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Research article

AMD-like retinopathy associated with intravenous iron

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ABSTRACT

Iron accumulation in the retina is associated with the development of age-related macular degeneration (AMD). IV iron is a common method to treat iron deficiency anemia in adults, and its retinal manifestations have not hitherto been identified. To assess whether IV iron formulations can be retina-toxic, we generated a mouse model for iron-induced retinal damage. Male C57BL/6J mice were randomized into groups receiving IV iron-sucrose (+Fe) or 30% sucrose (−Fe). Iron levels in neurosensory retina (NSR), retinal pigment epithelium (RPE), and choroid were assessed using immunofluorescence, quantitative PCR, and the Perls' iron stain. Iron levels were most increased in the RPE and choroid while levels in the NSR did not differ significantly in +Fe mice compared to controls. Eyes from +Fe mice shared histological features with AMD, including Bruch's membrane (BrM) thickening with complement C3 deposition, as well as RPE hypertrophy and vacuolization. This focal degeneration correlated with areas of high choroidal iron levels. Ultrastructural analysis provided further detail of the RPE/photoreceptor outer segment vacuolization and Bruch's membrane thickening. Findings were correlated with a clinical case of a 43-year-old patient who developed numerous retinal drusen, the hallmark of AMD, within 11 months of IV iron therapy. Our results suggest that IV iron therapy may have the potential to induce or exacerbate a form of retinal degeneration. This retinal degeneration shares features with AMD, indicating the need for further study of AMD risk in patients receiving IV iron treatment.

1. Introduction

Age-related macular degeneration (AMD) represents a leading cause of vision loss, affecting millions of elderly in the USA (Laatikainen and Hirvelä, 1995; The Eye Diseases Prevalence Research Group*, 2004). AMD is defined by the development of drusen – characteristic extracellular deposits on the basal side of the retinal pigment epithelium. AMD can cause death of RPE cells and photoreceptors in the macula (Hageman et al., 2001; Young, 1987). Vision loss associated with AMD can be irreversible. For this reason, efforts currently focus on measures to understand and thereby prevent the development of AMD.

While the exact pathogenesis of AMD is not currently known, it is likely a multifactorial process that is thought to develop from a combination of oxidative stress and inflammation (Anderson et al., 2002; Dunaief, 2006; Zarbin, 2004). Evidence for these mechanisms comes from known risk factors and protective agents. An antioxidant formulation including lutein, vitamin C, vitamin E, and zinc has been shown to reduce the risk of developing advanced AMD (Age-Related Eye Disease Study 2 Research Group, 2013). Polymorphisms in genes encoding several complement cascade inhibitors – including complement factor H, complement component 2, and complement factor B – have been shown to significantly increase the risk of developing AMD (Klein et al., 2005; Hageman et al., 2005; Gold et al., 2006; McKay et al., 2009). More evidence comes from pathological analysis of AMD retinas. Granules of lipofuscin, photo-responsive compounds known to generate reactive oxygen species, accumulate in the RPE cells of patients with AMD (Sarks et al., 1988). Similarly, complement cascade components such as complement factors C3b and C5 are known constituents of drusen (Johnson et al., 2001).

Iron is also implicated in AMD pathogenesis, and its mechanisms are currently under investigation. Studies have reported increased levels of iron in *post mortem* maculas of patients with AMD (Hahn et al., 2003). Specifically, iron has been shown to

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accumulate within Bruch's membrane and metal-binding melanosomes in the RPE (Biesemeier et al., 2015). Moreover, patients with increased retinal iron due to aceruloplasminemia tend to develop AMD-like disease at an earlier age than the general population (Cibis et al., 1959; Dunaief et al., 2005; Wolkow et al., 2011). Ceruloplasmin and hephaestin double knockout mice exhibit features of AMD, and demonstrate iron accumulation, oxidative stress, as well as increased complement activation in the retina (Hahn et al., 2004; Hadziahmetovic et al., 2008). Some of the mechanisms by which iron may mediate AMD pathology have already been studied. For example, recent investigations demonstrated that increased iron upregulates expression of C3 − a key protein involved in the complement cascade (Li et al., 2015). Furthermore, iron is a known source of free radicals by way of the Fenton reaction, and iron has been linked to oxidative stress in the retina by several studies (Dunaief, 2006; Song et al., 2014, 2012; Lukinova et al., 2009).

In a previous study, we presented evidence of subretinal iron injection causing inflammasome-mediated toxicity in the RPE (Gelfand et al., 2015). In contrast, this present study investigates whether an acute increase in systemic iron via *intravenous* injection leads to increased retinal iron, and whether these elevated iron levels are correlated with retinal degeneration. In particular, the IV route of iron administration is clinically relevant because IV iron therapy is commonly used to treat iron deficiency anemia. With evidence that iron excess may predispose to AMD, it is apparent that the ophthalmic risks of iron infusions need to be further researched. In this study, we not only test our hypothesis in mice, but also correlate our findings with a clinical case of a patient who experienced macular degeneration at age 43 after receiving IV iron therapy.

2. Methods

2.1. Animals

C57BL/6J male mice were randomized into two groups: iron-sucrose-injected (Venofer, American Regent, INC, NY) mice (+Fe, n = 6) and controls (−Fe, n = 5). The + Fe mice received weekly tail vein injections of 1.2 mg iron-sucrose diluted in normal saline, beginning at 2 months of age for a total of 12 injections. The −Fe mice were injected with IV sucrose (30%) with equivalent volumes at equivalent timings to the +Fe mice. All mice were housed in the same conditions and were fed a diet containing 300 ppm iron. Mice were sacrificed at 12 months of age for histologic studies.

A separate cohort of C57BL/6J male mice aged 10 weeks was used for initial quantitative PCR (qPCR) testing after two injections with either iron-sucrose ($n = 3$) or sucrose (30%) ($n = 3$). Mice were euthanized twenty-four hours after the last injection.

Euthanasia for all mice was carried out using i.p. phenobarbital injection followed by cervical dislocation. Mice were handled in accordance with the Institutional Animal Care and Use Committee of the University of Pennsylvania.

2.2. Quantitative real-time PCR

Gene expression in the mouse retinas was evaluated in the neurosensory retina (NSR) and RPE cells separately as described elsewhere (Wolkow et al., 2012). Taqman Probes (ABI, Grand Island, NY, USA) were used to detect ferritin light chain (*Ftl*, Mm03030144_g1) and transferrin receptor (*Tfrc*, Mm00441941_m1) expression, as indirect markers of iron. Eukaryotic 18S rRNA (Hs99999901_s1) was used as an endogenous control. qPCR was performed using a DNA amplification/detection system (Prism model 7500; ABI) with the ΔΔCT method, which provides normalized expression values. The amount of target mRNA was compared among the groups of interest. All reactions were performed in biological and technical triplicates (three qPCR replicates per biological sample).

2.3. Immunofluorescence

Enucleated eyes were fixed in 4% paraformaldehyde and the cornea, iris, and lens were removed to form eye cups. Eye cups were immersed in 30% sucrose overnight for dehydration and embedded in Tissue-Tek OCT (Sakura Fintek, Torrance, CA, USA) for freezing. 10 μm-thick sections were cut through the sagittal plane. Retinal ferritin levels were evaluated using immunofluorescence techniques detailed elsewhere (Dunaief et al., 2002). Rabbit anti-mouse L-ferritin (kind gift from P. Arosio, University of Brescia) was used as a primary antibody. Consecutive sections were tested with L-ferritin and no primary antibodies, the latter serving as a negative control. Anti-rabbit Cy3 was used as a secondary antibody on all sections. Sections were imaged with fluorescence microscopy under constant exposure parameters (Nikon 80i microscope, Nikon, Tokyo, Japan) and analyzed using NIS-Elements (Nikon). Quantification of signal intensity in the various retinal layers was determined by mean intensity measurements using ImageJ (Rasband, 2014). Three sections from each eye were imaged (two primary antibody and one no-primary antibody groups). Mean image intensities were calculated using images obtained by a masked observer from 4 control and 5 experimental mice.

2.4. Toluidine blue and Perls' stain

Enucleated eyes were fixed in 2% paraformaldehyde and 2% glutaraldehyde. Lens and cornea were removed to form eye cups. Eye cups were dehydrated in increasing concentrations of ethanol, and then embedded in plastic (JB4; Polysciences, Inc., Warrington, PA, USA). 3-μm thick plastic sections were cut through the sagittal plane. Plastic sections were stained with toluidine blue, as described elsewhere (Bhoiwala et al., 2015). To evaluate iron levels, plastic sections were stained using the hot Perls' protocol with bleaching (Theurl et al., 2016). Stained sections were evaluated under bright field microscopy (Nikon 80i).

2.5. Quantification of retinal degeneration

Two types of degeneration were studied: vacuolization in the RPE and photoreceptor layers, and thickening of Bruch's membrane. Analysis for both degeneration types was performed on plastic sections using bright field microscopy (Nikon 80i). Five slides, each with 14 consecutive plastic sections, were chosen for each eye. Two slides from temporal sections, one slide containing the optic nerve head, and two slides from nasal sections, were chosen. Slides were masked before analysis.

Vacuolization was evaluated using ImageJ analysis of photomicrographs. Measurements were taken from each slide and section and averaged among all the sections for a given eye. RPE was marked if it was either directly affected by vacuolization and/or adjacent to photoreceptors that were affected by vacuolization. The sum of the lengths of these affected RPE sections in a single image was then divided by the total length of the RPE in that image to calculate the proportion of RPE affected by vacuolization for individual eyes.

2.6. Measurement of iron accumulation in liver

To assess the systemic iron exposures of the mice, livers were harvested following sacrifice, and concentrations of non-heme iron