The retinal pigment epithelium in Sorsby Fundus Dystrophy shows increased sensitivity to oxidative stress-induced degeneration

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A B S T R A C T
Sorsby Fundus Dystrophy (SFD) is a rare inherited autosomal dominant macular degeneration caused by specific mutations in TIMP3. Patients with SFD present with pathophysiology similar to the more common Age-related Macular Degeneration (AMD) and loss of vision due to both choroidal neovascularization and geographic atrophy. Previously, it has been shown that RPE degeneration in AMD is due in part to oxidative stress. We hypothesized that similar mechanisms may be at play in SFD. The objective of this study was to evaluate whether mice carrying the S179C-Timp3 mutation, a variant commonly observed in SFD, showed increased sensitivity to oxidative stress. Antioxidant genes are increased at baseline in the RPE in SFD mouse models, but not in the retina. This suggests the presence of a pro-oxidant environment in the RPE in the presence of Timp3 mutations. To determine if the RPE of Timp3 mutant mice is more susceptible to degeneration when exposed to low levels of oxidative stress, mice were injected with low doses of sodium iodate. The RPE and photoreceptors in Timp3 mutant mice degenerated at low doses of sodium iodate, which had no effect in wildtype control mice. These studies suggest that TIMP3 mutations may result in a dysregulation of pro-oxidant—antioxidant homeostasis in the RPE, leading to RPE degeneration in SFD.

1. Introduction

Sorsby Fundus Dystrophy (SFD) [1] is an autosomal dominant, fully penetrant, degenerative disease of the macula [2]. SFD is manifested by symptoms of night blindness or sudden loss of visual acuity, usually in the third to fourth decades of life [3–5]. SFD is caused by specific mutations in the Tissue Inhibitor of Metalloproteinase-3 (TIMP3) gene [6–13]. The predominant histopathological feature in the eyes of patients with SFD are confluent, 20–30 μm thick, amorphous deposits found between the basement membrane of the retinal pigment epithelium (RPE) and the inner collagenous layer of Bruch’s membrane. While SFD is a rare disease, it closely resembles the more common age-related macular degeneration (AMD) in the occurrence of drusen, choroidal neovascular membranes, and central geographic atrophy. SFD, however, differentiates itself from AMD with earlier onset of symptoms, strong autosomal dominant inheritance pattern, and late involvement of peripheral chorio-retinal atrophy. The sub-retinal deposits in both SFD and AMD have been shown to be rich in TIMP3 [14–16].

The identification of rare coding variants in the TIMP3 gene when analyzing 16,144 AMD patients and 17,832 controls suggested that TIMP3 may play a role in AMD [17]. In addition, the AMD Consortium identified the first genetic association signal specific to wet AMD near matrix metalloproteinase-9 (MMP9), a substrate for TIMP3. The clinical and histopathological similarities between AMD and SFD, the identification of variants in the TIMP3 gene and matrix metalloproteinase pathway in AMD, and the observation that sub-retinal deposits in both SFD and AMD are rich in TIMP3 [14–16,18] suggest that similar downstream effectors might be in play in both conditions.

Produced constitutively by the RPE [2,19], TIMP3 is a normal component of Bruch’s membrane [20], which binds to sulfated
glycosaminoglycans of the extracellular matrix [21,22]. While mice expressing S179C-Timp3 (one of the mutations identified in SFD patients), showed only slight abnormalities in the inner aspect of Bruch’s membrane and RPE microvilli [23] there was a clear absence of typical SFD-related pathology in mutant mice on a C57BL/6 background. One possibility that has been suggested to explain this discrepancy between humans and mice is that the SFD-TIMP3 modification predisposes the retina or RPE/choroid to be more vulnerable to environmental or epigenetic stress, which may be absent in the inbred mouse strain housed under controlled conditions of the vivarium. We have recently reported that SFD mutant mice are more susceptible to laser-induced choroidal neovascularization [24]. The purpose of this study is to investigate whether mice carrying the S179C-Timp3 mutation are more susceptible to RPE damage induced by NaIO₃, an oxidizing agent that has been widely used as a model for AMD as it induces oxidative stress and subsequent damage exclusively in the RPE [25,26].

2. Materials and methods

2.1. Mice

All mice utilized in this study were housed in the Cole Eye Institute vivarium under approved Institutional Animal Care and Use Committee (IACUC) protocols. All procedures on the mice were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and conformed to the National Institutes of Health Guide for the Care and Use of Animals in Research. Heterozygous breeding of Timp3+/S179C [23] produced homozygous Timp3S179C/S179C mice and age-matched, wildtype littermate controls on a C57BL/6J background. Experiments were carried out in 4 month-old, homozygous Timp3S179C/S179C mice. Mice were injected via tail vein with a 1% soIV/S179C mice and RPE/choroid to be more vulnerable to environmental or epigenetic stress, which may be absent in the inbred mouse strain housed under controlled conditions of the vivarium. We have recently reported that SFD mutant mice are more susceptible to laser-induced choroidal neovascularization [24]. The purpose of this study is to investigate whether mice carrying the S179C-Timp3 mutation are more susceptible to RPE damage induced by NaIO₃, an oxidizing agent that has been widely used as a model for AMD as it induces oxidative stress and subsequent damage exclusively in the RPE [25,26].

2.2. Isolation of RNA

Eyes were enucleated following euthanasia and immediately dissected. Retinas were removed, immediately frozen, and stored at −80 °C until use. RPE was dissociated as previously described [27]. Both eye cups (without retinas) from the same mouse were combined and incubated with RNAProtection Cell Reagent (Qiagen, Hilden, Germany) for 10 min at room temperature with occasional agitation in order to dissociate the RPE from the choroid and sclera. After incubation, the choroid/sclera was removed from the tube and RPE was lysed by passing through 27½ G needle and syringe. Lysates were clarified by centrifugation. Protein content was quantified in each lysate using the Micro BCA Protein Assay Kit (Thermo Scientific, WA, USA). Western blots were performed as previously described [28]. The protein levels were quantified using Image Studio V5.2 software (LI-COR Biosciences, Lincoln, NE, USA). The following antibodies were used: β-actin—mouse monoclonal IgG, #8H10D10, Cell Signaling Technology, Danvers, MA, USA); DJ-1—(Rabbit polyclonal IgG, #NB300-270, Novus Biologicals, Littleton, CO, USA); Sod1—(Mouse monoclonal IgG, #SC-101523, Santa Cruz Biotechnology, Dallas, TX, USA).

2.3. Quantitative PCR

2.4. Western blotting and quantifications

After euthanasia, the dorsal side of the eyes were labeled for orientation and the eyes subsequently enucleated. Whole eyes were immediately placed in cold fixative solution (2% PFA (Electron Microscopy Sciences, Hatfield, PA, USA), 2.5% glutaraldehyde (Polysciences, Warminster, PA, USA), 0.1 M sodium cacodylate (Electron Microscopy Sciences, Hatfield, PA, USA), 2.5% glutaraldehyde (Polysciences, Warminster, PA, USA), 0.0011% calcium chloride (Millipore Sigma, Burlington, MA, USA)). After initial fixation, incisions were made just below the limbus and the eyes were then returned to fixative for the duration of the incubation. After fixation, the anterior segment, lens, and vitreous were dissected away from the posterior eye cups. The eye cups were washed with cold 0.1 M sodium cacodylate, post-fixed in osmium tetroxide (Electron Microscopy Sciences, Hatfield, PA, USA) (1% OsO₄, 0.1 M sodium cacodylate), and washed again in 0.1 M sodium cacodylate. The eye cups were dehydrated by incubating them in sequentially increasing concentrations of cold ethanol, infiltrated with propylene oxide (Thermo Fisher Scientific, Waltham, MA, USA), and embedded in epon. Resin blocks were cut into 0.75 μm sections and stained with toluidine blue (Thermo Fisher Scientific, #T161, Waltham, MA, USA). Sections were imaged with Zeiss Axiosmager. Z1 using MRC5 camera (Zeiss, Oberkochen, Germany) at 40X.

2.5. Plastic sections and bright field imaging

Eyes were enucleated immediately after euthanasia and immediately dissected to remove anterior segment and retina. Both eye cups (without retinas) from the same mouse were placed in the same tube with RIPA buffer (Alfa Aesar, Havernill, MA, USA) + protease/phosphatase inhibitors (Sigma Aldrich, St. Louis, MO, USA) and incubated on ice for 30 min with agitation every 5 min to dissociate the RPE from the choroid/sclera. After incubation, the choroid/sclera was removed from the tube and RPE was lysed by passing through 27½ G needle and syringe. Lysates were clarified by centrifugation. Protein content was quantified in each lysate using the Micro BCA Protein Assay Kit (Thermo Scientific, WA, USA). Western blots were performed as previously described [28]. The protein levels were quantified using Image Studio V5.2 software (LI-COR Biosciences, Lincoln, NE, USA). The following antibodies were used: β-actin—mouse monoclonal IgG, #8H10D10, Cell Signaling Technology, Danvers, MA, USA); DJ-1—(Rabbit polyclonal IgG, #NB300-270, Novus Biologicals, Littleton, CO, USA); Sod1—(Mouse monoclonal IgG, #SC-101523, Santa Cruz Biotechnology, Dallas, TX, USA).

2.6. Flat mounts, imaging, and quantification of RPE degeneration

Enucleated eyes were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA), after which retinas were mechanically detached from the RPE/choroid under a dissecting microscope. The flat mounts with exposed RPE were then processed for labeling with phalloidin-TRITC (1:1000; Sigma-Aldrich, #P1951, St. Louis, MO, USA) as previously described [29]. RPE/choroid flat mounts were mounted on glass slides and images were acquired using a laser scanning confocal microscope (TCS-SP8, Leica, Wetzlar, Germany). Serial Z-stacks and tiles of the flat-mounted RPE were collected and merged into a three-dimensional projection of the entire flat-mount.
Images were acquired using identical settings. Quantification of RPE degeneration was calculated using ImageJ2 [30]. Micrographs were calibrated from a reference scale embedded in the image. Quantifications were performed as previously described [28]. Briefly, the outline of the whole RPE/choroid area and of the degenerated area were delineated using the freehand line tool in ImageJ2. In cases where not all areas of degeneration were contiguous, separate areas of degeneration were summed to determine the cumulative area of degeneration. This area was divided by the total area of the flat-mounted RPE to determine percent degeneration. The edges of degenerated area were defined as regions in which the RPE cells are present but their morphology is not the typical hexagonal shape as shown by TRITC-phalloidin staining.

2.7. Statistical analysis

All data were analyzed using GraphPad Prism v7.0 (GraphPad Software, La Jolla, CA, USA). All data were normally distributed and are presented as mean ± SEM. Groups for qPCR were compared using unpaired, two-tailed Student’s t-test. Similarly, comparisons of RPE degeneration within groups was performed using unpaired, two-tailed Student’s t-test. Significance threshold (α) was set to 0.05.

3. Results

3.1. The RPE has increased expression of antioxidant genes in mice carrying the S179C-Timp3 mutation

To evaluate the expression of antioxidant genes in the retina and RPE, eyes from 4-month old, homozygous S179C-Timp3 mice and wildtype (WT) littermate mice were enucleated and RNA was isolated from retinas and RPE for expression analysis. To ensure contaminant-free isolation of RNA from each tissue, relative enrichment of retina and RPE marker genes were analyzed. RPE65 is expressly expressed by RPE [31] and in our samples was approximately 70-fold enriched in RNA from RPE compared to RNA isolated from retinas (Fig. 1A). Similarly, rhodopsin (Rhod) is expressed predominantly in photoreceptors [32] and was found to be enriched approximately 3-fold in retinal RNA isolations compared to RPE RNA isolations (Fig. 1B).

Next, the expression of four major antioxidant genes was analyzed in the RPE from age-matched S179C-Timp3 mice and WT controls from at least 4 male and 4 female mice (n = 8 total) from each group. Nrf2, Park7 (more commonly referred to as DJ-1), Sod1, and Sod2 have previously been characterized to be upregulated in response to oxidative stress [33–36]. Nrf2 (Fig. 2A), Park7 (DJ-1) (Fig. 2B), Sod1 (Fig. 2C), and Sod2 (Fig. 2D) genes were upregulated at least 2 fold in RPE from S179C-Timp3 mice compared to WT controls (Fig. 2). However, there are no changes in the expression of Sod1 and DJ-1 at the protein level in WT or S179C-Timp3 mice (Supplemental Fig. 1).

In contrast, the retina showed no significant changes in antioxidant gene expression (Nrf2 (Fig. 3A), Park7 (Fig. 3B), Sod1 (Fig. 3C), and Sod2 (Fig. 3D)) between S179C-Timp3 mice and WT controls (Fig. 3). This suggests that there may be a RPE-specific oxidative stress in SFD or that oxidative stress response is an integral part of a typical RPE reaction to diseases or pathology.

3.2. Mice carrying S179C-Timp3 mutation are more susceptible to NaIO3-induced retinal degeneration

Due to increased expression of antioxidant genes in S179C-Timp3 mice, we hypothesized that the RPE of S179C-Timp3 mice would be more susceptible to damage from lower levels of oxidative stress. Sodium iodate has been widely used as a model for oxidative stress-induced RPE degeneration [37–43].

To evaluate the relative susceptibility of mice carrying the S179C-Timp3 mutation to RPE degeneration, WT and S179C-Timp3 mice were injected intravenously with either low dose (10 mg/kg body weight) or high dose (20 mg/kg body weight) NaIO3. One week after injection, the eyes of experimental mice were evaluated by histology (Fig. 4). Both WT and S179C-Timp3 groups had normal retinas with PBS control injections (Fig. 4A–a, F–f). Upon injection with low dose of NaIO3, retinas from WT mice remained intact (Fig. 4B–b, c–c) while retinas from S179C-Timp3 mice began showing signs of RPE degeneration and disorganization of the outer nuclear layer (Fig. 4G–g, H–h). At high doses of NaIO3, both WT and S179C-Timp3 mice had severe degeneration of the RPE and outer retina (Fig. 4D–d, E–e, F–f, J–j). This indicates that WT mice are still susceptible to damage from oxidative stress as induced by NaIO3, but much more oxidative stress is required in order to see degeneration comparable to that of S179C-Timp3 mice at low doses.

3.2.1. S179C-Timp3 mice have increased area of RPE degeneration induced by NaIO3

To analyze the effect of NaIO3 specifically on the RPE, RPE/choroid flat mounts of experimental mice were analyzed for degeneration after staining with rhodamine-phalloidin, a reagent that binds filamentous actin (Fig. 5). As before, two doses of NaIO3 were used (10 mg/kg and 20 mg/kg) and one week passed between injection and enucleation. Both WT and S179C-Timp3 mice injected with PBS had normal RPE morphology (Fig. 5A–a, D–d). However, after injection with low dose of NaIO3, degeneration began in the central region of the RPE of S179C-Timp3 mice (Fig. 5E–e) while no degeneration occurred in the RPE of WT mice (Fig. 5B–b). Both WT and S179C-Timp3 mice had severe loss of RPE after injection with high doses of NaIO3 (Fig. 5C–c, F–f).

The area of degeneration was centralized around the optic nerve and was quantified as percent of total area of the flat mount (Fig. 5G). These

Fig. 1. RNA isolated from retina and RPE shows enrichment in marker genes specific for each tissue. A) RPE65 was enriched in RNA extracted from RPE compared to RNA extracted from retinas. B) Rhod was enriched in RNA extracted from retinas compared to RNA extracted from RPE. A-B) Fold changes are shown relative to retina. Comparisons were analyzed using Student’s unpaired t-test. n > 8 for each analysis.
Fig. 2. Antioxidant gene expression is increased in RPE from S179C-Timp3 mice. A) Nrf2, B) Park7, C) Sod1, and D) Sod2 were analyzed by quantitative PCR using RNA extracted from RPE from S179C-Timp3 mice and WT littermate controls. A-D) Fold changes are shown relative to WT samples. Comparisons were analyzed using Student’s unpaired t-test. n = 8 for each analysis.

Fig. 3. Antioxidant gene expression is unaltered in retina from S179C-Timp3 mice. A) Nrf2, B) Park7, C) Sod1, and D) Sod2 were analyzed by quantitative PCR using RNA extracted from retinas from S179C-TIMP3 mice and WT littermate controls. A-D) Fold changes are shown relative to WT samples. Comparisons were analyzed using Student’s unpaired t-test. n = 8 for each analysis.
areas of degeneration were defined as cells that deviated from the normal hexagonal morphology by becoming larger, elongated cells with stress fibers detected by TRITC-phalloidin (Fig. 5 C–c, E–e, F–f, arrowheads). Areas without any TRITC-phalloidin staining were indicative of complete loss of RPE (Fig. 5 C–c, E–e, F–f asterisks). There was no significant difference in area of degeneration between WT and S179C-Timp3 mice given high doses of NaIO₃ (Fig. 5 G, right). However, there was a significant increase in area of RPE degeneration in S179C-Timp3 mice after injection with low dose of NaIO₃ (Fig. 5 G, middle).

4. Discussion

Oxidation-reduction (redox) homeostasis plays a critical role in a number of physiological processes. The concept of oxidative stress was first proposed in 1985 as “a disturbance in the prooxidant-antioxidant balance in favor of the former” [44] and subsequently, the regulation of redox signaling has been the subject of intense investigation [45–47]. Oxidative stress has been hypothesized to play an important role in the pathogenesis of a number of retinal dystrophies including AMD [48]. Many studies have suggested that the severe RPE degeneration in geographic atrophy is a consequence of a loss of antioxidant functions [49–54]. Whether oxidative stress is a factor in the pathogenesis of Sorsby Fundus Dystrophy has not been investigated prior to this study.

The RPE, which constitutes the outer blood-retinal barrier, sits as a monolayer of cuboidal, polarized pigmented cells on Bruch’s membrane, between the choroid and the neural retina. In addition to its barrier function, the RPE is involved in retinoid storage and metabolism and provides the machinery for phagocytosis of the outer segments of photoreceptors. The RPE is highly metabolically active and exposed to high levels of oxygen and age-pigment, such as lipofuscin, which makes it increasingly susceptible to oxidative stress.

A number of molecular redox switches have been identified that control redox signaling and/or redox sensing [44]. Nuclear Factor-E2-related Factor 2 (Nrf2) belongs to a small family of redox sensitive transcription factors that has been postulated to be a key defensive mechanism against oxidative stress [55]. Four key antioxidant genes were analyzed in this study: Nrf2, Park7/DJ-1 [29,56], Sod1, and Sod2 [57]. We found increased antioxidant gene expression in the RPE (Fig. 2), but not in the retina (Fig. 3) of S179C-Timp3 mice at baseline. While we did not see significant changes at the protein level

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**Fig. 4.** S179C-Timp3 mice have increased degeneration of RPE and outer retina after injection with low doses of sodium iodate. Plastic sections of WT (A–E) and S179C-Timp3 (F–J) mice show varying levels of degeneration after injections with NaIO₃. n > 3 for each group. Whole-retina images taken at 40x (A–J) and magnified images from insets (a–j) showing degeneration of RPE and photoreceptors. A–a, F–f) Sections from mice injected with PBS only had no degeneration. G–g, H–h) S179C-Timp3 mice injected with 10 mg/kg NaIO₃ showed degeneration of the RPE and outer retina, but WT mice with the same dose did not (B–b, C–c). D–d, E–e, I–i, J–j) Both WT and S179C-Timp3 mice had severe degeneration after injection with 20 mg/kg NaIO₃. Black scale bars in images represent 40 μm.
(Supplemental Fig. 1), the increase at the mRNA level remains a critical observation because it suggests that oxidative stress sensing mechanisms are at play in the RPE of S179C-Timp3 mice. Moreover, studies have shown that mRNA levels do not always correlate to protein levels in post-mitotic cells, such as RPE [58]. Lack of correlation between mRNA and protein has been reported in many systems and ascribed to a number of mechanisms including post-transcriptional mechanisms, half-life of proteins, protein degradation, etc [59]. Together, these data suggest that in SFD, the RPE is the initial target of oxidative stress, and by extension, the initial target of disease. This is not surprising as TIMP3 has previously been shown to be expressed primarily in the RPE and choroid [2].

The observation of increased antioxidant gene expression in the RPE of SFD mice also suggests that the presence of mutant Timp3 likely creates a pro-oxidant environment in the RPE. Whether this is caused by an MMP-dependent or –independent pathway has yet to be determined. We cannot rule out the possibility that the antioxidant machinery (at least the ones we tested: Nrf2, DJ-1, Sod1, and Sod2) may be working appropriately in SFD, but that under identical baseline oxidative stress conditions, wildtype mice may simply generate a more robust antioxidant response. However, our data suggest specific RPE sensitivity to oxidative stress that are unique to the S179C-Timp3 mice. This will be the focus of future studies as we have yet to identify the pro-oxidant pathways at play in SFD.

We hypothesize that the baseline pro-oxidant environment of the RPE in S179C-Timp3 mice render them more susceptible to low levels of oxidative stress. This was seen in the development of pathological changes in the RPE of mutant mice exposed to low doses of NaIO\textsubscript{3}. There was a significant loss of RPE and photoreceptors one week after injection with low doses of NaIO\textsubscript{3} in the mutant mice but not in WT mice, as shown by histology (Figs. 4 and 5). The area of RPE degeneration after injection with low dose of NaIO\textsubscript{3} in S179C-Timp3 mice (Fig. 5) consistently began in the central retina adjacent to the optic nerve and spread outwards, likely due to the intravenous delivery of NaIO\textsubscript{3}. Bordering the areas of RPE loss, the RPE was elongated with filamentous actin stress fibers, suggesting that RPE stress precedes cell death (Fig. 5, arrowheads), similar to what has been hypothesized to occur in geographic atrophy in AMD.

The idea that patients with AMD have a chronic low level oxidative stress in their retinas has led to the proposal of antioxidant therapy as a treatment for AMD [60]. Supplementation with vitamin A, a potent antioxidant, is often prescribed for AMD patients [61, 62]. Our study points to the possibility of antioxidant therapy being potentially beneficial for patients with SFD. Future studies will investigate if antioxidant therapy mitigates RPE degeneration due to oxidative stress in S179C-Timp3 mice.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found at https://doi.org/10.1016/j.redox.2020.101681.

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