Deletion of Efemp1 Is Protective Against the Development of Sub-RPE Deposits in Mouse Eyes

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PURPOSE. EFEMP1 (fibulin-3) is mutated in Malattia Leventinese/Doyne’s honeycomb retinal dystrophy (ML/DHRD), an inherited macular dystrophy similar to AMD. Both ML/DHRD and AMD are characterized by the presence of sub-RPE deposits. Efemp1 knockout mice do not develop sub-RPE deposits. This study was to test whether sub-RPE deposits can be induced in Efemp1 knockout mice by experimentally applied stress conditions that cause wild-type mice to develop sub-RPE deposits.

METHODS. Efemp1 knockout and control mice at 6, 18, or 24 months old were fed with a synthetic high-fat diet (HFD). Beginning 1 month after starting the HFD, one group of mice was exposed to cigarette smoke daily for 1 month, and another group of mice was subjected to photochemical injury every other day for 2 weeks from a 488-nm argon laser. After the treatments, histologic analysis was performed to assess whether sub-RPE deposits were induced.

RESULTS. Basal laminar deposits (BLamDs), a form of sub-RPE deposits, were observed in the 18- and 24-month-old wild-type mice but not in Efemp1 knockout mice in any age groups after exposure to HFD and cigarette smoke or laser injury.

CONCLUSIONS. Mice lacking fibulin-3 do not develop sub-RPE deposits. Environmental oxidative stressors (HFD/cigarette smoke or HFD/laser) known to cause BLamD formation in wild-type mice failed to induce BLamD formation in Efemp1 knockout mice. These results suggest that fibulin-3 is a central player in the development of BLamD, and deletion of fibulin-3 is protective against the development of BLamD.

Keywords: Efemp1, sub-RPE deposits, macular degeneration
Deletion of Efemp1 is Protective Against BLamD

Methods

Mice

Efemp1+/- and Efemp1-/- mice were generated previously. EFEMP1 protects mice and their wild-type littermates at 6, 18, or 24 months of age were used. Efemp1+/- mice at the same age groups were used as controls. Both male and female mice were used for all the genotypes, age groups, and treatments. All mice were handled in accordance with the standards of humane animal care described by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, using protocols approved by the Institutional Animal Care and Use Committee of the University of Arizona or Mayo Clinic. Animals were housed under standard conditions and maintained on a 12-hour light/dark cycle with free access to water and food. Mice from three age groups and three genotypes were divided into four groups for treatments (Table 1). Group C is the control group. Mice in this group were fed with standard laboratory rodent diet without HFD, cigarette smoke exposure, or laser exposure. Group H is the HFD group where mice were fed with HFD without cigarette smoke or laser exposure. Group HS is the combinational treatment where mice were fed with HFD and exposed to cigarette smoke. Group HL is the combinational treatment where mice were fed with HFD and exposed to argon laser treatment.

CTD patients and Efemp1+/-/+ mice. Although there is no fibulin-3 mutation found in AMD, fibulin-3 also accumulates in Bruch’s membrane and sub-RPE deposits in AMD. Higher amounts of normal or mutant fibulin-3 may alter the basement membrane structural homeostasis through increased enzyme inhibitory activities or other means. This may in turn inhibit the turnover of basement membrane material and entrap shed microvesicle membranous debris to form sub-RPE deposits. In support of this hypothesis, Efemp1 knockout (Efemp1-/-) mice do not express fibulin-3 exhibit premature aging, hernia, and other symptoms associated with heightened ECM enzyme activities and never develop sub-RPE deposits throughout their lifespan. In contrast, wild-type mice often develop sub-RPE deposits at an advanced age. These findings suggest that the presence of fibulin-3 may be required for sub-RPE deposit formation.

Experimentally applied stress conditions have been shown to cause the formation of sub-RPE deposits in wild-type mouse models. Exposure of mice on a high-fat diet (HFD) to whole cigarette smoke causes BLamD formation. A combinational treatment of HFD and laser photochemical injury also induces BLamD-like deposits in mice that are similar to the deposits in AMD. Here we report the results of the study to test whether sub-RPE deposits develop in Efemp1-/- mice on a HFD after exposure to whole cigarette smoke or laser photochemical injury.

HFD Treatment

Groups H, HS, and HL mice were fed with a synthetic HFD (TD.88051; Envigo, Indianapolis, IN, USA) containing 15.8% fat, 1.25% cholesterol, and 0.5% sodium cholate. Group C mice were fed with standard laboratory rodent diet. After 1 month on the HFD, groups HS and HL were subjected to smoke or laser exposure while still on the HFD. Group H mice were continuously on the HFD until the experimental endpoints of groups HS and HL.

Cigarette Smoke Exposure

Beginning 1 month after starting the HFD, group HS mice were exposed to cigarette smoke daily for 1 month using a Teague Enterprises mouse smoking system (TE-10; Teague Enterprises, Davis, CA, USA). The cigarette smoke was generated from Kentucky Research 3R4F Reference Cigarettes (University of Kentucky, Lexington, KY, USA). These cigarette standards have 0.73 mg nicotine/cigarette and total particulate matter of 11.0 mg/cigarette. Forty-eight hours prior to use, the cigarettes were placed in a closed chamber at room temperature along with a solution of glycerin/water mixed in a ratio of 0.76/0.26 to establish a relative humidity of 60%. Total suspended particulate (TSP) in the exposure chamber was calculated using the system calibration. Mice were placed in cages and exposed to smoke in sealed exposure chambers. Smoke dosage was increased gradually over 5 days to allow mice an adaptation period. Mice were exposed daily for 30 days to smoke generated from 10 lit cigarettes for 2 hours each time. The TSP of the chamber was ~200 mg/m³.

Laser Exposure

After 1 month on the HFD, group HL mice were subjected to photochemical injury every other day for 2 weeks with a 488-nm blue argon laser system Stellar-Pro 488/50 (Modu-Laser, Centerville, UT, USA). Mice were anesthetized with tribromoethanol and placed in a mouse holder in the laser path. The same eye of each mouse was exposed to 30-mJ blue laser for 5 seconds once per day, every other day for 2 weeks. This setting did not cause thermal or phototoxic injury to mouse eyes.

Plasma Cholesterol Measurement

At the end of the experiments, mice were euthanized, and blood samples were taken. Plasma was obtained by centrifugation at 4°C. Total cholesterol was determined using an enzymatic colorimetric method for the quantitative determination of total cholesterol in serum (Wako Diagnostics, Richmond, VA, USA).

Ocular Phenotype Analysis

Following the stress treatments, mice were euthanized by CO₂ asphyxiation, and eyes were collected for histologic analysis as previously described to assess whether sub-RPE deposits developed in any of the mouse eyes. For transmission electron microscopy, the eyes were fixed overnight in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.2. After postfixation with 1% osmium tetroxide, the eyes were stained in 2% tannic acid, dehydrated in a graded series of alcohols, and embedded in epoxy resin. One limitation of this postfixation is that it is not optimal for lipid preservation. Thin sections were cut on a Reichert Ultracut microtome (Leica Microsystems, Inc., Buffalo Grove, IL, USA) and stained with uranyl acetate and lead citrate. Samples were examined and photographed using a Philips CM-12 electron microscopic system.
microscope equipped with an AMT CCD camera (Advanced Microscopy Techniques Corp., Danvers, MA, USA).

Basal laminar deposit severity and frequency in mice were graded based on a semiquantitative grading system introduced by Cousins et al.40 Mild BLamD referred to the presence of any discrete focal nodule of homogenous deposit between the RPE cell membrane and its basement membrane in at least one micrograph (of at least 10) within a section from an individual specimen. Moderate BLamD was defined as the presence of continuous deposit underlying two or more RPE cells, presence of banded structures, or deposit thickness ≥20% of RPE cell cross-sectional height in at least three micrographs with a section from an individual specimen. Severe BLamD contained continuous sheets of deposits underlying 10 or more RPE cells and deposit thickness >30% of RPE cell height in at least five micrographs within a section from an individual specimen.

RESULTS

Mice at 6, 18, or 24 months of age from three different genotypes were divided into four groups to be fed with a normal diet or HFD with or without exposure to cigarette smoke or argon laser (Tables 1, 2): group C were fed with standard laboratory rodent diet, group H were fed with an HFD, group HS were fed with an HFD and exposed to cigarette smoke, and group HL were fed with an HFD and exposed to argon laser treatment. Mice fed with an HFD became noticeably heavier at the end of 1 month. The general appearance of the mice was not otherwise affected by the HFD, cigarette smoke, or laser treatment to their eyes. No obvious difference was observed between male and female mice. The morphology of the eyes and the retina appeared to be well preserved. We did not observe obvious differences in choroid and the outer retina between treatment and control groups.

Total Plasma Cholesterol Level

Mice in the three groups (H, HS, HL) fed with an HFD had more than twofold higher total plasma cholesterol levels than the control group mice (C) with corresponding ages fed with a standard rodent diet (Fig. 1). Mice treated with HFD and cocaine smoke (HS) or argon laser (HL) had similar levels of cholesterol to mice fed with HFD (H) without smoke or laser treatment. There was no significant difference in total plasma cholesterol levels in the same group of mice with different ages.

No Effects on Sub-RPE Deposits by HFD Alone

In the control group C, BLamD was not observed in 6- or 18-month-old wild-type Efemp1+/+ mice (Fig. 2). No BLamD was observed in Efemp1+/− mice at any age (Fig. 2). Consistent with our previous observation, isolated small BLamDs were observed in 24-month-old Efemp1+/− mice.55 Basal laminar deposits were also observed in Efemp1+/− mice at 6, 18, and 24 months of age (Fig. 2).

With only an HFD feeding in group H, similar to those in group C, BLamD was not observed in 6- or 18-month-old Efemp1+/− mice or in Efemp1+/− mice at any age. Basal laminar deposits were observed in 24-month-old Efemp1+/− mice and in Efemp1+/− mice at all three ages (Table 3).
Effects of HFD in Combination With Cigarette Smoke on Sub-RPE Deposits

With a combinational treatment of the HFD and cigarette smoke exposure in group HS, BLamDs were observed in Efemp1<sup>+/+</sup> mice at 18 and 24 months of age but not at 6 months of age (Table 3). We observed moderate BLamDs in Efemp1<sup>+/−</sup> mice at 18 months of age (Fig. 3B) and severe BLamDs at 24 months of age. In Efemp1<sup>−/−</sup> mice, BLamDs were observed at all three ages (6, 18, and 24 months). The severity of BLamDs in treated Efemp<sup>1−/−</sup> mice was similar to that of untreated Efemp<sup>1−/−</sup> mice at the same age. The combinational treatment of HFD and smoke exposure did not appear to augment BLamDs in Efemp<sup>1−/−</sup> mice. In contrast, no BLamD was observed in Efemp<sup>1−/−</sup> mice at any of the three age groups under the same combinational treatment. No other form of sub-RPE deposits was observed in these mice either (Fig. 3B). This finding indicates that Efemp<sup>1−/−</sup> mice are resistant to these stress conditions in the development of BLamDs.

Effects of HFD in Combination With Argon Laser on Sub-RPE Deposits

With a combinational treatment of HFD and argon laser exposure of the eyes in group HL, BLamDs were also observed in Efemp1<sup>+/+</sup> mice at 18 and 24 months of age, but not at 6 months of age (Table 3). Similar to those treated with HFD and smoke, moderate severe and severe BLamDs were observed in group HL mice at both 18 and 24 months of age (Fig. 4B). In Efemp1<sup>−/−</sup> mice BLamDs were observed at all three ages. The combinational treatment of HFD and laser exposure did not appear to augment BLamDs in Efemp<sup>1−/−</sup> mice. However, no BLamD or any other form of sub-RPE deposits was observed in Efemp<sup>1−/−</sup> mice at any age under the combinational treatment of HFD and laser (Fig. 4B). This finding indicates that...
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### DISCUSSION

In a previous study, we noted that Efemp1-/- mice (which do not express fibulin-3) do not develop BLamDs as they age. 37 The results of this study show that no sub-RPE deposit is induced in Efemp1-/- mice even after being fed with a HFD and exposed to cigarette smoke or argon laser photochemical injury. The same stress conditions have induced BLamDs in wild-type mice.35,39 It is not known how the interaction between HFD and cigarette smoke or HFD and argon laser causes BLamDs to develop in younger animals, presumably when damaged materials exceed the capacity of a fibulin-3-controlled turnover system. EFEMP1-/- mice where fibulin-3 is absent, The RPE basement membrane turnover process is active and damaged materials associated with the basement membrane are promptly removed. Even when the burden increases under extra stress stimulation of HFD/cigarette smoke or HFD/laser, the turnover system can still handle it.

Any of the HFD, cigarette smoke, or argon laser photochemical injury in isolation does not appear to be sufficient to induce BLamD or other forms of sub-RPE deposits in wild-type mice.58,59 It is not known how the interaction between HFD and cigarette smoke or HFD and argon laser causes BLamD. Although our study shows clearly that deletion of fibulin-3 is protective against the development of BLamDs induced by HFD/cigarette smoke or HFD/argon laser in mice, we do not know whether deletion of fibulin-3 is protective against BLamDs caused by other factors. For example, knockout mice lacking collagen XVIII develop BLamDs.55 Transgenic mice expressing an enzymatically inactive form of cathepsin D in RPE cells also develop BLamDs.46 If deleting fibulin-3 prevents these mice from developing sub-RPE deposits, it would add evidence that fibulin-3 is a central player in sub-RPE deposit formation. Sub-RPE deposits are a consistent early finding in ML/DHRD and dry AMD. Currently, there is no treatment for these diseases. Our study suggests that fibulin-3 is a promising target for developing treatments for these diseases.

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### References


