

# Age-Related Retinal Pigment Epithelium and Bruch's Membrane Degeneration in Senescence-Accelerated Mouse

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**PURPOSE.** To investigate age-related changes in the retinal pigment epithelium (RPE), Bruch's membrane, and choriocapillaris in the senescence-accelerated mouse (SAM).

**METHODS.** The external and eyecup features and light and electron microscopic findings were examined in three male and two female mice of a senescence-prone mouse strain (SAM P<sub>8</sub>) monthly for 12 months. These results were compared with those in age-matched mice of similar background but senescence resistant (SAM R<sub>1</sub>). Choroidal vascular casts were prepared at 12 months in seven mice each of the SAM P<sub>8</sub> and SAM R<sub>1</sub> strains. Quantitative analysis of area of choriocapillaris was performed by automated image analysis, and the results were analyzed by paired Student's *t*-test.

**RESULTS.** We found in the SAM P<sub>8</sub> strain that hair loss, coarseness of hair texture, and ulceration of skin appeared and increased as the age advanced (at approximately 5-9 months). Eyecup examination showed no differences. Light and electron microscopy revealed progressively more prominent abnormalities in the RPE and Bruch's membrane mice older than 10 months. Two of the five SAM P<sub>8</sub> mice older than 11 months showed what appeared to be intra-Bruch's membrane choroidal neovascularization. The RPE and Bruch's membrane appeared normal in the SAM R<sub>1</sub> strain. In the SAM P<sub>8</sub>, vascular casts of the choriocapillaris showed a mild but significant decrease in vascular area when compared with the SAM R<sub>1</sub> strain at 12 months ( $P = 0.011$ ).

**CONCLUSIONS.** Senescence accelerated mice develop progressive age-related changes in the RPE-Bruch's-choriocapillaris complex that have features that may be relevant in the study of age-related macular changes in humans. (*Invest Ophthalmol Vis Sci.* 2000;41:3936-3942)

Age-related macular degeneration (AMD) is a leading cause of blindness in Western society. Although incompletely understood, AMD is associated with a progressive degeneration of photoreceptors, retinal pigment epithelium (RPE) cell layer, and choriocapillaris. Drusen are a hallmark of the disease. Intra-Bruch's membrane neovascularization is rare (occurring in less than 10% of cases) but is often associated with severe visual loss. One severe limiting factor in studying AMD is the unavailability of a relevant animal model to test hypotheses related to cause and treatments. Most of the models developed to date have concentrated on the development of choroidal neovascularization, for example, by using laser photocoagulation in primates, rabbits, and rats or subretinal neovascularization in transgenic mice.<sup>1-4</sup> Also, changes in the choriocapillaris have been studied in animal models by inducing RPE atrophy by surgical or pharmacologic means.<sup>5-11</sup>

There are few reports available on animal models showing macular lesions associated with aging akin to human disease.<sup>12</sup> This is in part due to the limited number of animals that have macular differentiation and the cost of the animals that do. Another problem in studying age-related changes is the time that these changes take to develop. Although it is generally agreed that in AMD the macular changes are the most severe and significant, most agree that the RPE-Bruch's-choriocapillaris changes are not limited to the macular region. Drusen, choroidal neovascularization, and RPE atrophy occur outside the macular region. Because of these problems, the identification of a rapidly aging inexpensive animal in which a progressive age-related RPE-Bruch's-choriocapillaris degeneration (with occasional intra-Bruch's membrane choroidal neovascularization) develops could be useful in the study of AMD.<sup>12-16</sup> The senescence-accelerated mouse (SAM) has some of these characteristics.<sup>15-16</sup> It was developed from AKR/J mouse strains, and consists of nine accelerated senescence-prone (SAM P) strains and four senescence resistant (SAM R) strains. The SAM P strains have an early onset and more rapid advancement of the senescence feature after a normal process of development and a significant shorter life span than do the SAM R strains. They are shown to have senescence-associated systemic and ocular changes.<sup>17-34</sup> The pathologic phenotypes reported in the SAM strains are shown in Table 1.

In the past, the SAM P<sub>1</sub> strain was shown to exhibit changes only in the Bruch's membrane and RPE.<sup>29-31</sup> The SAM P<sub>2</sub> and SAM P<sub>3</sub> strains were studied but did not show any

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Supported by a Core Grant from the National Eye Institute to The Johns Hopkins University (PO1-EY01765).

Submitted for publication July 6, 1999; revised March 16 and June 21, 2000; accepted July 13, 2000.

Commercial relationships policy: N.

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TABLE 1. Pathological Phenotypes Reported in SAM Strains

Strain	Feature
SAM P strains	
P <sub>1</sub>	Senile amyloidosis, impaired immune response, hyperinflation of lungs, hearing impairment, cataract, corneal ulcer, periocular lesions, RPE changes, thickening of Bruch's membrane
P <sub>2</sub>	Senile and secondary amyloidosis, impaired immune responsiveness, periocular lesions, corneal infiltration
P <sub>3</sub>	Degenerative disease of temporomandibular joint, cataract, periocular lesions, corneal infiltration
P <sub>6</sub>	Senile osteoporosis
P <sub>7</sub>	Senile amyloidosis, thymoma
P <sub>8</sub>	Deficits in learning and memory
P <sub>9</sub>	Cataract, persistent hyaloid artery
P <sub>10</sub>	Deficits in learning and memory, brain atrophy
P <sub>11</sub>	Senile amyloidosis and contracted kidney
SAM R strains	
R <sub>1</sub>	Nonthymic lymphoma and histiocytic neoplasma ovarian cyst, periocular lesions
R <sub>3</sub>	Late-appearing cataract, periocular lesions
R <sub>4</sub>	Deficits in learning and memory, nonthymic lymphoma, histiocytic sarcoma
R <sub>5</sub>	Colitis

retinal changes.<sup>27</sup> SAM P<sub>6</sub> and SAM P<sub>9</sub> have a genetic background in common with the R-series mice, and they are not expected to have retinal changes.<sup>15,16</sup> We chose the SAM P<sub>8</sub> strain for this study based on the following features: SAM P<sub>8</sub> is among the senescence-prone strains that have shorter life spans,<sup>15,16</sup> and the mice have deficits in learning and memory but no brain atrophy.<sup>20</sup> It has also been demonstrated that there were periodic-acid Schiff (PAS)-positive granular structures in the brain of the SAM P<sub>8</sub> as well as behavioral changes.<sup>19,23</sup> However, little is known about the changes in the retina, RPE, and choroid of the SAM P<sub>8</sub>. In this study, we chose the SAM P<sub>8</sub> to evaluate the age-related systemic and histologic changes in the retina-RPE-Bruch's-choroidal vasculature and the SAM R<sub>1</sub> as the control.

## METHODS

The SAM-P<sub>8</sub> strain was studied between the ages of 1 and 12 months. The SAM R<sub>1</sub> strain served as a comparison. All procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Both SAM strains were obtained as breeding pairs from Takeda Chemical Industries (Osaka, Japan). All mice were bred

and housed in a microisolation facility with filtered air and controlled humidity. The cages and bedding material were sterilized before use. Biosafety cabinets and sterile techniques were used to keep the animals pathogen free. Light and temperature were controlled (68–72°F and with a light-dark cycle with lights on from 7:00 A.M. to 9:00 P.M.). The mice had access to food (supplied by PMI Nutrition, St. Louis, MO) ad libitum and water (pH 2.5) purified by a reverse osmosis system. SAM used in this present study were derived from this stock and maintained in the animal holding center of Johns Hopkins Hospital. Thus, designations for these strains of mice are officially SAM P<sub>8</sub>/Ta and SAM R<sub>1</sub>/Ta.

External photographs were taken monthly from 1 to 12 months. The grading system described by Hosokawa was followed.<sup>21</sup> General features such as skin glossiness, coarseness of hair, hair loss, skin ulceration, and lordokyphosis were evaluated. The presence or absence of each feature and time of first appearance were noted in the two strains.

Three male and two female mice were examined histologically at each month from 1 month to 12 months. The mice were sedated and killed with an overdose of intravenous pentobarbital. The eyes were enucleated and the eyecups were then immersed overnight in 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer at 4°C. The eyecups were photographed with a microscope (Carl Zeiss; Thornwood, NY) at ×10 magnification. The eyecups were then dissected into halves; one half was archived, and the second half was used for light and electron microscopy. At 12 months seven mice (four male and three female) of the SAM-P<sub>8</sub> series and seven mice (four male and three female) of the SAM-R<sub>1</sub> series were used for choroidal vascular cast preparation.

## Light and Electron Microscopy

One half the eyecups obtained for light and electron microscopy were postfixed for 2 hours in 2% osmium tetroxide in phosphate buffer, alcohol dehydrated, and embedded in epoxy resin. Two-micrometer-thick sections, stained with toluidine blue, were used for light microscopy evaluation. Thin sections were stained with lead citrate and uranyl acetate and examined by electron microscope (JEM-100 CX; JEOL, Tokyo, Japan). Changes in photoreceptors, RPE, Bruch's membrane, and choriocapillaris were examined in both the SAM-P<sub>8</sub> and the SAM R<sub>1</sub> series.

## Choroidal Vascular Casts

At 12 months, seven mice each of the SAM P<sub>8</sub> and SAM R<sub>1</sub> series were used to make choroidal vascular casts. These casts were then studied for analysis of the choriocapillaris surface area. The mice were anesthetized with intramuscular injection of 50 mg/kg body weight ketamine and xylazine. The left ventricle was perfused with 50 ml of heparinized lactated Ringer's solution. The mice were killed before the injection of Mercox solution (Ladd Research Industries, Burlington, VT) through the left ventricle. The eyes were enucleated, the anterior segment separated by microdissection, and corrosion casts made of the posterior segment in 0.1 M KOH. After complete bleaching of the tissues, the retinal vessels were separated from the choroidal vasculature by careful microdissection under water. Choroidal vascular casts were mounted on aluminum tubs (Ted Pella, Irvine, CA) and sputter coated with gold palladium before scanning electron microscopy

(JSM-840A; JEOL). Three random areas in the posterior pole were recorded in each eye at  $\times 400$  magnification for quantitative analysis of the choroidal vascular bed.

An image analysis program (Microplan II; Laboratory Computer Systems, Cambridge, MA) was used for measuring the area of the choroidal vascular bed in each  $\times 400$  micrograph by tracing the area of the choriocapillaris. The resultant values were tabulated and analyzed by paired Student's *t*-test.  $P < 0.05$  was regarded as significant.

## RESULTS

### External Features

Mice in both the SAM R<sub>1</sub> and SAM P<sub>8</sub> series appeared similar up to 4 months in general features and activity. Skin changes (coarseness and hair loss) appeared at 5 months of age in the SAM P<sub>8</sub> series mice, frank skin ulceration appeared at 9 months of age, and lordokyphosis at 11 months. Skin ulceration mostly appeared around the face, hind limbs, and back part of the body in this series. Mice in the SAM R<sub>1</sub> series maintained good skin luster until the end of the study period (Figs. 1A, 1B, 1C). The mice in the SAM P<sub>8</sub> group showed a decrease in general activity after the age of 9 months in comparison with those in the SAM R<sub>1</sub> group.

### Gross Examination of Eyecups

The eyecups were examined at  $\times 10$  magnification at each time point of death. The eyecups were transparent because of the absence of melanin pigment, resulting in poor contrast. Retinal vessels appeared similar in both the groups at all time intervals. No gross changes such as drusen were observed.

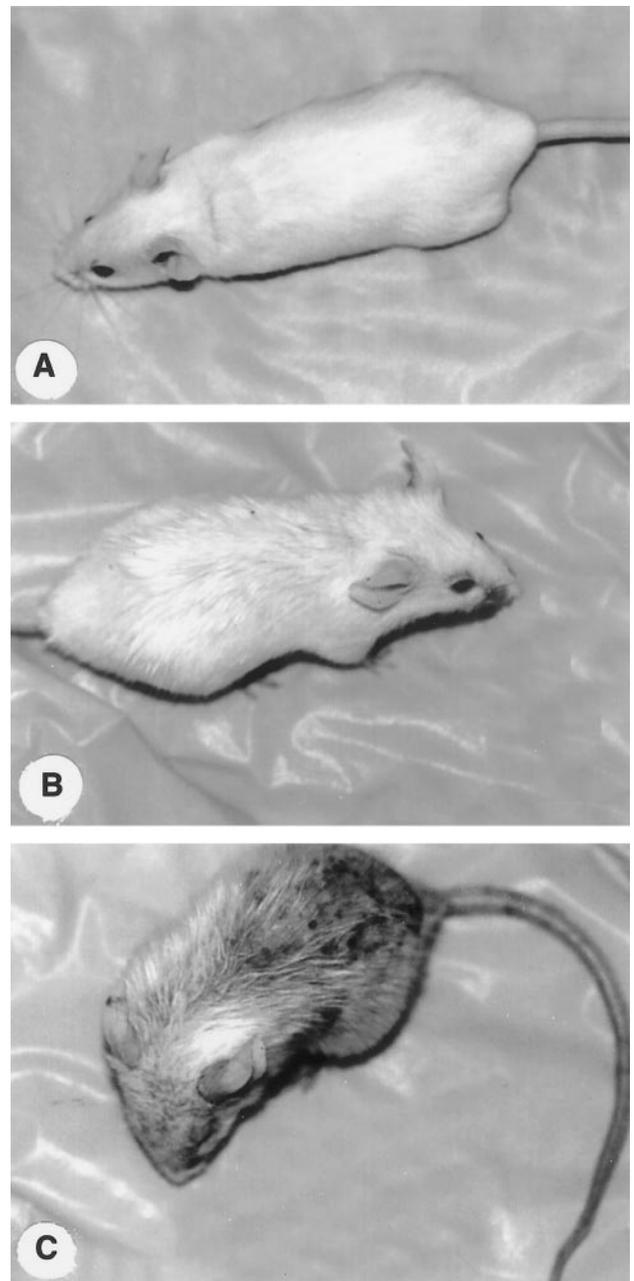
### Light Microscopy

In the SAM P<sub>8</sub> series, light microscopy revealed changes mainly in the RPE and Bruch's membrane. In the initial months the retinal photoreceptors, RPE, Bruch's membrane, and choriocapillaris appeared normal and were comparable to those in mice aged 12 months in the SAM R<sub>1</sub> series. In the SAM P<sub>8</sub> series mice older than 10 months, progressively severe changes appeared in the RPE-Bruch's membrane complex. The changes in Bruch's membrane were mainly in the form of localized areas of thickening on the RPE side, and in addition there was a uniformly increased thickness of Bruch's membrane in the other areas. The RPE cells showed some cellular variation in thickness and atrophy and appearance of increased numbers of lipoidal degeneration cells. The choriocapillaris did not appear abnormal on light microscopy at 12 months. The photoreceptor layer did not show any significant abnormality over the areas of abnormal RPE cells (Fig. 2A, 2B, 2C).

In SAM R<sub>1</sub> series eyes light microscopy did not show any photoreceptor, RPE, Bruch's membrane, or choriocapillaris abnormalities during the study period (Fig. 2D).

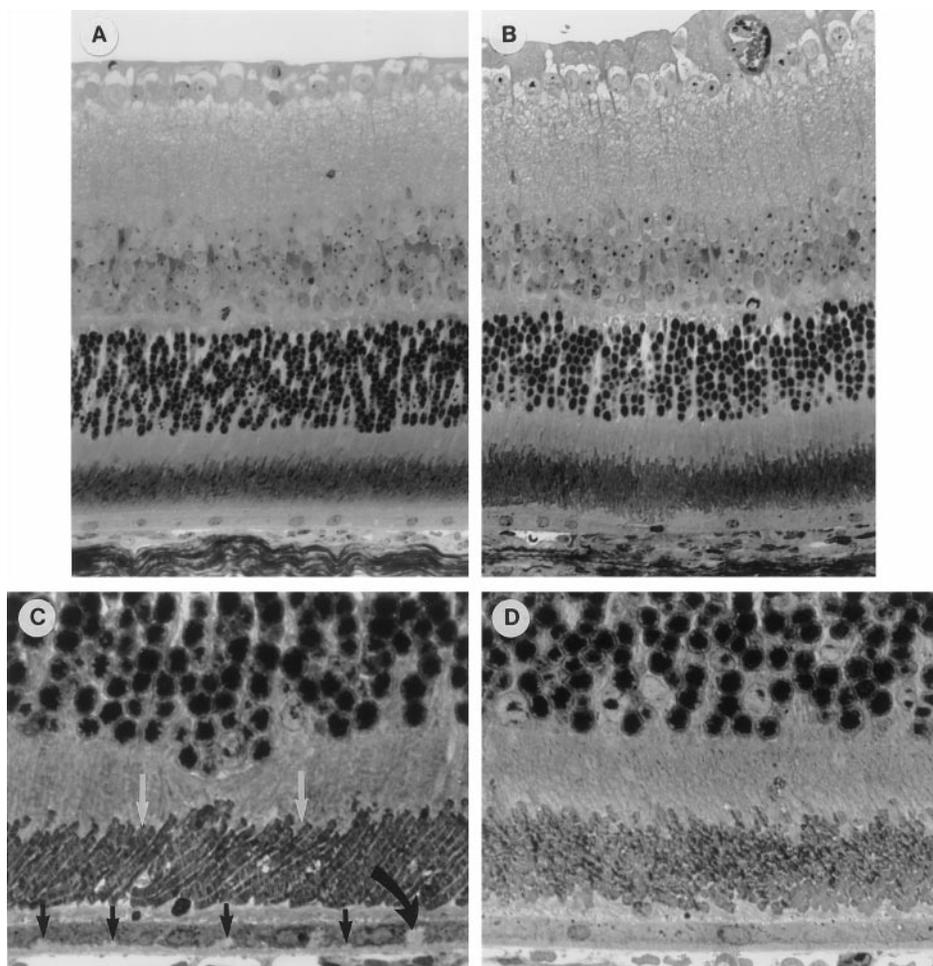
### Electron Microscopy

Electron microscopy at 2 months revealed normal photoreceptors, RPE, Bruch's membrane, and choriocapillaris in SAM P<sub>8</sub> series mice (Fig. 3A). Early disruption of basal microvilli of RPE could be seen by 8 months of age (Fig. 3B). Severe changes appeared in the RPE at 12 months (Fig. 3C), with marked disruption of basal microvilli and derangement of cellular com-



**FIGURE 1.** Comparative external photographs showing skin changes. (A) SAM R<sub>1</sub> series at 9 months. SAM P<sub>8</sub> series showing (B) loss of hair and lack-luster skin at 5 months and (C) skin ulceration at 9 months.

ponents. Bruch's membrane showed uniformly increased thickness in mice aged more than 8 months (Fig. 3F). Bruch's membrane measured three to four times more in thickness in the SAM P<sub>8</sub> series after 8 months of age when compared with mice of the same age group in the SAM R<sub>1</sub> series. We also observed localized areas of increased thickness in Bruch's membrane and abnormal deposits of amorphous material in the sub-RPE space, similar to the basal laminar deposits of human AMD (Figs. 3H, 3I). The deposits were localized to the RPE side of Bruch's membrane. At 11 months, there were fingerlike extensions of the choriocapillaris into the thickened Bruch's membrane with amorphous material surrounding such extensions. Intra-Bruch's membrane extensions were seen in



**FIGURE 2.** Light microscopy photomicrographs showing normal-appearing photoreceptors, RPE, Bruch's membrane, and choriocapillaris in (A) a SAM P<sub>8</sub> eye at 2 months and (D) a SAM R<sub>1</sub> eye at 12 months. (B) SAM P<sub>8</sub> eyes at 6 months showed minimal RPE changes. (C) At 12 months, the SAM P<sub>8</sub> series eyes showed increased thickness and localized thickening in Bruch's membrane (*black arrows*) an increased number of degenerated cells in RPE (*curved black arrows*). The photoreceptor layer appeared normal (*white arrows*). Magnification, (A, B)  $\times 400$ ; (C, D)  $\times 1000$ .

two of five eyes examined in the SAM P<sub>8</sub> series after month 11 (Figs. 3D, 3E). Intra-Bruch's membrane neovascularization assumed different shapes as shown in Figures 3D and 3E. These extensions showed no lumen (Fig. 3D) or very narrow lumens (Fig. 3E). The RPE did not show any abnormality over the areas with intra-Bruch's membrane vascular extensions. The photoreceptors in this model did not show any degenerative changes during the period of the study.

The photoreceptors, RPE, and choriocapillaris in the SAM R<sub>1</sub> series mice did not show any abnormality up to 12 months (Fig. 3B). Bruch's membrane showed minimal increase in thickness with age (Fig. 3G) when compared with the SAM P<sub>8</sub> series mice of the same age (Fig. 3F).

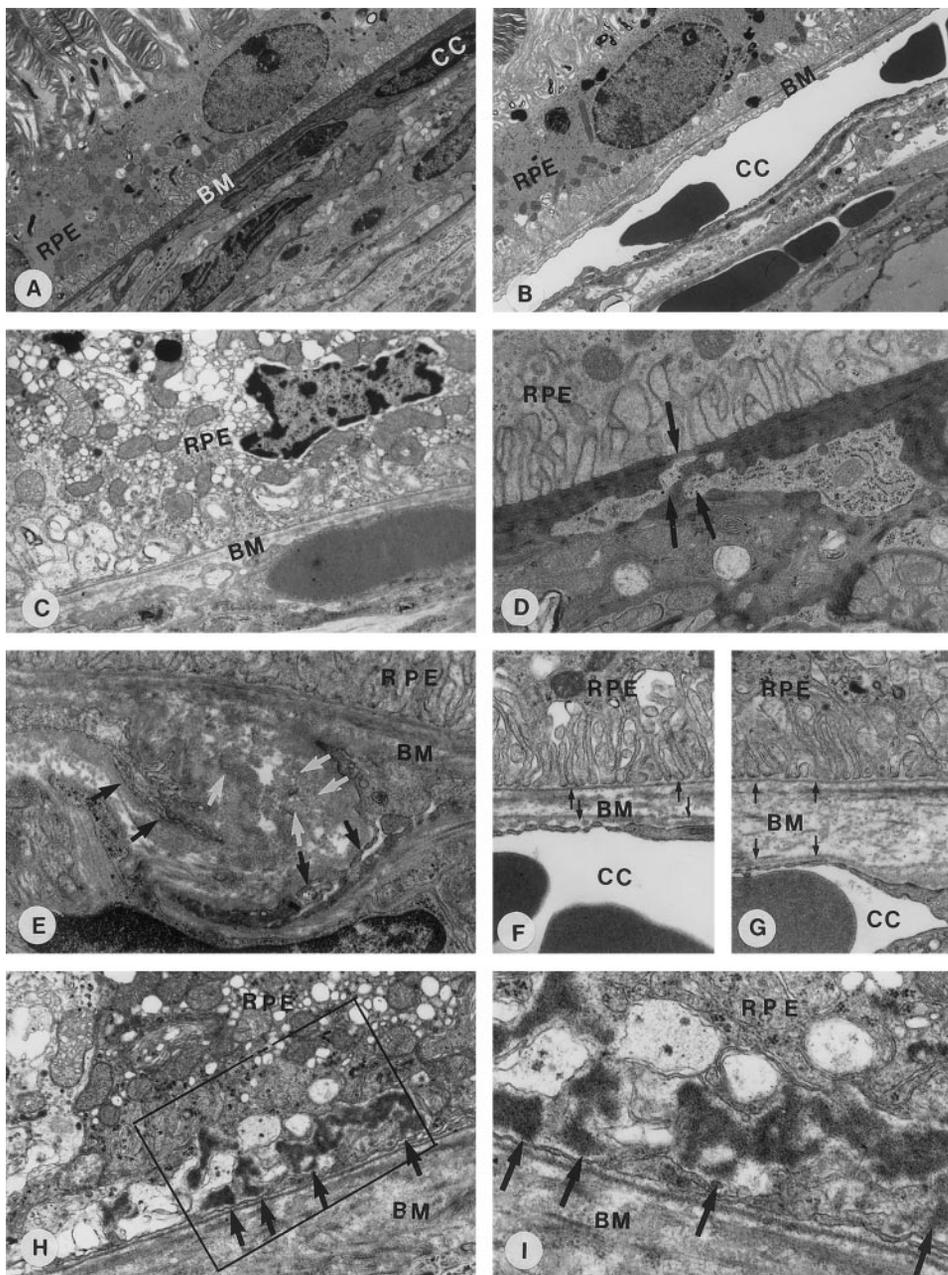
**Scanning Electron Microscopy of Choroidal Vascular Casts.** The choroidal vascular casts of SAM R<sub>1</sub> series at 12 months showed uniformly distributed choriocapillaris in the posterior pole as well as the equatorial region, no grossly atrophic areas were found (Figs. 4A, 4C). The choriocapillaris in the SAM P<sub>8</sub> series at 12 months showed areas of atrophy with reduced density of the choriocapillaris. There was no visible difference between the posterior pole and the equator (Figs. 4B, 4D).

**Quantitative Analysis of the Choroid Vascular Casts.** Analysis showed a mean area of choriocapillaris in the SAM R<sub>1</sub> series at 12 months of  $0.0562 \pm 0.002 \text{ mm}^2 \text{ mm}$  ( $n = 14$ ) and in the SAM P<sub>8</sub> series at 12 months  $0.054 \pm 0.0038 \text{ mm}^2$  ( $n = 14$ ). The mean area of choriocapillaris was minimally reduced

in the SAM P<sub>8</sub> series when compared with the SAM R<sub>1</sub> series ( $P = 0.011$ ).

## DISCUSSION

AMD is of multifactorial origin with both environmental and genetic factors contributing to its onset. The complexity of the disease has hampered the development of relevant animal models to test environmental and genetic causative factors. Also of importance, there is no good model to obtain reasonable preclinical data on the efficacy of a variety of treatments to prevent the changes associated with macular degeneration. The SAM P<sub>8</sub> strain had certain features that may make it relevant to understanding and potentially preventing several of the changes that are associated with AMD. The mouse had a progressive and age-related degeneration of the RPE-Bruch's-choriocapillaris complex. This degeneration had certain characteristics that resemble early stages of AMD. There was a degeneration and loss of RPE as evidenced by the presence of cell that had lost most of their intracellular details. There were abnormal deposits of amorphous material (resembling basal laminar deposits) in the sub-RPE space, some with a microfibrillar structure. There was a progressive and dramatic (three- to fourfold) thickening of Bruch's membrane. There was a mild but significant atrophy of the choriocapillaris. Finally, in two of five animals examined after 11 months we found vascular

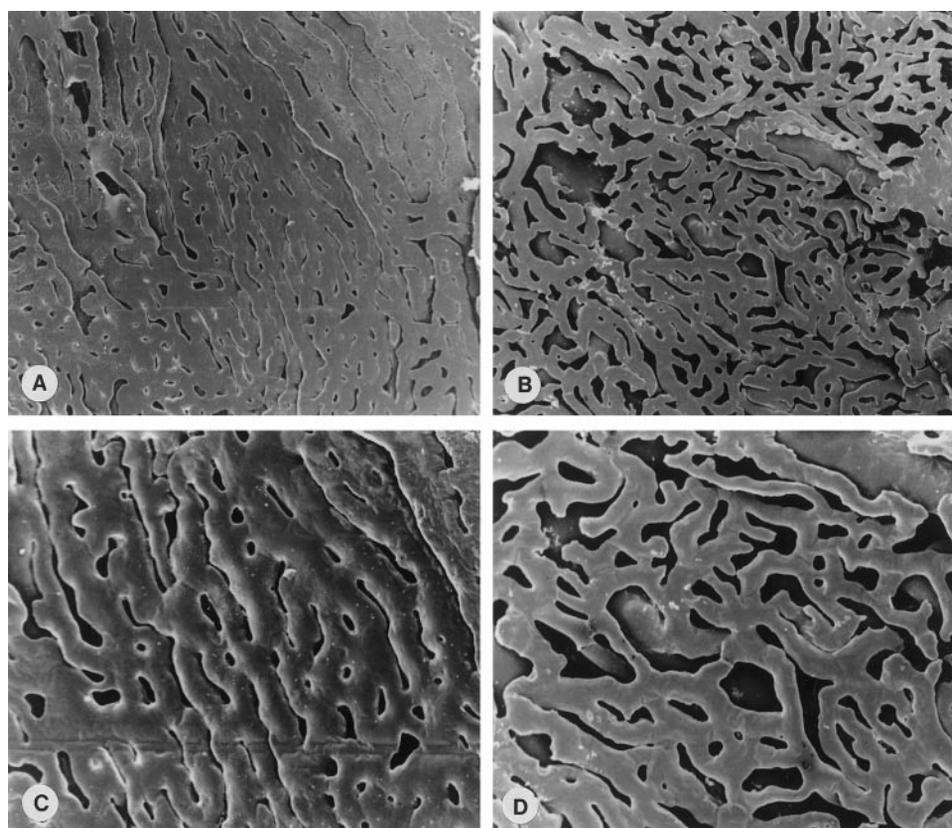


**FIGURE 3.** Electron photomicrographs show normal-appearing photoreceptor (PR) outer segments, RPE, Bruch's membrane (BM), and choriocapillaris (CC) in (A) a SAM P<sub>8</sub> series eye at 2 months and a SAM R<sub>1</sub> series eye at 12 months (B). At 12 months, SAM P<sub>8</sub> eyes (C) showed severe RPE changes in some areas. Different forms of intra-Bruch's invasion of vessels (D; black arrows) and thickened Bruch's membrane due to deposition of amorphous material (E; white arrows) observed in a SAM P<sub>8</sub> series eyes in two mice more than 8 months of age. Compare thickness of Bruch's membrane in (F) SAM P<sub>8</sub> and (G) SAM R<sub>1</sub> mice at 12 months. Localized thickening of Bruch's membrane appeared on the RPE side (H, I; arrows) in an SAM P<sub>8</sub> eye. Original magnification, (A, B)  $\times 3,375$ ; (C)  $\times 6,250$ ; (D through G)  $\times 12,500$ ; (H)  $\times 16,000$ ; (I)  $\times 40,000$ .

invasion of the thickened Bruch's membrane. Although no choroidal neovascularization was evident in the vascular cast studies, this may have been due either to the method of vascular casting or simply to the rarity of the neovascularization (similar to human disease). The accelerated aging is an additional advantage, shortening the time that experiments must take. Because there are multiple strains of the SAM with genetically similar control animals, comparison experiments are possible.

Human AMD is mainly classified as the dry or wet disease type.<sup>35</sup> The dry form of the disease constitutes abnormalities in the RPE and Bruch's membrane. The neovascular activity in AMD has been successfully produced in experimental animals.<sup>5-11</sup> Conclusions of such studies tend not to be typical of the choroidal neovascularization seen in AMD and should be tested in other animal models.<sup>36</sup>

The SAM model is naturally occurring, with different substrains manifesting a spectrum of diseases associated with senescence (Table 1).<sup>13-34</sup> Light microscopy (Fig. 2C) and electron microscopy (Figs. 3H, 3I) showed deposits with characteristics similar to basal laminar material seen in AMD.<sup>37</sup> RPE and Bruch's membrane changes observed in our study have also been reported in several studies.<sup>29-34</sup> They have shown swelling of basal infoldings, extension of intercellular space, and accumulation of lipofuscin granules in RPE. Bruch's membrane was shown to have discontinuation of elastic layer and abnormal increase in fine fibrils in the outer collagenous layer. They also showed increased staining of type IV and anti-heparin sulfate proteoglycan (HSPG) antibodies. In our study we observed localized bumps in the Bruch's membrane (akin to basal laminar deposits found in human AMD) as well as a generalized increase in the thickness of Bruch's membrane in the



**FIGURE 4.** Choroidal vascular casts showing visible atrophy of choriocapillaris in (B, D) SAM P<sub>8</sub> series eyes compared with (A, C) SAM R<sub>1</sub> series eyes. Magnification, (A, B)  $\times 200$ ; (C, D)  $\times 400$ .

SAM model. Bruch's membrane increased in thickness by three- to fourfold in the SAM P<sub>8</sub> compared with the SAM R<sub>1</sub> strain. This may be an indication of generalized degenerative changes in the choroid-RPE complex. We did not observe any photoreceptor changes in these mice during the study period.

A surprising and potentially important observation made in this study is intra-Bruch's membrane neovascularization (Figs. 3D, 3E; akin to the early wet form of AMD). We have observed this in more than two of five eyes at ages of more than 11 months in the SAM P<sub>8</sub> series. Intra-Bruch's membrane neovascularization observed in this model is similar to that described in human cadaveric eyes.<sup>38-45</sup> Although intra-Bruch's membrane neovascularization was observed on histology, choroidal vascular casts failed to demonstrate similar findings. This may have been because the lumen was absent or very narrow in the vascular extensions or because the Mercor solution we used for the vascular casts was thick and failed to enter the lumens. Intra-Bruch's membrane neovascularization is a significant finding in this model and, if common, would be helpful in developing studies to better understand the natural course of subretinal neovascularization.

The choroidal vascular cast preparations showed significant atrophy of the choriocapillaris in the SAM P<sub>8</sub> series compared with the SAM R<sub>1</sub> series at 12 months. Studies have shown severe choriocapillaris atrophy in eyes with geographic atrophy in humans (Gerard Luty, unpublished results, March 1998). Retinal degeneration animals (*rd* mouse, RCS rats) also show choriocapillaris atrophy. This finding is consistent with AMD, but its importance is unknown.

Unlike previous models of RPE atrophy with choriocapillaris degeneration by pharmacological and surgical means, this model is a naturally progressive degeneration with the changes

that are associated with senescence, simulating the human disease.

### Acknowledgments

The authors thank Takeda Chemical Industries, Ltd., Osaka, Japan, for the animals used in the study.

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