Ceruloplasmin/Hephaestin Knockout Mice Model Morphologic and Molecular Features of AMD

Majda Hadziabmetovic,1 Tzvet Dencchev,1 Ying Song,1 Nadine Haddad,1 Ximing He,1 Paul Habn,1 Domenico Pratico,2 Rong Wen,2 Z. Leah Harris,4 John D. Lambris,5 John Beard,6 and Joshua L. Dunaief1

PURPOSE. Iron is an essential element in human metabolism but also is a potent generator of oxidative damage with levels that increase with age. Several studies suggest that iron accumulation may be a factor in age-related macular degeneration (AMD). In prior studies, both iron overload and features of AMD were identified in mice deficient in the ferrooxidase ceruloplasmin (Cp) and its homologue hephaestin (Heph) (double knockout, DKO). In this study, the location and timing of iron accumulation, the rate and reproducibility of retinal degeneration, and the roles of oxidative stress and complement activation were determined.

METHODS. Morphologic analysis and histochemical iron detection by Perls' staining was performed on retina sections from DKO and control mice. Immunofluorescence and immunohistochemistry were performed with antibodies detecting activated complement factor C3, transferrin receptor, L-ferritin, and macrophages. Tissue iron levels were measured by atomic absorption spectrophotometry. Isoprostane F2α-VI, a specific marker of oxidative stress, was quantified in the tissue by gas chromatography/mass spectrometry.

RESULTS. DKO exhibited highly reproducible age-dependent iron overload, which plateaued at 6 months of age, with subsequent progressive retinal degeneration continuing to at least 12 months. The degeneration shared some features of AMD, including RPE hypertrophy and hyperplasia, photoreceptor degeneration, subretinal neovascularization, RPE lipofuscin accumulation, oxidative stress, and complement activation.

CONCLUSIONS. DKO mice have age-dependent iron accumulation followed by retinal degeneration modeling some of the morphologic and molecular features of AMD. Therefore, these mice are a good platform on which to test therapeutic agents for AMD, such as antioxidants, iron chelators, and antiangiogenic agents. (Invest Ophthalmol Vis Sci. 2008;49:2728-2736) DOI:10.1167/iovs.07-1472

Iron is a trace element that functions as a component of many proteins and enzymes, including oxygen-carrying proteins hemoglobin and myoglobin, cytochromes, and other enzymes that are involved in oxidation or reduction. Although iron is an essential metabolic component, it is also a potent generator of damaging free radicals that can cause oxidative stress. Regulation of ferrous iron levels is critical for meeting physiologic demand while preventing the toxicity associated with iron overload.1

Iron absorption in the intestine generally exceeds iron elimination from the body, leading to an age-dependent increase in iron levels in many tissues, including the retina.2 With aging, the levels of serum ferritin, a measure of total body stores, increase.3 The age-associated increase in iron may contribute to age-related degenerative diseases.

Iron is absorbed in the intestine and then delivered to target tissues by the plasma iron transport protein transferrin (Tf). Transferrin-iron complexes bind to transferrin receptor (TIR)-1 on the cell membrane and undergo internalization by receptor-mediated endocytosis.4 Iron is then used for cellular metabolism, and excess iron is stored in the storage protein ferritin. Intracellular levels of ferritin are posttranscriptionally controlled by the iron regulatory proteins IRP-1 and -2. Increased iron leads to increased cytosolic ferritin mRNA translation, which in turn increases ferritin levels.5

Iron export from the cells is facilitated by ceruloplasmin (Cp), a multicopper ferrooxidase. Cp oxidizes ferrous to ferric iron, the only form that can be taken up by the serum transport protein transferrin.6,7 Heph (Heph) is another multicopper ferroxidase with 50% identity to Cp that also facilitates iron export.8 Heph is naturally mutated in sex-linked anemia (Sla) mice.9

Accumulation of body iron stores may be important in both aging and age-related diseases, including retinal diseases.10 Patients with the disease aceruloplasminemia, a hereditary deficiency in Cp, have defective export of iron from some tissues including the retina,11,12 resulting in macular degeneration beginning in the fourth decade.13 Previously, we have found that AMD-affected maculas have significantly increased total iron concentration,14 compared with age-matched controls, suggesting that iron accumulation may play a role in this disease.

Age-related macular degeneration (AMD) is the most common cause of vision loss in the United States and other developed nations among people 65 years of age and older.15 Although the pathogenesis of AMD is incompletely understood, evidence suggests that oxidative stress and inflammation may mediate or exacerbate macular degeneration.1 Because iron overload has been implicated in age-related neurodegenerative diseases such as Alzheimer's and Parkinson's disease,16,17 and also in the macular degeneration occurring in patients with the rare autosomal recessive disease aceruloplasminemia,1 we investigated iron's contribution to AMD.

From the 1F. M. Kirby Center for Molecular Ophthalmology, Scheie Eye Institute, and the 2Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, Pennsylvania; the 3Department of Pharmacology, Temple University, Philadelphia, Pennsylvania; the 4Department of Ophthalmology, Bascom Palmer Eye Institute, University of Miami, Miami, Florida; the 5Department of Pediatric Anesthesia and Critical Care, The Johns Hopkins School of Medicine, Baltimore, Maryland; and the 6Department of Nutrition, College of Health and Human Development, Pennsylvania State University, University Park, Pennsylvania.

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Corresponding author: Joshua L. Dunaief, 305 Stellar-Chance Labs, 422 Curie Boulevard, Philadelphia, PA, 19104; jldunaief@mail.med.upenn.edu.

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To test the hypothesis that deficiency in both Cp and Heph would induce retinal iron overload and neurodegeneration, we generated mice harboring both the Cp knockout allele and a Heph mutation from Sla mice and refer to them herein as double knockout (DKO). Previously, we found that combined deficiency in Cp and Heph results in retinal iron accumulation with increases in several forms of the iron storage protein ferritin, and ultimately, retinal degeneration. This degeneration shares many features with AMD, including photoreceptor and RPE death, sub-RPE deposits, and subretinal neovascularization. The viability of the DKO’s is limited by an age-dependent movement disorder, and our initial report on these mice was limited in number and age (9 months and younger). We now report details of retinal degeneration in a study of a larger number of DKO’s, including some that lived to 13 months of age—to date, the longest-lived DKO’s. In the present study, we performed a detailed analysis of the rate of iron accumulation and found that iron levels in the retina and RPE/choroid continued to increase until 6 months of age, followed by progressive retinal degeneration. Further, we provide evidence that oxidative stress and activation of the complement cascade may be involved in the retinal degeneration. Finally, we report iron accumulation and elevated ferritin in the DKO ciliary body.

**Materials and Methods**

**Animals**

C57BL/6 wild-type mice, C57BL/6 mice with a targeted mutation in the Cp gene (Cp<sup>−/−</sup>), and naturally occurring Sla mutation in the Heph gene (Heph<sup>−/−</sup> or Heph<sup>+/−</sup>) were used. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania and complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

The eyes were enucleated immediately after the death of the animal or were either fixed overnight in 2% paraformaldehyde (PFA) and 2% glutaraldehyde for histochemical iron detection and morphologic analysis or were fixed in 2% paraformaldehyde (PFA) for immunohistochemistry. For morphology, n = 6 for mice younger than 6 months, n = 15 for mice 6 to 9 months, and n = 4 for mice 12 to 13 months old.

**Quantitative Iron Detection**

After enucleation, the eyes from wild-type and DKO mice were fixed in 4% PFA for several days. Eyecups were made by removing the anterior segment. The ciliary body was removed with a curved scalpel blade, and the neurosensory retina was then detached from the underlying RPE/choroid tissue, taking care to minimize disruption of the RPE. The retina and RPE/choroid (with sclera) were placed in separate tubes, and dried for 5 days at room temperature. Iron in these tissues was measured by graphite furnace atomic absorption spectrophotometry (model 5100 AA; Perkin Elmer, Boston, MA), according to standard methods.

**Isoprostane Quantification**

For biochemical analysis of isoprostane F<sub>2α</sub>VI levels, a chemically stable, specific marker of oxidative stress, mice were killed, and the retinas were isolated immediately and collected on dry ice. The retinas were homogenized, and total lipids were extracted as previously published. After mass spectrometry, quantification was performed by using the peak area ratio. Statistical Analysis

The mean ± SE of iron levels in retina and RPE/choroid were calculated for wild-type and knockout groups. The means between wild-type and knockout mice of the same age were compared using the two-tailed t-test. P < 0.05 was considered to be statistically significant. All statistical analysis was performed with commercial software (SAS, ver. 9.1; SAS Institute, Inc., Cary, NC).

**Immunofluorescence and Immunohistochemistry**

The fixed globes were rinsed in PBS and the eyecups were dissected. The eyecups were cryoprotected overnight in 30% sucrose and embedded in optimal cutting temperature compound (OCT; Tissue-Tek; Sakura Finetek, Torrance, CA). Immunofluorescence was performed on cryosections 10 μm thick, as previously published. Primary antibodies used were rat anti-mouse C3b/C3b/C3c (1:10 dilution; the generous gift of John Lambris, University of Pennsylvania, Philadelphia), which detects the cleavage products of complement factor C3; rabbit anti-human TfR antibodies (1:100; Zymed Laboratories, Inc., San Francisco, CA); rabbit anti-light ferritin (F17) antibodies (1:2500; the kind gift of Paolo Santambrogio and Paolo Arosio, IRCCS, Milan, Italy), rat anti-mouse F4/80 (1:50 dilution; Serotec, Martinsried, Germany). Primary antibody reactivity was detected with fluorophore-labeled secondary antibodies (Jackson ImmunolResearch Laboratories, Inc., West Grove, PA). The control sections were treated identically but with omission of the primary antibody. The sections were analyzed by fluorescence microscopy with identical exposure parameters. Immunohistochemistry was performed according to the manufacturer’s instructions (Vectastain ABC-AP Kit; Vector Laboratories, Inc.). The primary antibodies were rat anti-mouse CD11b (1:100 dilution; BD Biosciences, San Jose, CA). Chromogenic visualization was performed using a biotinylated polyclonal anti-rat IgG, together with the BCIP/NBT detection system (Vector Laboratories, Inc.) for 15 minutes. The control sections were treated identically but with omission of the primary antibody. The sections were analyzed by bright-field microscopy.

**Fluorescent Blood Vessel Labeling**

Blood vessels were directly labeled with a solution containing a fluorescent carbocyanine dye DiI (1,1-dioctadecyl-3,3,3’,3’-tetramethyl-indocarbocyanine perchlorate; Sigma-Aldrich), as described by Zhao et al. The mice were killed and perfused with PBS (4–5 mL), followed by a solution containing 160 mM DiI (4–5 mL). They were subsequently perfused with 4% paraformaldehyde (20 mL in 0.1 M phosphate buffer; pH 7.4), and the eyes were harvested and the anterior segments removed. The eyecups were examined with a confocal microscope (Stemi SV11; Carl Zeiss Meditec, Dublin, CA) and imaged with a digital camera (AxioCam; Carl Zeiss Meditec).

**Results**

**Perls’ Staining for Iron in the Retina and Ciliary Body in Wild-Type and Cp<sup>−/−</sup>–Heph<sup>−/−</sup>/N (DKO) Mice**

As previously published, at the age of 5 to 6 months DKO retinas had increased iron, with the highest levels in present
the RPE. In this study, we also Perls’ stained the ciliary body. Because iron tends to accumulate in the retina with age,\textsuperscript{10} we tested whether 16-month-old wild-type mice had any iron accumulation in the retina or ciliary body. Neither a 16-month-old (Fig. 1A) nor 7-month-old wild-type retina (not shown) had any Perls’ label in the ciliary body or retina. In contrast, the 7-month-old DKO ciliary body had a strong granular Perls’ stain (Fig. 1B) in the nonpigmented ciliary epithelium. Perls’ label was also detected in the RPE of the 7-month-old DKO.

**Levels of L-Ferritin in DKO Ciliary Bodies**

The levels of ferritin light chain (L-ferritin) are controlled by intracellular iron levels through the iron regulatory protein.\textsuperscript{25} To determine whether L-ferritin levels are altered in the ciliary body of the DKO, we immunolabeled the retina and ciliary body with anti-L-ferritin. Levels of L-ferritin increased in the nonpigmented ciliary epithelium of 7-month-old DKO mice in comparison to those in age-matched wild-type or single knockout Cpo or Heph mice (Figs. 1C–F).

**Levels of Transferrin Receptor in DKO Retinas**

Transferrin receptor mediates cellular iron uptake. Like ferritin, its levels are controlled by the iron regulatory proteins in response to cellular iron levels and move in the opposite direction from ferritin levels; in response to increased iron, TfR levels decrease. To test whether the DKO retinas have altered TfR levels, we immunolabeled 7-month-old DKO and age-matched wild-type retinas with anti-TfR. As expected in iron-overloaded tissue, TfR, while present in all retinal cell layers in the wild-type, was undetectable in the DKO, except for a thin layer near the junction of photoreceptor inner and outer segments (Fig. 2).

**Iron Quantification by Atomic Absorption in the Retinas and RPE/Choroid of DKO and Wild-Type Eyes with Age**

Neurosensory retinas (without RPE) of 6-month-old DKO mice (n = 6) had significantly higher iron levels in comparison with age-matched wild-type (Fig. 3A; n = 4). Also, iron levels were significantly higher in 6-month-old DKO neurosensory retinas than in 3-month-old DKO (n = 6). Iron accumulation in the DKO retinas reached a plateau at 6 months, and there was a slight, nonsignificant increase at 9 months. At 6 months, the iron levels were increased in the DKO relative to wild-type by approximately 2.5-fold.

In RPE/choroid samples (Fig. 3B), a significant difference in iron levels was found among the following groups: 3-month-old DKO (n = 6) versus wild-type (n = 6) mice, and 6-month-old DKO (n = 6) versus wild-type (n = 4) mice, as well as 3- versus 6-month-old DKO. As in the retinas, it was apparent that the iron levels in the RPE/choroid samples plateaued at ~6 months of age, as the slight increase in iron between 6 and 9 (n = 4) months in DKO mice was not significant. At 6 months, iron levels increased by approximately 4.5-fold in the DKO relative to wild-type.

**Isoprostane F2α-VI Levels in DKO Mice in Comparison to Age-Matched Wild-Type**

Isoprostane F2α-VI is a product of nonenzymatic oxidation of polyunsaturated fatty acids by reactive oxygen species. It serves as a quantitative, specific marker of oxidative stress in the retina.\textsuperscript{14} The levels were significantly increased (Fig. 3C) in 6-month-old DKO retinas (n = 4) in comparison with 6-month-old wild-type (n = 10).

**Age-Dependent Retinal Degeneration with Neovascularization in DKO Mice**

Retinas from DKOs younger than 6 months appeared normal. In comparison with wild-type (Fig. 4A), 7-month-old DKO mice had focal areas of retinal degeneration generally involving less than 10% of the retina. The degeneration consisted of RPE hyperplasia (Fig. 4B), RPE hypertrophy, and focal photoreceptor degeneration characterized by thinning of the ONL, inner segment vacuolization, and loss of outer segments. Sparse macrophages were present between the RPE and outer segments (Fig. 4C). In DKO mice at the age of 9 months, the retinal degeneration was generally more severe than at 7 months. There were focal areas of hypertrophic RPE cells with loss of overlying photoreceptor outer segments and thinning of the ONL (Fig. 4D). Hypertrophic RPE cells were found in as much as a quarter of the retinal length. Nine-month-old DKOs also tended to have more macrophage infiltration than at 7 months of age (Fig. 4E). In 12- to 13-month-old DKOs, the degeneration was more severe. Hypertrophic RPE cells were evident in 90% of the total retinal length, along with loss of inner and outer segments, thinning of the ONL (Fig. 4F), and subretinal macrophage infiltration. Focal areas of neovascularization occurred in three-fourths of the 12- to 13-month-old mice and approximately half of mice ranging from 7- to 9-month-old. The neovascularization was detected by using cardiac perfusion with Dil followed by epifluorescence microscopy of the eyecups. While wild-types had no neovascularization (Fig. 4G), DKOs had focal areas of hyperfluorescence (Fig. 4H). These areas corresponded on histologic sections to focal RPE disruption with vessels extending from the basal side of the RPE though the photoreceptor layer (Fig. 4I).

**Subretinal Macrophage Infiltration in DKO Mice**

The frequency and distribution of macrophage infiltrates and their association with neovascularization, atrophy of the retinal pigment epithelium, and the breakdown of Bruch’s membrane suggest that AMD has a chronic inflammatory component.\textsuperscript{24} Macrophages have also been associated with phagocytosis of wide-spreaded collagen, which may be a stimulus for inflammation.\textsuperscript{25} Although the age-matched wild-type mouse retinas had no macrophage infiltration (data not shown), 9- and 13-month-old DKOs had sparse macrophage infiltration present between the RPE and outer segments (Fig. 5).

**Accumulation of Lipofuscin-like Material in the DKO RPE with Age**

Lipofuscin accumulation has been described in the aging human eye and in AMD. Although the aged-matched wild-type mouse retinas had no detectable autofluorescent lipofuscin in the RPE (Fig. 6A, 6C), DKOs had RPE autofluorescence. Focal areas of autofluorescent hypertrophic RPE were present in 7- to 9-month-old DKO retinas (Figs. 6B, 6D), and most of the RPE cells in the 12-month-old DKO were autofluorescent (Fig. 6E). Like human RPE lipofuscin, the spectrum of autofluorescence was broad, including emission in the green spectrum (excitation = 460–500 nm, barrier filter = 510–560 nm) when excited with blue light (Figs. 6B, 6E) and emission in the red spectrum (Fig. 6D; excitation = 530–560 nm, barrier filter = 575–648 nm) when excited with green light.

**Activated Complement Components in DKO Bruch’s Membrane**

The complement cascade has been implicated in the pathogenesis of AMD by histologic and genetic studies.\textsuperscript{20–30} Activated complement components have been detected in drusen from human donor AMD retinas. To determine whether the pathogenesis of...
retinal degeneration in the DKO mice may involve complement activation, we immunolabeled DKO retinas with anti-C3b/iC3b/C3c, which is specific for activated complement. While age-matched wild-type retinas had no detectable label (Fig. 7A), 9-month-old DKO had sub-RPE label within Bruch’s membrane in focal stretches (Fig. 7B), each as long as 200 μm.

DISCUSSION

In wild-type mice, both ferroxidases Cp and Heph are present in RPE and neurosensory retina, where they are believed to facilitate iron export from the cells by oxidizing ferrous to ferric iron, the only form that can be taken up by the transport
protein transferrin. Mice deficient in both ferroxidases (DKOs) developed age-dependent retinal iron overload and ultimately retinal degeneration. Elevations in iron levels in DKO retinas resulted in increased levels of the cytosolic iron storage protein ferritin.

In this study, increased L-ferritin was observed in the DKO ciliary body, which was consistent with elevated iron levels in this location detected by Perls' stain. The functions of the ciliary body include secretion of aqueous humor, glycoproteins of the vitreous body, antioxidant enzymes, and neuropeptides. The aqueous humor is almost completely isolated from the blood in the ciliary body stroma by an epithelium. The ciliary body is composed of two polarized neuroepithelial cell layers: pigmented and nonpigmented. Tight junctions in the ciliary epithelium are restricted to the apical plasma membrane of the nonpigmented cell layer establishing a functional blood–aqueous barrier. Several studies revealed that the ciliary body expresses transferrin and ceruloplasmin. It has been suggested that these two metalloproteins could act synergistically to decrease formation of reactive oxygen intermediates (ROIs) in the aqueous and vitreous, which are in close contact with the retina, lens, trabecular meshwork, and cornea. The reason for iron accumulation in the nonpigmented epithelial layer in the DKO is unclear, but suggests that iron normally passes through this layer, and the transit is dependent on Cp and Heph. It is possible that iron is normally transported by the nonpigmented epithelium into the aqueous, where it would bind transferrin and supply the iron needs of the nonvascularized ocular tissues: the lens epithelium and the cornea. In support of this hypothesis, there is strong immunoreactivity to TfR antibodies in the human lens epithelium and corneal endothelium.

The iron-overloaded DKO retinas had a decrease in levels of TfR. Intracellular iron concentrations are regulated by two iron regulatory proteins: IRP1 and -2. When cytoplasmic iron is low, IRPs bind to the iron regulatory element (IRE) in the 3′-untranslated region of the TfR's mRNA, stabilizing the mRNA. Conversely, when iron levels are high, the IRP binds iron and dissociates from the TfR's mRNA, leading to degradation of the mRNA and decreased TfR protein levels. Thus, the decreased levels of TfR in DKO retinas most likely represent an appropriate regulatory response to iron overload, preventing further iron uptake in iron-overloaded retinas.

Mice deficient in Cp and Heph showed age-dependent retinal iron accumulation followed by degeneration. This degeneration shared many features with AMD, including RPE hypertrophy and hyperplasia, photoreceptor degeneration, RPE lipofuscin accumulation, subretinal neovascularization, and sub-RPE deposits. These deposits have been observed at the electron microscopic level, but are not large enough to be detected at the light microscopic level (unlike the drusen and basal linear deposits seen in AMD eyes) representing a difference between human AMD and the DKO mice. Perhaps larger deposits will develop in conditional Cp/Heph-knockout mice, which should live a full lifespan.

The retinal degeneration in the DKO mice was first evident at 6 months of age, and progressed at a variable rate. Mice that survived until 12 to 13 months all had RPE hypertrophy involving 90% of the retina, and most (75%) had subretinal neovascularization. Sparse macrophage infiltration was seen in 90% of 6- to 9-month-old DKO, and neovascularization was often associated with macrophages, suggesting that the macrophages may promote the neovascularization. New blood vessels most likely originated in the choroidal vasculature, as image analysis from thin plastic sections suggested connections between the choroid and the neovascularization. No direct connections between the retinal vasculature and the neovascularization were found, although some sections in

![Graphs of iron and isoprostane quantification in the retinas and RPE/choroid of DKO and wild-type eyes with age. Total iron in nanograms per neurosensory retina (A) measured by atomic-absorption spectrometry (AAS) is shown for age and genotype. Total iron in nanograms per RPE/choroid measured by AAS (B) is shown for age and genotype. Isoprostane F2α-VI levels (C) measured by mass spectrometry are shown for 6-month-old DKO in comparison to 6-month-old wild-type neurosensory retinas. *Significant difference (P < 0.05).](image-url)
which NV extended up through the ONL suggest this may occur. Qualitatively, the morphologic changes within each age group were similar, but some variability in the rate and extent of degeneration was found. This variability may be due to exogenous factors such as iron in the diet or maternal genotype, but further investigation will provide insight into the factors influencing these differences.

Quantification of retinal iron by atomic absorption disclosed the location and rate of retina iron build-up in DKO mice. Neurosensory retinas of 6-month-old DKO mice at the age of 9 months (D, E) had focal areas of significantly hypertrophic RPE cells with loss of overlying photoreceptor outer segments and thinning of the ONL. Nine-month-old DKO mice had more macrophage infiltration than did the 6-month-old DKO (E, arrow). Twelve-month-old DKO mice (F) had hypertrophic RPE cells, loss of inner and outer segments, and thinning of the ONL. Epifluorescence microscopy of eyecups from Dil-perfused mice show that 9-month-old wild-type mice had no neovascularization (G), whereas age-matched DKO (H) had focal areas of hyperfluorescence (arrows). These areas correspond to focal RPE disruption, with a vessel passing through the photoreceptor layer, as seen in a 6-month-old DKO retina (I, arrow). RPE, retinal pigment epithelium; OS, photoreceptor outer segment; IS, photoreceptor inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer. Scale bar, 50 μm.

FIGURE 4. DKO mice had age-dependent retinal degeneration with neovascularization. Bright-field micrographs of plastic sections show that relative to 16-month-old WT mice (A) 7-month-old DKO mice had focal areas of RPE hyperplasia (B, arrow) and focal photoreceptor degeneration consisting of thinning of the outer nuclear layer (ONL), inner segment vacuolization, and loss of outer segments. Macrophage infiltration was also present (C, arrow) in 7-month-old DKO. DKO mice at the age of 9 months (D, E) had focal areas of significantly hypertrophic RPE cells with loss of overlying photoreceptor outer segments and thinning of the ONL. Nine-month-old DKO mice had more macrophage infiltration than did the 7-month-old DKO (E, arrow). Twelve-month-old DKO mice (F) had hypertrophic RPE cells, loss of inner and outer segments, and thinning of the ONL. Epifluorescence microscopy of eyecups from Dil-perfused mice show that 9-month-old wild-type mice had no neovascularization (G), whereas age-matched DKO (H) had focal areas of hyperfluorescence (arrows). These areas correspond to focal RPE disruption, with a vessel passing through the photoreceptor layer, as seen in a 7-month-old DKO retina (I, arrow). RPE, retinal pigment epithelium; OS, photoreceptor outer segment; IS, photoreceptor inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer. Scale bar, 50 μm.

FIGURE 5. Macrophage infiltration in DKO retinas. Fluorescence photomicrograph of 9-month-old DKO (A) immunolabeled with an anti-F4/80 antibody shows subretinal immunoreactivity (arrow; red fluorescence) specific for this glycoprotein expressed by macrophages. Bright-field photomicrographs of 9- and 13-month-old DKO retinas (B, C) labeled with anti-CD11b antibodies show Mac-1-positive cells (arrows, blue chromogen) specific for macrophages. RPE, retinal pigment epithelium; ONL, outer nuclear layer. Scale bar: (A) 50 μm; (B, C) 25 μm.
present at 6 months. This continued retinal degeneration in DKOs older than 9 months may be coming from iron-facilitated oxidative stress. The highly reproducible rate of iron accumulation in DKOs measured by atomic absorption should facilitate further investigations of iron chelators.

Since iron can mediate the generation of free radicals via the Fenton reaction, iron-overloaded retinas from DKO mice are likely to experience a higher amount of oxidative stress. Because isoprostane has been found to be a reliable measure of oxidative stress, this indicator was used in the present study. Results showed negative correlation between Cp and Heph expression and isoprostane levels, suggesting that iron mediated oxidative stress is likely to contribute to the retinal degeneration.

With aging, the autofluorescent pigment lipofuscin accumulates in the cytoplasm of many cell types including the RPE. Lipofuscin is not just a harmless hallmark of aging, since lipofuscin-loaded cells have decreased capacity to phagocytose rod outer segments, and have increased susceptibility to light toxicity. One component of lipofuscin, the bis-retinoid pigment A2E may promote retinal degeneration. In this study, we found that autofluorescent lipofuscin-like material accumulates in RPE of DKO mice with age, implying that retinal degeneration in our model may also be promoted or exacerbated by age-related pigment accumulation. The components of lipofuscin can vary from tissue to tissue and within a specific cell type over time, and so there is no strict definition of lipofuscin. The autofluorescence in the DKO RPE meets several of the criteria generally associated with lipofuscin: It has a broad spectrum; is bleached by 5 mM CuSO₄ but not H₂O₂, KMnO₄, KMnO₄ plus oxalic acid, or NaBH₄ (data not shown); and observed in hypertrophic RPE cells, the cytoplasm of which is packed with lysosomes/endosomes. Studies on the biochemical composition of the lipofuscin within the DKO RPE must await generation of additional 12-month-old DKOs. Since they rarely live to this age, RPE-specific conditional knockouts, which have normal viability, would facilitate this analysis.

An increasing number of studies propose local inflammation and activation of the complement cascade in the pathogenesis of AMD. The complement system plays an important role in a variety of disease processes. Under normal conditions local complement activation is protective against pathogens. Uncontrolled activation of complement can damage host cells and tissues and contribute to disease progression. It has been suggested that photooxidation of RPE lipofuscin continues over time and contributes to inflammation in AMD. Photooxidation products then could be recognized by the complement system which would lead to its activation and to low-grade inflammation. Also, it has been suggested that drusen form or enlarge

**FIGURE 6.** Lipofuscin accumulated in the DKO RPE with age. Whereas the aged-matched wild-type retinas had no detectable autofluorescent lipofuscin in the RPE (A, C), DKOs had RPE autofluorescence (B, D, E, arrows). Focal areas of autofluorescent hypertrophic RPE were present in 7-month-old (B, arrow) and 9-month-old (D, arrow) DKO retinas. In 12-month DKOs (E, arrow) most of the RPE cells were autofluorescent. RPE, retinal pigment epithelium; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar, 50 μm.

**FIGURE 7.** Activated complement components were present in DKO Bruch’s membrane. Fluorescence photomicrograph of 9-month-old WT immunolabeled with anti-C3b/iC3b/C3c antibodies showed no immunoreactivity, whereas there was sub-RPE immunoreactivity (arrow) in a 9-month-old DKO RPE, retinal pigment epithelium. ONL, outer nuclear layer. Scale bar, 50 μm.
as a product of a local inflammatory processes, as they contain numerous inflammatory proteins. Many of these proteins are associated with complement system activation and regulation. In this study, we found sub-RPE immune-reactivity for activated complement, suggesting that in our model, retinal degeneration may be associated with inflammatory processes. Complement activation has also been observed in other mouse models of AMD caused by various upstream mechanisms including defects in macrophage recruitment and mutation of fibulin-3, an extracellular matrix component.

Iron is an essential element in human metabolism but also is a very potent generator of oxidative stress. Evidence supports a role of iron overload in AMD. Earlier, we found that iron levels within the human retina increase with age. Further, it has been shown that relative to age-matched control retinas, retinas of patients with AMD have elevated iron levels that may contribute to retinal degeneration. Patients with hereditary retinas of patients with AMD have elevated iron levels that may be associated with inflammatory processes. Future development of conditional Heph knockout mice should facilitate these studies, both by providing information about the cell-autonomous function of Heph, and by providing a more abundant supply of mice, which unlike the current DKO models, will be fertile and have a normal lifespan.

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