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Drusenoid maculopathy in rhesus monkeys (*Macaca mulatta*): Effects of age and gender

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Abstract

Purpose—To compare drusenoid maculopathy in monkeys with human age-related macular degeneration and evaluate the influence of age, gender and caloric restriction.

Methods—Examination by indirect ophthalmoscopy, slit lamp biomicroscopy and fundus photography, including in some cases fluorescein angiography, was performed on 61 male and 60 female rhesus macaques of ages 10–39 years. Fifty-four of the monkeys were maintained on a calorically restricted diet (approximately 30% lower than control levels) and 67 on an approximately ad libitum diet for 2–19 years, with all other environmental factors held constant. Maculopathies were graded on a 5-point scale and the effects of age, sex, and diet on prevalence and severity were examined. The retinas of 6 monkeys with macular drusen, 19–28 years old, were examined histologically.

Results—Rhesus monkeys showed a high prevalence (61 %) of drusenoid maculopathy. The prevalence and severity of the maculopathy increased with age ($p=0.012$). Fully half of all monkeys aged 10–12 years had some detectable degree of drusen. This high prevalence in young adulthood indicates that drusen develop much earlier in rhesus monkeys than in humans, who develop early

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maculopathy most rapidly at 50-60 years of age, even when correcting for the 3-fold difference in lifespan. No neovascularization or geographic atrophy was found. Females had a higher prevalence and severity than males ($p=0.019$). Calorically restricted monkeys had a slightly lower prevalence and severity at 10-12 years than controls, but the difference was not statistically significant. This is an on-going project and differences between the caloric restricted and ad-lib groups may emerge as the animals age. Some monkeys developed severe maculopathy in their 20s with others unaffected in their 30s. The histology of drusen resembled those in human retina.

Conclusion—Drusenoid maculopathy is common in rhesus monkeys even in young adult life. Half of the rhesus monkeys examined have drusen at a much younger age than in humans. Severity of maculopathy was greater in female monkeys, a gender difference not consistently found in humans. No differences were detected due to caloric restriction, but a definitive test of this intervention will require a larger sample, longer period of observation, and/or an earlier institution of caloric restriction. Genetic factors are implied because with similar environments, some monkeys are affected at an early age, while older ones are not.

Keywords

macula; age-related macular degeneration; drusen; caloric restriction; diet; monkey

Introduction

Age-related macular degeneration is the leading cause of vision loss in the elderly. The macula is a structure unique to humans, apes, and monkeys, making nonhuman primates a potentially valuable model for the study of macular disease. Several studies have documented similar pathology, including drusen and pigmentary changes, in monkeys and baboons that resemble that found in humans [1-3,5-8,12,14,18,22,25-30]. However, there is also evidence that the diseases may be different. For example, proteins found in human drusen may be absent in monkey drusen [19], and some monkey drusen visible by ophthalmoscopy may actually be pigment epithelial cells filled with vacuoles and lipid droplets [7,8]. In addition, neovascularization, a defining complication of late-stage human age-related macular degeneration, is not found in monkeys except in rare cases [12], some with the added complication of myopia [25]. Therefore, further study is warranted to determine the relevance of the nonhuman primate maculopathy to the human disease.

Because this degeneration is age-related, factors that can reduce the effects of aging could influence its progression. The one proven means of counteracting aging is caloric restriction, a finding replicated in several species including yeast, flies, fish, dogs, rodents, and monkeys [17,24]. The effects of caloric restriction may be due to multiple tissue-dependent mechanisms, including reduced oxidative stress, lower glucose and insulin levels, increased DNA repair capacity, altered gene expression and lower metabolic rate. To test whether caloric restriction influences age-related maculopathy, we examined the retinas of rhesus monkeys that were maintained for 2-19 years on a calorically restricted diet (approximately 30% reduction in energy intake compared to control animals matched for gender, age, and initial body weight), and compared these findings with control animals on an approximate ad libitum diet. Some monkeys were euthanized for experimental purposes or due to declining health, and in these cases eyes were obtained to study macular pathology.

Materials and Methods

The study involved 121 rhesus monkeys (*Macaca mulatta*), 61 males and 60 females, maintained at the National Institutes of Health facility in Poolesville, Maryland or at the Oregon National Primate Research Center (ONPRC) in Beaverton, Oregon. Ages ranged from 10 to

39 years; a large fraction (64%) were 20 years of age or older, a relatively old age for monkeys. Table 1 provides details of the experimental groups. The mean lifespan of rhesus monkeys has been estimated to be 25 years, with a maximum lifespan of 40 years; thus, one year in the monkey's lifetime can be considered equivalent to approximately 3 human years [21]. All monkeys were part of a long-term study sponsored by the National Institute on Aging to evaluate the effects of caloric restriction on longevity, health, and a variety of indices of aging [17]. All monkeys were fed the same specially formulated diet enriched with extra vitamins and minerals. As previously described [13], a control group of 67 monkeys was fed two meals a day of approximately ad libitum amounts, while the calorie restricted group of 54 monkeys received the same diet but at amounts about 30% less compared to levels provided to monkeys of equivalent age and body weight. The range of exposure to calorie restriction ranged from 2 to 19 years. The diets were supplemented with limited amounts of fresh fruits or vegetables (10-14 kcal/day) and filtered drinking water was available at all times. Animals were housed indoors individually in visual contact with other monkeys in temperature controlled rooms with lights on for 12 hours per day. The dietary regimens and environments were similar at both sites.

Each monkey was examined by indirect ophthalmoscopy, slit lamp biomicroscopy and fundus photography and in selected cases, fluorescein angiography. Examinations were performed under anesthesia with ketamine (10 mg/kg IM) or telazol (2-4 mg/kg IM) or, for fluorescein angiography, with isoflurane (2-3% in 100% oxygen). The pupils were dilated with 1-3 drops each of 1% tropicamide and 2.5% phenylephrine hydrochloride. Drusen were identified as round whitish areas which presented either solitary or coalescing into compound structures comprising several neighboring drusen.

Based on ophthalmic examination and the review of color stereophotographs, the severity of the drusenoid maculopathy was graded into five categories: 0 = normal; 1 = 5 or fewer drusen; 2 = 6 to 15 drusen; 3 = more than 15 drusen and/or any large drusen (>200 microns in diameter); 4 = choroidal neovascularization or geographic atrophy. When the two eyes of an individual fell into different categories, the two grades were averaged to obtain a severity score.

Six monkeys were euthanized including 3 females, ages 19, 20 and 26 years, and 3 males, ages 23, 25 and 27 years. Five had been fed an approximately ad libitum diet, and one, the 20-year-old female, had been on a 30% calorically restricted diet for 3 years. The eyes were enucleated and fixed by immersion in 3 % glutaraldehyde or 4% paraformaldehyde in phosphate buffered saline. The globes were pierced at the equator, and 1 ml of fixative was infused to facilitate penetration of the fixative. After storage for several weeks in fixative, the eyes were washed in buffer and dissected with the aid of a surgical microscope. The anterior segment was removed and stored in buffer solution. The posterior segment was dissected into segments including the macula, the paramacula on both nasal and temporal sides of the macula, the perimacula, the superior retina, the inferior retina and the optic nerve head. In the current experiments, only the macular area was examined, and the other segments stored in buffer solution for future studies. The macula was identified by the retinal vascular pattern and a dark foveal zone. Its nasal border was formed near the edge of the optic nerve head. The macula was considered to have a diameter of about 6 mm centered at the fovea; it was cut into 2-3 × 5-6 mm rectangular segments, which were postfixed in 1% osmium tetroxide for 1 hour, dehydrated and embedded in epon. The segments were sectioned semi-serially and the sections stained by toluidine blue and examined by light microscopy. At the site of selected drusen, ultra-thin sections were post-stained with uranyl acetate and lead citrate and examined by electron microscopy (Zeiss EM 10C/CR); these results are being reported separately. All procedures were approved by the Institutional Animal Care and Use Committee of the ONPRC/Oregon Health and Science University and the Gerontology Research Center, NIA, and conformed to the NIH "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, amended 2002) and the U.S. Animal Welfare Act, as amended.

The effects of age, sex, and diet on the prevalence of maculopathy were examined by chi-square analyses. For these analyses the five drusen grades were reduced to three categories: grade 0 (no drusen), grade 1, and grades 2 and 3 combined. Grade 4 was excluded because no examples were found. The effects of age, sex, and diet on average severity scores were tested by 3-way ANOVA followed up for the age variable with pair-wise Bonferroni-Dunn post-hoc tests.

Results

Figure 1 shows fundus photographs of monkeys with relatively severe drusenoid maculopathies. Many roundish, white drusen are concentrated in and around the fovea. A relatively large soft druse is located directly in the fovea in Figure 1C. A fluorescein angiogram of this fundus revealed fluorescein staining but no leakage (Figure 2), indicating intact retinal epithelium over this relatively large druse. Histology of this druse showed a detachment of the retinal epithelial layer directly under the fovea, which extended about 300 microns and was elevated about 30 microns from Bruch's membrane (Figure 3). No degeneration of the photoreceptors was apparent, since the width of the outer nuclear layer was normal, although there was disorganization of the outer segments contacting the elevated epithelial layer. Several small drusen were seen adjacent to the large one. No obvious inflammatory cells were visible within or around these drusen. Figure 4 shows several drusen obtained from the monkey retinas shown in Figure 1. There was a great variety in size from relatively large drusen to some undetectable clinically (Figure 4E&F). All, however, had similar shapes with much bilateral symmetry, resembling "blebs" in the epithelial layer if viewed in three dimensions. Evidence of overt inflammation was absent in all of the drusen when examined by light microscopy but not when examined by electron microscopy, as reported in a companion paper [9].

Over half (61%) of the monkeys had some drusenoid maculopathy and 14% (17/121) had a moderately severe form, grade 2 or greater by our classification scheme. Figure 5 shows the prevalence of drusenoid retinopathy in different age groups among these monkeys and compares it to human data of maculopathy, which included subjects with and without vision loss [31,32]. Although the prevalence of maculopathy is similar, it develops at a younger age in monkeys than in man even when allowing for a 3:1 conversion factor for human:monkey age. As in humans, about 30% of the population does not develop maculopathy even in old age.

Figure 6 compares the prevalence of drusenoid maculopathy of any grade in monkeys within each diet $\times$ gender subgroup across three age categories. Prevalence increased with age, particularly for females ($p=0.032$). Figure 7 similarly illustrates the prevalence of moderately severe maculopathy (grade ≥ 2 by our criteria), which increased significantly with age for all groups combined ($p=0.049$). When average severity scores of the maculopathy (Figure 8) were examined by ANOVA, there was a significant increase with age ($p=0.003$ for main effect of age; the 17-23 and 24-39 year age categories both had significantly higher scores than the 10-16 year old group, $p=0.025$ and $p=0.002$, respectively). In addition, females had significantly higher scores than males ($p=0.012$). No overall effect of caloric restriction was found ($p=0.49$); calorically restricted females appeared to show a reduced severity compared to those on the control diet, but this difference was not statistically significant ($p=0.18$). Because of the large range in duration of caloric restriction, we examined its effect separately in the NIA cohorts that experienced a longer treatment duration (14-19 years). These subgroups also showed no significant effect of caloric restriction for all ages combined ($p=0.91$) or within each of the three age groups ($p=0.65$, 0.80 , and 0.67 for age groups 10-16, 17-23 and 24-39 years, respectively).

Discussion

We found a high prevalence of drusenoid maculopathy in rhesus monkeys. Over half (61%) of these monkeys had some evidence of maculopathy. This estimate resembles the prevalence obtained in several independent studies carried out on free-ranging colonies of rhesus monkeys at the Caribbean Primate Research Center in Puerto Rico: 50% [5], 75% [27] and 47% [12]. However, some studies of other rhesus populations, including many housed indoors, have obtained a lower prevalence ranging from 31% [18] to 6% [3,26]. These differences may be due to several factors such as exposure to sunlight versus indoor lighting, average age in the colonies, gender distribution, genetic differences among colonies, the methods of examination, and the criteria used to define maculopathy. There are also differences in prevalence in human studies of age-related macular degeneration. For example, the Chesapeake Bay Waterman, the Beaver Dam and the Blue Mountain studies [16] obtained prevalence values of 28, 30, and 28 %, respectively, in elderly subjects (ages 79, 75 and 85 years and over, respectively); whereas, the Rotterdam [32], Barbados [16] and Copenhagen [31] studies obtained values of 56, 50 and 45 %, respectively (ages 85, 80, 75 and over, respectively). The differences in these human studies are due mainly to the criteria used to determine the presence of maculopathy.

The most striking difference between the human and monkey maculopathies is the time scale during which they develop. The maculopathy in *Macaca mulatta* begins at a relatively early age with a prevalence of 50% by 10-12 years of age (equivalent to 30-36 human years). There are even reports of severe maculopathy in monkeys 1-3 years of age [6]. This differs from the time course of the human disease, which begins around 50 years of age and advances most rapidly after about 60 years of age. This difference appears to reflect a greater susceptibility for the disease in monkeys together with their faster rate of senescence. The rapid time course of the maculopathy must reflect differences in genetic factors between monkeys and man. That genetic factors play a role in this degeneration is supported by evidence that under similar environmental conditions some monkeys remain drusen free at old age, while younger ones can be severely affected.

A genetic factor appears to be involved in the drusenoid maculopathy of another species of macaque monkeys, *Macaca fascicularis*, the cynomolgus monkey [28,29]. In this species there is evidence for two different forms of the macular degeneration, one occurring in young monkeys at about age two and another manifest in older monkeys. The early onset maculopathy appears to have an autosomal dominant pattern of inheritance [29]. It would be useful to have quantitative data on the time course of both maculopathies in cynomolgus monkeys because of the rapidity at which the maculopathy develops in *Macaca mulatta*.

Despite other similarities to human maculopathy, we found no evidence of geographic atrophy or choroidal neovascularization in these monkeys. These relatively late events in the human form of the disease represent only a fraction of the cases. The relative scarcity of old monkeys to examine may explain the difficulty in detecting this late complication. Lack of progression to advanced stages may also be due to genetic differences and/or the absence of dietary and environmental factors, such as high fat diets and smoking.

In our sample, the severity of maculopathy in female monkeys was greater than in males. Others have also noted a higher prevalence of maculopathy in female monkeys [6,12]. However, a gender difference has not been strongly supported in most human studies [31] and was not found in *Macaca fascicularis* [29]. Further study of this gender-related factor is necessary. An interesting question addressed by our experiment is whether caloric restriction has any influence on the prevalence and severity of this age-related disease. Caloric restriction is the only proven strategy that can increase the lifespan of many animals [17,24], but its effect on longevity and the susceptibility to age-related disease in larger animals, including monkeys, is

unclear. In our study there is a suggestion that caloric restriction reduced the severity of the maculopathy in the more severely affected female monkeys, but this difference was not statistically significant. Attempts to follow-up on this trend will require a larger sample of monkeys and/or a longer period of study. In the current sample, there were monkeys exposed to calorie restriction ranging from 2-19 years, and with ages of induction ranging from 1-23 years of age. The evidence from our study that the maculopathy begins at a very early age in monkeys implies that in order to be optimally effective in reducing disease incidence, a calorically restricted diet should be started early in life. This is a long term project and differences between caloric restricted and ad-lib groups may appear as these animals age.

The light microscopy of the drusen found in these monkeys resembles that described for human age-related macular degeneration [10,23]. All the drusen encountered, regardless of their size, tended to have radial symmetry with a dome-like separation of the retinal epithelial layer from Bruch's membrane. In three dimensions, these structures must resemble a "bleb" in the epithelial layer, implying a similar force factor that breaks the attachment of the epithelial cell basal lamina to Bruch's membrane. In all of the drusen we examined, including the largest ones, the epithelial layer over the dome of the drusen was intact, and this was supported by the lack of fluorescein leakage into the subretinal space. There was also no evidence from light microscopy of inflammatory cells either within or around these drusen. However, we have found processes of macrophage-like cells in Bruch's membrane in close proximity to drusen when these same areas were examined by electron microscopy [9], confirming similar observations of others in human maculopathy [4,11,15,20].

In summary, drusenoid maculopathy is a common affliction of monkeys, which begins at a surprisingly early age. Its clinical course and histopathology are sufficiently similar to human age-related macular degeneration to offer a valuable animal model of this disease.

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Figure 1.

Figure 1 shows fundus photographs of the maculas of three monkeys. **b** and **c** are from both eyes of the same monkey. **a** and **d** are from different monkeys.

Figure 2.

Figure 2 is a fluorescein angiogram of the retina from the monkey, the macula of which is shown in Figure 1A. **a** is an early and **b** is a late stage in the angiogram. The foveal druse shows much staining but no leakage in the late stage angiogram.

Figure 3. Figure 3 shows a histological section through the foveal druse of Figures 1a and 2. The section is stained with toluidine blue.

Figure 4.

Figure 4 shows histological sections through drusen of different sizes. The sections shown in **e** and **f** are considered to be too small to be detected clinically. The sections are stained with toluidine blue.

Figure 5.

Figure 5 shows the prevalence of maculopathy at different ages in the monkey colony. The data are compared with independent Danish (31) and Dutch (32) studies of human subjects.

Figure 6.

Figure 6 shows the prevalence of drusenoid maculopathy of any grade within each diet $\times$ gender subgroup across three age categories. The number of monkeys in each subgroup is shown adjacent to the corresponding data point.

Figure 7.

Figure 7 shows the prevalence of moderate-to-severe drusenoid maculopathy (grade >2) within each diet $\times$ gender subgroup as a function of age. The number of monkeys in each subgroup is shown adjacent to the corresponding data point.

Figure 8.

Figure 8 compares the average drusen severity grade at different ages for each diet $\times$ gender subgroup. Drusen grade increased significantly with age ($p=0.003$) and was higher for females than for males ($p=0.012$). No difference was found due to caloric restriction.

Table 1
Numbers and characteristics of monkeys included in the study at each site

	Diets	n	Age	CR Duration
<u>Oregon groups</u>				
Young males	CR	6	10-11	3-5 years
	AL	6	11-12	
Old males	CR	3	26-29	5-6 years
	AL	5	24-28	
Young females	CR	5	11-13	4-5 years
	AL	5	12-13	
Old females	CR	9	18-25	2-3 years
	AL	9	18-25	
Total		48		
<u>Maryland groups</u>				
Middle-aged males	CR	14	19-24	18-19 years
	AL	17	19-24	
Old males	CR	4	34-38	18-19 years
	AL	7	33-39	
Middle-aged females	CR	11	15-21	14 years
	AL	15	14-21	
Old females	CR	1	32	14 years
	AL	4	26-33	
Total		73		

CR: Caloric restriction group; AL: Ad libitum control group