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Polyethylene glycol induced mouse model of retinal degeneration

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ABSTRACT

Age-related macular degeneration (AMD) is a leading cause of irreversible blindness. This study was done to characterize dry AMD-like changes in mouse retinal pigment epithelium (RPE) and retina after polyethylene glycol (PEG) treatment. We injected male C57BL/6 mice subretinally with PBS, 0.025, 0.25, 0.5 and 1.0 mg of PEG-400 and the animals were sacrificed on day 5. Eyes were harvested and processed for histological analysis. In all other experiments 0.5 mg PEG was injected and animals were sacrificed on days 1, 3, 5 or 14. Paraffin, 5 μm and plastic, 1 μm and 80 nm sections were used for further analysis. Subretinal injection of 0.5 mg PEG induced a 32% reduction of outer nuclear layer (ONL) thickness, 61% decrease of photoreceptor outer and inner segment length, 49% decrease of nuclear density in the ONL and 31% increase of RPE cell density by day 5 after injection. The maximum level of TUNEL positive nuclei in the ONL (6.8 + 1.99%) was detected at day 5 after PEG injection and co-localized with Casp3act. Histological signs of apoptosis were observed in the ONL by light or electron microscopy. Degeneration of RPE cells was found in PEG injected eyes. Gene expression data identified several genes reported to be involved in human AMD. C3, Cfi, Serping1, Mmp9, Htra1 and Lpl were up-regulated in PEG injected eyes compared to PBS controls. PEG leads to morphological and gene expression changes in RPE and retina consistent with dry AMD. This model will be useful to investigate dry AMD pathogenesis and treatment.

1. Introduction

Age-related macular degeneration (AMD) is the leading cause of blindness in the United States as well as around the world. AMD accounts for more than 50% of blindness in Caucasian Americans (Friedman et al., 2004; Congdon et al., 2004). AMD is characterized by drusen deposits, RPE cell changes, geographic atrophy of the RPE cells and choroid capillaries and neovascular maculopathy. There are two clinical subtypes of AMD – dry and wet. These subtypes also represent the pathological stages of AMD. Features of dry AMD can be seen in the "early stage" of AMD that is characterized by accumulation of lipofuscin in RPE, decrease in number of RPE cells and thickening and loss of structure of Bruch's membrane (Coleman et al., 2008; Sarks et al., 1999; Zarbin, 2004). The hallmark of dry AMD is the formation of drusen. Studies have shown that drusen contain acute phase proteins, components of the complement system (C3, C5, C5b-9, CFH and others), lipids, sialic acid and other biochemical components (Coleman et al., 2008; Klein et al., 2005; Nozaki et al., 2006). The "advanced stage" of dry AMD (also known as geographic atrophy) is characterized by hypertrophied RPE cells and calcified Bruch's membrane. Formation of new blood vessels originating from choroid (neovascularization) is the hallmark of wet AMD. Although several studies have focused on treating wet AMD, few studies have reported on treatment for dry AMD (Gehrs et al., 2010; Tan et al., 2008; Yehoshua et al., 2011). Understanding the mechanisms responsible for dry AMD during its initial phases will be highly beneficial in designing new therapeutic strategies for the treatment of dry AMD. For this purpose, availability of a clinically relevant animal model is highly desirable.

We have previously shown that PEG can induce choroidal neovascularization in mouse (Lyzogubov et al., 2011). In the present communication we will describe effects of subretinal injection of PEG (0.5 mg) on retina and choroid. The aim of this study was to investigate PEG-induced RPE and retinal degeneration and to develop a simple and effective animal model of dry AMD.

Abbreviations: AMD, Age-related macular degeneration; RPE, retinal pigment epithelium; Casp3act, active Caspase 3; PCNA, proliferating cell nuclear antigen; ONL, outer nuclear layer; IACUC, Institutional Animal Care and Use Committee; IHC, immunohistochemistry; PIS&POS, photoreceptor inner and outer segment; OPL, outer plexiform layer; OLM, outer limiting membrane; CK18, cytokeratin 18; MMP9, matrix metalloproteinase 9; HTRA1, high-temperature requirement A serine peptidase 1; ONC, optic nerve crush; ChC, choroidal capillaries.

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2. Material and methods

2.1. Animals

Male C57BL/6 mice (6–8 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, ME). Animals were kept under 12-h dark/12-h light conditions. This study was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Arkansas for Medical Sciences, Little Rock, AR.

2.2. Subretinal injection of PEG

Subretinal injection of PEG was performed as previously described (Lyzogubov et al., 2011). Details are given in Supplemental material. To test the dose dependent effect of PEG-400 (Polyethylene glycol with mean molecular weight 400, Spectrum Chemicals and Laboratory Products, Gardena, CA) we divided animals into 5 groups ($n = 5$ animals in each group). We injected PBS, 0.025, 0.25, 0.5 or 1.0 mg of PEG (PEG diluted in PBS). Volume of each injection was 2 μL. Subretinal bleb formation was considered as successful subretinal injection. About 20% retinal detachment was observed immediately after injection however this detachment was further reduced after 24 h of the injection. We have observed that (while injecting) the injected PEG is diffused (spill over) into untreated space and affects photoreceptors and RPE i.e. akin to geographical atrophy (GA). Eyes were harvested at day 5 post-injection. In another experiment we injected 0.5 mg of PEG ($n = 5$ mice) or PBS ($n = 5$ mice) and sacrificed animals at days 1, 3, 5 and 14 post-injection. At each time point we fixed 5 eyes in formalin and 5 eyes were processed for electron microscopy.

2.3. Tissue processing for light and electron microscopy

We prepared formalin fixed paraffin embedded section and plastic sections for light and electron microscopy using routine methods described in details in the Supplemental materials. We stained paraffin sections with hematoxylin and eosin (H&E) or used the sections for immunohistochemistry (IHC). Semi-thin sections were stained using epoxy tissue stain containing toluidine blue and basic fuchsin (Electron Microscopy Sciences, Hatfield, PA). Thin sections were counterstained with uranyl acetate and lead citrate (both from Polysciences Inc., Warrington, PA) and examined using a FEI Tecnai G2 TF20 transmission electron microscope (FEI Worldwide Corporate Headquarters, Hillsboro, Oregon).

2.4. Morphometry

Three H&E stained paraffin sections of each eye were chosen for analysis. We captured 3 images from each eye close to site of the injection between optic nerve and ciliary body. Site of the injection was identified by the presence of tiny retinal damage caused by the needle. We used the ImageJ program (NIH, Bethesda, MD) to measure thickness of the outer nuclear layer (ONL), length of photoreceptor inner and outer segment (PIS&POS), density of nuclei in the ONL and number of RPE cells in the RPE layer. Three measurements were performed for each image. All measurements were performed in a blinded manner by two investigators.

2.5. TUNEL assay and immunohistochemistry

The terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) method was used to detect apoptotic cells. The paraffin sections were stained with the TdT in Situ Apoptosis Detection Kit – Fluorescein TUNEL-based Apoptosis Detection Assay (R&D Systems, Minneapolis, MN).

We stained paraffin sections using a mouse monoclonal anti-PCNA antibody (Ab) (Cell Signaling, Danvers, MA), a rabbit anti-Caspase 3 active (Biovision, Milpitas, CA), rabbit anti-Atg12 (Cell Signaling, Danvers, MA), mouse monoclonal anti-Cytokeratin 18 (CK18) Ab (ABR, Golden, CO), sheep anti-Rhodopsin (Abcam, Eugene, OR); AF488-conjugated goat anti-mouse IgG (H + L); AF594-conjugated donkey anti-rabbit IgG (H + L), AF594-conjugated donkey anti-sheep IgG (H + L) (all from Molecular Probes, Eugene, OR). The mouse on mouse (M.O.M.) Kit (Invitrogen, Valencia, CA) was used to block possible non-specific binding of mouse Ab to mouse IgGs localized in mouse tissue. Negative control sections were stained with isotype matched control Ab at identical concentrations to those of the primary Ab or without primary or secondary Ab. Nuclei were stained using ProLong antifade reagent with DAPI (Invitrogen, Grand Island, NY). The Olympus Vanox-S AH-2 fluorescent microscope (Olympus Optical, Japan) was used to count TUNEL-positive nuclei in the ONL. Representative images were captured using the laser confocal microscope LSM510.

2.6. Total RNA extraction

Mouse eyes, injected with PBS ($n = 5$) or 0.5 mg PEG ($n = 5$) were collected on day 5 after injection. Total RNA was purified separately from retina and RPE-choroid using RNasy protect minikit (Qiagen, Valencia, CA). The RNA from retina and RPE-choroid was shipped to the DNA Facility Core Lab, University of Iowa, IA for gene expression analysis.

2.7. Statistical analysis

Data were analyzed and compared using ANOVA or Student *t*-test, and differences were considered statistically significant with $P < 0.05$. Data are presented as mean value (M) ± standard error (SE).

3. Results

3.1. Dose- and time-dependent effect of PEG on the thickness of ONL

In a first experiment we injected PBS, 0.025, 0.25, 0.5 and 1.0 mg of PEG subretinally to investigate the dose-dependent effect of PEG on thickness of the ONL of the retina. The mice were sacrificed on day 5 post-PEG injection ($n = 5$ mice/group). Eyes were harvested and paraffin (5 μm) sections were stained with H&E. We measured ONL thickness and found that 0.5 and 1.0 mg of PEG significantly (ANOVA test, $p < 0.05$) reduced thickness of the ONL (Fig. 1A–F). In a second experiment, the minimal effective dose (0.5 mg) of PEG or PBS was injected (subretinal) to investigate the time-dependent effect of PEG on thickness of the retinal ONL. The PEG and PBS injected animals ($n = 5$ mice/group) were sacrificed at days 1, 3, 5 and 14 after injection. We measured ONL thickness in paraffin sections stained with H&E and found significant (ANOVA test, $p < 0.05$) reduction of ONL thickness on days 5 and 14 in PEG injected animals compared to PBS treated controls (Fig. 1G). Based on these findings we used subretinal injection of 0.5 mg of PEG for further experiments, and all subsequent animals were sacrificed on day 5 after injection.

3.2. PEG-induced caspase-dependent apoptosis of photoreceptors

Photoreceptor death is a characteristic of dry AMD (Zarbin, 2004). We hypothesized that reduction of ONL thickness after subretinal injection of PEG was caused by death of photoreceptors. This photoreceptor cell death may be caused by apoptosis. To test this hypothesis we performed TUNEL assays and IHC for Casp3act