and economically.

Current proposed strategies for visual rehabilitation include neural retina transplantation, delivery of corrective genes or growth factors to diseased photoreceptors, and electrical stimulation of inner retinal neurons via a retinal prosthesis.4,8,13,21 Paramount to the success of these treatments is the survival of neurons in the inner retina. After photoreceptor death, however, it is hypothesized that secondary visual neurons undergo transneuronal degeneration due to the withdrawal of synaptic input or trophic factors.

Two morphometric studies of RP maculas have demonstrated that neurons are lost in the inner nuclear layer (INL) and ganglion cell layer (GCL).5,10 However, at least 30% to 75% of nuclei in the GCL and as much as 78% to 88% of the nuclei in the INL were preserved. In the present study, the degree of preservation of the outer nuclear layer (ONL), INL, and GCL was determined for the extramacular regions of the same RP retinas.

Morphometric analysis from the extramacular regions in RP eyes is important for a number of reasons. First, with previous data from the macular region on hand, it should increase our understanding of the effect of RP on the entire retina. Second, in RP (rod-cone variant, the type studied here), patients first have a loss of peripheral vision. Given that the earliest and most severe loss of photoreceptors appears to occur in the extramacular regions, we wanted to determine whether the transneuronal degeneration was more pronounced in the extramacular regions than in the macular regions. Last, depending on the degree of inner retinal preservation, such an analysis might target certain retinal regions over others for proposed treatment strategies.

Methods
Selection of Postmortem Eyes
The RP eyes were obtained through the Foundation Fighting Blindness (Hunt Valley, MD). The eyes had no retinal condition other than RP. Eyes were categorized into either a moderate or severe RP group as follows. Before death, the eyes in the...
TABLE 1. Characteristics of Study Eyes with Moderate Retinitis Pigmentosa

<table>
<thead>
<tr>
<th>Patient/Age (y)/Sex</th>
<th>FFB Donor No.</th>
<th>RP Type</th>
<th>Visual Acuity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/62/F</td>
<td>357</td>
<td>Simplex</td>
<td>20/200</td>
</tr>
<tr>
<td>2/63/M</td>
<td>328</td>
<td>Simplex</td>
<td>20/50</td>
</tr>
<tr>
<td>3/81/F</td>
<td>368</td>
<td>Simplex</td>
<td>20/100</td>
</tr>
<tr>
<td>4/76/F</td>
<td>370</td>
<td>AD</td>
<td>20/200</td>
</tr>
<tr>
<td>5/82/M</td>
<td>377</td>
<td>AD</td>
<td>20/50</td>
</tr>
<tr>
<td>6/82/F</td>
<td>247</td>
<td>AD</td>
<td>20/400</td>
</tr>
<tr>
<td>7/83/F</td>
<td>299</td>
<td>AD</td>
<td>20/80</td>
</tr>
</tbody>
</table>

FFB, Foundation Fighting Blindness; RP, retinitis pigmentosa; AD, autosomal dominant.

TABLE 2. Characteristics of Study Eyes with Severe Retinitis Pigmentosa

<table>
<thead>
<tr>
<th>Patient/Age (y)/Sex</th>
<th>FFB Donor No.</th>
<th>RP Type</th>
<th>Visual Acuity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/84/F</td>
<td>288</td>
<td>Simplex</td>
<td>NLP</td>
</tr>
<tr>
<td>2/88/M</td>
<td>338</td>
<td>Simplex</td>
<td>LP</td>
</tr>
<tr>
<td>3/76/F</td>
<td>378</td>
<td>Simplex</td>
<td>NLP</td>
</tr>
<tr>
<td>4/78/M</td>
<td>379</td>
<td>Simplex</td>
<td>NLP</td>
</tr>
<tr>
<td>5/68/M</td>
<td>180</td>
<td>Simplex</td>
<td>NLP</td>
</tr>
<tr>
<td>6/62/M</td>
<td>441</td>
<td>AD</td>
<td>NLP</td>
</tr>
<tr>
<td>7/89/F</td>
<td>364</td>
<td>AD</td>
<td>LP</td>
</tr>
<tr>
<td>8/73/M</td>
<td>242</td>
<td>XL</td>
<td>NLP</td>
</tr>
<tr>
<td>9/65/M</td>
<td>297</td>
<td>XL</td>
<td>LP</td>
</tr>
<tr>
<td>10/69/M</td>
<td>105</td>
<td>XL</td>
<td>NLP</td>
</tr>
<tr>
<td>11/81/M</td>
<td>402</td>
<td>AR</td>
<td>LP</td>
</tr>
</tbody>
</table>

FFB, Foundation Fighting Blindness; RP, retinitis pigmentosa; AD, autosomal dominant; AR, autosomal recessive; XL, X-linked; NLP, no light perception; LP, light perception.

RESULTS

Mean ages (in years) for the control, moderate, and severe groups were 74.27, 75.57, and 75.73, respectively. The mean ages for the control, autosomal dominant, X-linked, and simplex groups were 74.27, 79, 69, and 75 years, respectively. There were no statistically significant differences between mean ages by severity group ($P = 0.95$) or by genetic type ($P = 0.46$).

The mean postmortem intervals (in hours) for the moderate and severe groups were 17.4 ± 22.4 and 5.5 ± 3.5, respectively. The mean postmortem intervals for the autosomal dominant, X-linked, and simplex groups were 9.5 ± 10.9, 5.3 ± 1.3, and 13.3 ± 20.5 hours, respectively. There were no statistically significant differences between mean postmortem intervals by severity group ($P = 0.10$) or by inheritance type ($P = 0.75$). Postmortem intervals ranged from 1 to 15 hours for all control eyes. The mixed-effects model takes into account that several measurements are obtained from the same eye and that these measurements are expected to be correlated among themselves. The first-order autoregressive correlation structure simply assigns a higher correlation (or higher weight) to cell count measurements obtained at adjacent eccentricities and gives counts obtained from other eccentricities located farther away a weight that decreases exponentially with an increase in the distance from the original eccentricity.

Scheffe’s multiple comparisons tests were used to test for pair-wise differences in mean levels among the two severity groups and the control group and to examine pair-wise differences among the different inheritance types and the control group and between each other.

The percentage of nuclei preserved in each layer was determined with respect to the control group by dividing cell counts of each severity group or genetic type by the mean cell count of the control group at each eccentricity and for each cell layer. Differences in these percentages were also analyzed using mixed-effects models. ANOVAs were used to compare mean age differences and mean postmortem interval differences between severity groups and inheritance types.

Light Microscopy

Eyes were slit at the pars plana and placed in a fixative (0.5% to 4% glutaraldehyde, 0.5% to 4% paraformaldehyde) in 0.1 M phosphate buffer. Retinitis pigmentosa maculae and adjacent extramacular retinal areas were dissected; embedded in glycol methacrylate, epoxy resin, or ornithine carbamoyltransferase cryoprotectant; and sectioned at 1 to 2 µm. Sections were stained with Richardson’s methylene blue/azure II mixture. Control eyes were similarly sectioned and stained with either Richardson’s methylene blue/azure II mixture or hematoxylin and cosin. With a calibrated reticle, adjacent 125-µm-wide segments from each section were evaluated. The number of nuclei was counted within each cell layer (ONL, INL, and GCL). ONL counts were performed at eight 125-µm segments located 4.0 mm to 5.0 mm from the fovea or center of the macula. ONL counts were not systematically performed beyond 5.0 mm because they were uniformly zero in all RP eyes. INL and GCL counts were performed at 48 125-µm segments at 4.0 mm to 10.0 mm eccentricity from the fovea.

Statistical Analysis

Mixed-effects models with a first-order autoregressive correlation structure were used to determine whether the severity of RP or the RP inheritance type correlated with differences in the mean cell counts for each cell layer compared with the moderate RP group had visual acuities of 20/400 or better, whereas those in the severe RP group had light perception or worse vision. Extramacular sections (4 -10 mm from the fovea) from 18 postmortem RP eyes were examined by morphometry. Seven of the 18 eyes belonged to the moderate group and 11 to the severe group. For each eye, the patient’s age, sex, donor number, genetic type of RP, and visual acuity were recorded. Tables 1 and 2 show these data for the moderate and severe groups, respectively.

Retinitis pigmentosa inheritance types were simplex ($n = 8$), autosomal dominant ($ADRP; n = 6$), X-linked ($XLRP; n = 3$), and autosomal recessive ($n = 1$). Because only one eye from the autosomal recessive group was available, an analysis of this inheritance type was not possible. Control postmortem eyes with normal retinas ($n = 11$) were obtained from either the Wilmer Eye Pathology Laboratory (Baltimore, MD) or from the University of Washington RP Histopathology Laboratory (Seattle, WA). The control eyes were selected to match the age distribution of the RP eyes by decade.

There were no statistically significant differences between mean postmortem intervals by severity group ($P = 0.95$) or by genetic type ($P = 0.46$). The mean postmortem intervals for the autosomal dominant, X-linked, and simplex groups were 20/400 or better, whereas those in the severe RP group had light perception or worse vision.
FIGURE 1. (A) Extramacular region of retina of a normal 71-year-old male eye donor. A single layer of nuclei is retained in the ganglion cell layer; 3 to 4 layers of nuclei are present in the inner nuclear layer, and 5 to 6 layers of nuclei are found in the outer nuclear layer. (B) Extramacular region of retina of an 81-year-old male eye donor with severe retinitis pigmentosa. There is a single layer of nuclei in the ganglion cell layer, 2 to 3 layers of nuclei in the inner nuclear layer, and total loss of cells from the outer nuclear layer. The retinal pigment epithelium cells are flattened and atrophic in some areas and clumped in others. Magnification, ×400.

but two eyes, those with postmortem intervals of 31 and 63 hours. All mixed-model analyses described were repeated after excluding measurements for these two eyes, and all results remained virtually unchanged. Moreover, these two eyes had good retinal preservation, allowing accurate nuclear counts.

Figure 1A is a photomicrograph of the extramacular region of the retina from a control 71-year-old male eye donor. There is a single layer of nuclei in the GCL, 3 to 4 layers of nuclei in the INL, and 5 to 6 layers of nuclei in the ONL. There is a slight detachment of the outer segments, and some autolysis is apparent. Figure 1B is a photomicrograph of the extramacular region of the retina from an 81-year-old male eye donor with severe RP. There is a single layer of nuclei in the GCL, in which the nuclei counts are slightly diminished relative to counts from a normal eye. There are only 2 to 3 layers of nuclei in the INL and no nuclei in the ONL. Flattened and atrophic cells of the retinal pigment epithelium (RPE) are seen in some areas, whereas other areas show RPE cell clumping.

Figures 2 and 3 show the actual mean cell counts for the INL and GCL at each eccentricity level for the different severity and inheritance groups. These counts are superimposed on linear fits obtained by using the mixed-effects model for mean cell counts for the INL and GCL at each eccentricity level for the different severity and inheritance groups. A mixed-effects model revealed that the decrease in cell count with increasing eccentricity is statistically significant (∑ < 0.0001 for INL, GCL, and ONL), regardless of severity group or inheritance type. The model also showed an overall significant decrease in mean cell counts for each of the cell layers when the severity groups were compared to those of control group (∑ < 0.0001 for INL, GCL, and ONL). Mean cell counts for the moderate and severe groups, when averaged for all the eccentricities, were not significantly different (∑ < 0.14 for INL, ∑ < 0.17 for GCL; Fig. 2). Similarly, even at the eccentricities closest to the macula in which it appeared that there might be a difference between the moderate and severe groups, this was not the case. For example, at 4.0 mm eccentricity, the probability values were 0.36 and 0.59, respectively, when the INL and GCL of the moderate group was compared with those of the severe group.

Using the methodology described above, the percentage of retained nuclei was determined for each group. On average, 40% of the INL nuclei were preserved in the moderate and severe groups. A smaller percentage of GCL nuclei was preserved in the moderate and severe groups (i.e., 30% for the moderate group and 20% for the severe group). For the ONL, only slightly more than 3% of the nuclei were preserved for the moderate group at 4.0 mm eccentricity, dropping rapidly to near zero at 5.0 mm eccentricity, whereas no ONL nuclei were preserved at any of the eccentricities studied in the severe group.

Similarly, when the data were analyzed by inheritance type, mean nuclei counts for all inheritance groups were significantly lower than for the control group (∑ < 0.0001 for the INL, GCL, and ONL). Results from the INL counts for the...
different inheritance types of RP showed a higher overall mean percentage of cells preserved for the ADRP group compared with the other two groups ($P = 0.0032$ ADRP versus simplex; $P = 0.0033$ ADRP versus XLRP). Simplex and XLRP groups showed no difference ($P = 0.47$). GCL counts showed a difference between mean percent preservation in the ADRP versus XLRP ($P = 0.02$), but no differences were found between mean percent preservation for ADRP versus simplex RP, or simplex versus XLRP types ($P = 0.26$, and $P = 0.15$, respectively). Finally, the ONL had $2\%$ or fewer nuclei preserved. The mean percentages of ONL nuclei did not differ among the different groups: ADRP versus simplex RP, $P = 0.93$; ADRP versus XLRP, $P = 0.96$; and simplex versus XLRP, $P = 0.91$.

**DISCUSSION**

Morphometric analysis revealed some preservation of the inner retinal nuclei in extramacular regions of RP retinas, but the preservation of the INL and GCL was less than that found in the macular region. Consistent with the macular morphometric data, the INL of the extramacular regions showed greater preservation of nuclei than the GCL. The analysis of RP eyes by inheritance types showed that ADRP eyes had greater INL preservation than simplex or XLRP, and greater GCL preservation than XLRP.

The data from the extramacular regions in this study add to our published results from morphometric analysis of the maculas of the same retinas. Although some inner retinal cell preservation was found in the extramacular regions, it was less evident than in the macular region. This finding correlates well with our electrical stimulation data in blind RP patients. In our experience, to elicit a visual percept by electrical stimulation of the remaining inner retinal neurons, the extramacular regions have always had higher threshold current requirements than the macular regions.

There are several possibilities for the observed difference in the number of inner retinal nuclei between the macular and extramacular regions. First, all our patients had rod-cone RP in which the rods are affected earlier and more severely than cones. There are more rods than cones in the extramacular regions, and thus one would expect to see an earlier and more extensive effect in the extramacular regions. Second, the synaptic connections are more elaborate and widespread in the extramacular regions than in the macula. Loss of input from a similar-sized area in the extramacular regions could have a more widespread effect on the synaptic input to inner retinal neurons than found in the macula. Third, the difference could result from the progressive decrease in the numbers of inner retinal nuclei per unit area with increasing eccentricity from the fovea; loss of a few nuclei from an extramacular region,
Morphometric Analysis of Extramacular RP Retina

with fewer cells at baseline, could have a greater percentage effect than the same number of cells lost from the more densely populated macular region.

The greater preservation of the INL, than the GCL, in the extramacular regions is similar to our findings from the macular regions of the same eyes. Better preservation of the INL than the GCL may result from a number of possible causes. First, the GCL may be more affected by thickened and occluded retinal blood vessels, whereas the INL might continue to be supplied by the choroidal blood flow when ONL cells are decreased or absent. Second, Müller cells proliferate and hypertrophy in retinal degeneration, including retinal detachment, including retinal detachment and advanced RP. Because the proliferating Müller cells may no longer have the characteristic angular-appearing nuclei, they may have been included in the INL counts. Without specific Müller cell markers, this issue is difficult to resolve with absolute certainty, but we know from the retinal detachment studies that the hypertrophied Müller cell nuclei migrate toward the RPE and do not remain in the INL. Such migration of the Müller cell nuclei may remove them from INL counts.

Other than the aforementioned references, histologic analysis of retinal degeneration with attention to the preservation of inner retinal neurons is sparse for human retinas. More complete studies have been performed in the retinal degenerate (rd) mouse and the Royal College of Surgeons (RCS) rat. Results from these studies have been conflicting and have shown different outcomes, ranging from little inner retinal loss to loss of 20% or more of the inner retinal neurons. Moreover, in the RCS rat, new vessel growth has been noted around areas of RPE cell migration. In these areas, the new vessels dip toward the outer retina, and there is severe disorganization of the entire retina. Similar areas have been seen in some peripheral areas of human RP retinas (personal communication, Raymond Lund, 1998). We did see translocated RPE cells in the human RP retinas that had deposited thick layers of extracellular matrix, causing narrowing and occlusion of retinal vascular lumina. However, we did not find areas of new vessel growth as noted in the RCS rat retinas.

Inner retinal cell preservation despite near total photoreceptor loss is important for retinal therapies such as gene therapy, photoreceptor transplantation, and retinal microchip implantation. These approaches require a viable inner retina to relay visual information to the brain. The strategy behind gene therapy for photoreceptor rescue is the insertion of a normal allele into a cell carrying a known mutation in that gene. However, if this intervention is carried out after most inner retinal neurons have died, it will fail short of its goal. Similarly, inner retinal preservation is important for photoreceptor trans-
plants, which need to form synapses on inner retinal neurons, and for microchips designed to electrically stimulate these cells. The question remains: What number of viable inner retinal neurons is required for any of these techniques to restore useful vision? At this time, this question is impossible to answer. Some data relevant to the retinal chip approach are available from the cochlear implant literature. Cochlear implants function by a paradigm similar to that envisioned for the retinal microchips (i.e., by bypassing distal damaged or absent receptors and electrically stimulating more proximal neurons). Cochlear implants have been clinically successful in patients who have lost as much as 90% of spiral ganglion cells. In extramacular regions of RP retinas, the remaining inner retinal neurons, as a percentage of normal cell counts, far outnumber the percentage of spiral ganglion cells required for a cochlear implant to restore auditory function.

Last, morphometric analysis of extramacular retinas from the different inheritance groups showed that ADRP eyes had significantly greater preservation of INL nuclei than XLRP or simplex RP, and greater GCL preservation than eyes with XLRP. This finding is not consistent with morphometric analysis from the macular regions of the same eyes, which did not show a difference between ADRP, XLRP, or simplex RP for any of the three layers. Because the extramacular regions have earlier, more severe photoreceptor loss, transneuronal degeneration secondary to photoreceptor degeneration may be more pronounced than in the macula, in which photoreceptor loss usually occurs later in RP.

Acknowledgment

The authors thank W. R. Green, Eye Pathology Laboratory at the Wilmer Eye Institute at Johns Hopkins University School of Medicine, Baltimore, Maryland, for providing normal eyes.

References