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Retina

Vitreous Biomarkers for Proliferative Vitreoretinopathy Prognostication in Patients Undergoing Primary Retinal Detachment Repair

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Purpose: To compare baseline levels of exploratory biomarkers in the vitreous fluid of patients with primary retinal detachment who subsequently develop proliferative vitreoretinopathy (PVR) versus those who do not.

Methods: In this exploratory case-control study, we evaluated the baseline protein biomarker levels from a biobank containing the vitreous fluid of patients who had undergone primary pars plana vitrectomy (PPV) for rhegmatogenous retinal detachment. Undiluted samples were collected at the time of PPV and stored at −80°C. Samples from 13 patients who developed PVR within 6 months (PVR group) and 13 age- and gender-matched controls who did not develop PVR (control group) were included. Protein abundance levels were evaluated using a proximity extension assay, and a confirmatory enzyme-linked immunosorbent assay (ELISA) was used to measure the concentration of vimentin.

Results: Baseline vimentin (Normalized Protein eXpression [NPX], 8.6 vs. 6.4, $P < 0.0001$) and heme oxygenase 1 (NPX 8.9 vs. 7.0, $P < 0.001$) levels were found to be elevated in vitreous fluid of patients who subsequently developed PVR compared to those who did not. Confirmatory analysis using ELISA demonstrated mean vimentin concentrations of 7254 vs. 2727 ng/mL in the PVR versus control groups ($P = 0.0152$). The odds ratio for developing PVR was 14 (confidence interval, 1.4–168; $P = 0.03$), assuming a baseline vimentin threshold of 7500 ng/mL.

Conclusions: Vimentin is an intermediate filament protein expressed by retinal glial cells, and our data combined with prior evidence suggest that it may serve as an early vitreous biomarker for subsequent PVR formation and reactive gliosis. Furthermore, we found, for the first time, elevated baseline levels of heme oxygenase 1, a measurable indicator of oxidative stress.

Translational Relevance: Our positive findings could impact clinical care for retinal detachment patients by facilitating risk stratification for targeted interventions or closer monitoring in those at the highest risk of developing PVR.

Introduction

Proliferative vitreoretinopathy (PVR) remains the most common reason for failure of retinal detachment surgery. It is clinically identified by the growth and contraction of fibrotic membranes. The traction from these membranes can lead to progressive retinal detachment, which may reopen previously treated retinal breaks, produce new retinal breaks, or distort the macula.[1]

The development of PVR follows a sequence of cellular and trophic responses. Retinal ischemia, which occurs immediately after retinal detachment, is followed by progressive photoreceptor apoptosis and the contraction of fibrotic epiretinal membranes.[2,3]

The initiation of PVR retinal fibrosis occurs due to fibroblasts that derive from retinal pigment epithelium (RPE) cells and undergo epithelial–mesenchymal transition, starting the process of collagen and extracellular matrix deposition. This process is orchestrated by a panel of dysregulated proinflammatory, chemotactic cytokines and mitogenic growth factors, inducing an exaggerated inflammatory reaction.[4] Early identification of inflammatory or fibrotic factors that predict the development of PVR, along with targeted treatments aimed at impeding or inhibiting PVR development after retinal reattachment surgery, would represent a significant clinical advance.

Currently, there is no approved preventative or pharmacologic treatment for PVR. Identification of prognostic biomarkers indicative of the likelihood of developing PVR after primary vitrectomy for rhegmatogenous retinal detachment (RRD) and prevention through the modification of the initial cue of the PVR process may thus be important to preclude further vision loss.

We aimed to utilize a vitreous biobank to explore and compare baseline levels of a host of protein biomarkers in the vitreous fluid of patients at the time of primary RRD to investigate baseline differences between patients who subsequently developed PVR versus those who did not.

Methods

We conducted a matched case-control study from the vitreous biobank obtained from vitrectomies performed at the University of California, San Francisco and the Zuckerberg San Francisco General Hospital and Trauma Center. The study was approved by the Institutional Review Board of the University of California, San Francisco and adhered to the tenets of the Declaration of Helsinki. Informed consent was obtained from all study participants.

Patients

Between September 2014 and September 2020, all patients who had undergone pars plana vitrectomy (PPV) for RRD repair and had vitreous samples stored at the biobank were screened. Preoperative demographics and pre-, intra-, and postoperative clinical findings were reviewed. Adult patients with primary RRD without PVR at presentation undergoing vitrectomy with at least 6 months of follow-up were included. Exclusion criteria consisted of clinical features of PVR being present at the time of initial surgery; history of rheumatologic and immunoregulatory diseases; local or systemic immunomodulatory or antiproliferative therapies, including corticosteroids; vitreous hemorrhage; uveitis; moderate to advanced glaucoma; concomitant retinal pathology; history of intraocular surgery other than uneventful phacoemulsification for senile cataract; or antecedent ocular trauma. A 1:1 matched control group was selected based on age and sex.

Proteomic Data

Undiluted vitreous samples were obtained from the center of the vitreous cavity at the onset of PPV under full visualization with a closed infusion line. Specimens were immediately flash-frozen and subsequently stored in the biobank freezer at –80°C until they were selected and sent for analysis. An exploratory investigation of protein abundance levels was initially performed using a proximity extension assay (PEA) from the Olink Immuno-Oncology[5] (v.3112) and Oncology II[6] (v.7004) panels. These panels were chosen based on their protein assay lists having the most overlap with potential biomarkers for predicting PVR after RRD and their unique inclusion of a variety of inflammatory cytokines and growth factors. A full list of potential protein biomarkers included in these panels and associated assay validation data (e.g., limit of detection [LOD], lower and upper limits of quantification, within- and between-run precision coefficient of variation) can be found on the manufacturer's website (https://www.olink.com) and Supplementary Tables S1 and S2.[5,6] Each panel simultaneously analyzes 92 proteins utilizing 1 µL of biological sample and reports Normalized Protein eXpression (NPX) values[7] (Olink Proteomics, Uppsala, Sweden). The NPX is an arbitrary unit on a log base 2 scale wherein higher NPX values correlate with higher protein concentrations, where each point difference is equivalent to a twofold change in protein concentration. Based on obtained abundance NPX values and cross-referencing with preexisting literature, we then performed a confirmatory analysis measuring the concentration of vimentin (ng/mL) using an enzyme-linked immunoassay (ELISA) technique (Abcam, Waltham, MA, USA). Vitreous samples were diluted in a 1:30 ratio. Then, 50 µL of the diluted samples was transferred to each well and tested in duplicate according to manufacturer's protocol. Samples from four patients yielded results above the range of detection. The remaining quantity of the vitreous samples from those four patients was diluted to a final ratio of 1:120 and then tested according to the manufacturer's protocol.

Data Analysis

Sample size calculations to determine appropriate group sizes were unable to be performed because of a lack of previously published data on the studied topic. Descriptive and inferential statistics were performed with GraphPad Prism version 9 (GraphPad Software, San Diego, CA, USA). Abundance levels of each protein were compared between PVR and control groups. A false discovery rate approach using the two-stage step-up method of Benjamini, Krieger, and Yekutieli was used to address multiplicity.[8] Proteins with data below the LOD for more than half of the study patients were excluded from the analysis. The *q*-value threshold for significance was set to 5%. Vimentin concentration levels of PVR and control groups were compared using unpaired Welch's *t*-test. The odds ratio for developing PVR was then calculated using an arbitrary cutoff value of 7500 ng/mL. $P < 0.05$ was considered statistically significant.

Results

Of 158 patients with RRD and baseline vitreous samples in the biobank, a total of 13 patients with primary RRD who developed PVR within 6 months (PVR group) and 13 age-, sex-, and ethnicity-matched controls who did not develop PVR (control group) were included in the final analysis. Baseline and surgical characteristics of study patients are summarized in the Table. Mean age and sex composition as well as extent of RRD and number of breaks were balanced between the groups.

A total of 156 proteins that had data above the LOD for more than half of the study patients were included in the final analysis. Supplementary Table S3 highlights the LOD values and percentage below the LOD, stratified by study groups. Details of individual NPX values for all analyzed proteins are included in Supplementary File 1. There were two discoveries, both of which were elevated in patients who ultimately developed PVR: vimentin levels were increased by over fourfold (mean NPX 8.6 vs. 6.4, $P < 0.0001$) (Fig.), and hemoxygenase 1 levels were increased by over threefold (mean NPX 8.9 vs. 7.0, $P < 0.001$) in vitreous fluid from the PVR group compared to controls.

A confirmatory analysis of vimentin levels using ELISA demonstrated a mean baseline concentration of 7254 ng/mL in patients who subsequently developed PVR vs. 2727 ng/mL in patients who did not, $P = 0.0152$ (Fig.). The odds ratio for developing PVR was 14 (confidence interval, 1.4–168; $P = 0.03$), assuming a baseline vimentin threshold of 7500 ng/mL.

Table. Baseline Characteristics of Study Patients at Time of Initial Surgery

Characteristic	Control ($n = 13$)	PVR ($n = 13$)
Age, mean ± SD, y	58 ± 6.2	59 ± 11.4
Sex, male/female, *n*	9/4	9/4
Ethnic background, *n* (%)		
Caucasian	6 (46)	4 (31)
Hispanic	4 (31)	4 (31)
Asian	3 (23)	2 (15)
Other	0	3 (23)
Lens status		
Phakic, *n* (%)	11 (85)	10 (77)
Pseudophakic, *n* (%)	2 (15)	3 (23)
Extent of detachment, mean ± SD, clock hours	7 ± 3.4	7 ± 2.8
Number of breaks, mean ± SD	3 ± 2.6	3 ± 2.1
Tamponade used, *n* (%)		
C_3F_8	11 (85)	11 (85)
SF_6	1 (7.5)	2 (15)
SO	1 (7.5)	0

C_3F_8, perfluoropropane; SF_6, sulfur hexafluoride; SO, silicone oil.

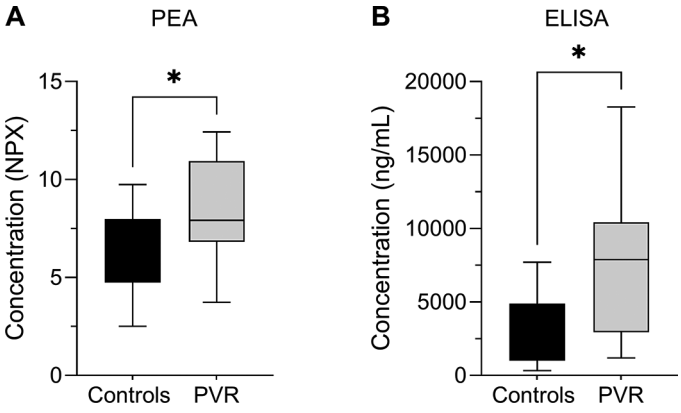

Figure. Comparison of mean baseline vimentin levels in patients undergoing vitrectomy for rhegmatogenous retinal detachment who subsequently developed PVR versus those who did not (control). (**A**) PEA. (**B**) ELISA. *$P < 0.05$.

Discussion

Prognostic molecular biomarkers are agents found in tissues that indicate the likelihood of developing a particular condition, regardless of whether the biomarker remains present or not. Prior studies on animal models have identified sets of proteins that help further our understanding of the pathogenesis of

RRD and its complications.[9] Biomarker profiling of baseline PVR risk has the potential to influence surgical technique and identify patients in whom novel prophylactic adjunctive anti-PVR therapies might be of use. Our study is the first to investigate prognostic vitreous biomarkers in humans prior to the development of PVR.

Increased levels of intermediate filaments (IFs) have previously been reported in the vitreous fluid of patients with retinal detachment (RD), and a positive correlation has been demonstrated with PVR scores.[10] Furthermore, a study of rabbits identified upregulation of vimentin by over twofold in eyes with retinal detachment with immunocytochemistry, confirming this to be primarily within the Müller cells.[11] Vimentin is among the most abundant type III IF proteins in the retina.[12] In mammalian retina, vimentin is primarily found in the two types of macroglia, Müller cells and astrocytes, and while primarily responsible for the cytoskeletal structure of retinal glial cells at the resting state of the retina, it has been found to increase dramatically in response to various types of retinal stress, injuries, and pathologic processes and to serve as a sensitive biomarker for reactive retinal gliosis.[13] However, its baseline expression profile prior to the development of the clinical features of PVR in humans has not been investigated.

A major pool of vimentin in the retina is present in the intracellular cytoskeletal network of retinal glial cells, which are characterized by their insolubility, and is assembled into IF polymers. Expression of these proteins is elevated following central nervous system and retinal injury and may have functional significance in the remodeling process of reactive glial cells.[14] From observation of the cerebrospinal fluid from acute or chronic neurodegenerative diseases, it has been speculated that degradation of the IF polymer causes the release of more soluble fragments of IF to the adjacent fluid compartments, as seen in multiple sclerosis or traumatic brain injury.[15] A previous study noted that vitreous vimentin was elevated in RD with various degrees of PVR,[10] although there have been no studies to date investigating baseline expression levels in relation to subsequent development and prior to onset of PVR. Animal studies have demonstrated that expression levels of the IF proteins such as vimentin are elevated following retinal injury and that they may have functional significance in the remodeling process of reactive glial cells. After injury, rodent Müller cells undergo a rapid change in the composition of their cytoskeleton with the upregulation of vimentin, a response indicative of a state of reactivity. The dynamic relationship between IFs and retinal glia may underscore a key mechanism in the rapid modification of Müller cell structure in response to retinal injury or other changes in the retinal milieu.[14]

To the best of our knowledge, the effect of heme oxygenase 1 (HO-1) expression has not previously been investigated in PVR. HO-1 is an enzyme that catalyzes the reaction of heme catabolism, converting heme to carbon monoxide (CO), biliverdin, and ferrous iron, which exerts anti-inflammatory and antioxidant effects.[16] While HO-1 is typically present at low levels in most tissues, it can be greatly induced by various stimuli.[7] This inducibility is significant, as HO-1 may be one of the most essential mechanisms for cellular protection that is activated during times of stress, including inflammation, ischemia, hypoxia, hyperoxia, hyperthermia, or radiation. The HO reaction may either exhibit cytoprotection by converting prooxidant hemoproteins and heme to the antioxidant bilirubin and biliverdin or, conversely, exacerbate oxidative stress by releasing ferrous iron and CO.[17] Maintenance of antioxidant/oxidant balance and prevention of vascular damage are thus believed to be key functions of HO-1.[18] Tang and colleagues[19] elucidated and validated that ferroptosis, as a novel cell death form induced by the upregulation of HO-1, is the major pathologic process in oxidative stress–mediated RPE death. They concluded that targeting HO-1–mediated RPE ferroptosis could serve as an effective retinal-protective strategy for degenerative retinal disease prevention. Ye et al.[20] found that elevated HO-1 expression contributes to transforming growth factor β1–induced lung myofibroblast differentiation through the activation of the serine/threonine kinase AKT pathway, suggesting that targeting E2F transcription factor 2/HO-1 might be a new therapeutic strategy to treat fibrotic diseases such as idiopathic pulmonary fibrosis. The role of HO-1 in the context of retinal detachment/fibrosis has yet to be elucidated.

Increased IFs and total protein present in the vitreous body may have more implications for PVR formation because, clinically, residual vitreous gel from incomplete removal of vitreous during vitrectomy has been postulated to be associated with recurrent retinal detachment with PVR formation. Additional studies are necessary to examine whether other altered proteins present in the vitreous body from the retinal detachment may contribute to PVR formation. Furthermore, identifying potential prognostic biomarkers may have therapeutic implications in an era when pharmacotherapy as an adjunct treatment method to improve anatomic success and postoperative visual acuity continues to be a very active area of investigation.[21,22] For example, inhibition of soluble

IFs has been shown to be a promising therapeutic target for diverse gliosis-dependent injuries in animal models using the vimentin-targeting small molecule withaferin A.[23]

The limitations of this study include the relatively small numbers of patients and the lack of reference physiologic values for the investigated vitreous biomarkers. PEA combines antibody-based immunoassays with DNA-based technologies, and the polymerase chain reaction–based amplification step allows for protein detection in small volumes and at very low concentrations (e.g., femtograms).[24] Analytes that are highly abundant in human plasma are usually not offered on standard PEA panels because they would require further sample dilution, which could affect the detection of other less abundant analytes. PEA is considered a useful resource for biomarker screening and target discovery, but it only provides relative protein levels.[7] With proteomic technologies, there can be substantial interstudy variability based on the analysis techniques, sensitivity, and abundance levels of the investigated proteins.[25] The use of appropriate samples, analytical platform, and bioinformatic method are essential factors to consider when undertaking such studies to ensure reliable results. At least one confirmatory test, commonly Western blotting, immunohistochemistry, or ELISA, all of which rely on antibody–antigen reactions, has been recommended.[26] The use of two different quantification techniques was also an attempt to implement confirmatory testing for vimentin, which had previously been studied in the context of PVR. Unfortunately, it was not possible to use ELISA to test the concentration of HO-1 on samples from those same patients because the vitreous volume remaining was insufficient. In the current study, an attempt was made to meticulously match study patients based on baseline demographic characteristics. Furthermore, the impact of storage time and processing of specimens in our measurements is unclear, although we anticipate that any potential impact would affect the two groups in a similar way.

In conclusion, vimentin is an IF protein expressed by retinal glial cells, and our data suggest that high levels of vitreous vimentin at the time of initial RRD repair (>7500 ng/mL) are associated with increased odds (odds ratio, 14) of subsequent PVR formation and may serve as a prognostic biomarker. We also report, for the first time, elevated baseline levels of HO-1, a measurable indicator of oxidative stress. Further studies are needed to evaluate validation data sets and to examine the functional role of other IFs and biomarkers in PVR formation and the role of prophylactic interventions.

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