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TITLE: Annexin A2 in Proliferative Vitreoretinopathy

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14. ABSTRACT Proliferative vitreoretinopathy (PVR) is a potentially blinding disorder that occurs in almost one-half of military personnel who have suffered a penetrating wound to the eye. PVR involves migration of pigmented retinal epithelial (RPE) cells from the outermost layer of the retina to the innermost layer at the point of retinal penetration, with subsequent epiretinal membrane or scar formation. We have discovered that when we induce an eye injury in mice that lack a protein called annexin A2 (ANXA2), the PVR process is greatly attenuated, and scar formation rarely occurs. In the past year, we have shown that RPE cells lacking ANXA2 fail to migrate in the presence of macrophages in an <i>in vitro</i> system. In addition, analysis of human retinal tissue from subjects undergoing ocular surgery for PVR reveals the presence of A2-immunoreactive cells within the PVR scar. In the third year of this project, we plan to explore the molecular mechanism whereby ANXA2 is required for RPE cell migration induced by macrophages. In addition, we will examine the effects of blockade of ANXA2 activity on macrophage-induced RPE cell migration <i>in vitro</i> , and PVR development in our mouse model. We hope that this work will lead to new approaches to the treatment of PVR in humans.					
15. SUBJECT TERMS Nothing listed					
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1. INTRODUCTION.

Proliferative vitreoretinopathy (PVR) is a potentially blinding disorder that occurs in almost one-half of military personnel who have suffered a penetrating wound to the eye. When there is a tear in the retina, cells inside the eye (retinal pigmented epithelial [RPE] cells) begin to proliferate and, over time, can form a scar that pulls the remaining retina away from the back of the eye, compromising vision. PVR involves migration of pigmented retinal epithelial (RPE) cells from the outermost layer of the retina to the innermost layer at the point of retinal penetration, with subsequent epiretinal membrane or scar formation. We have discovered that when we induce an eye injury in mice that lack a protein called annexin A2 (ANXA2), the PVR process is greatly attenuated, and scar formation rarely occurs. ANXA2 is a calcium-dependent, phospholipid-binding protein that mediates membrane remodeling events within cells. With our collaborators, we have shown that this protein supports repair of the lysosome's limiting membrane in innate immune cells such as macrophages, thus preventing activation of the inflammasome and release of inflammatory cytokines, such as, interleukin-1 β and interleukin-18. The current project is aimed at determining whether ANXA2 is required for PVR scar formation, and, if so, on which cells and also the mechanism by which it carries out this action. Data obtained in the past year confirm our hypothesis that ANXA2 is required for the full expression of PVR. In the dispase model in the mouse, we see minimal migration of RPE cells in *Anxa2*^{-/-} compared with *Anxa2*^{+/+} mice. In addition, we have used methods newly developed in our lab for the harvest of mouse RPE cells to show that RPE cells lacking ANXA2 fail to migrate in the presence of macrophages. Finally, analysis of human retinal tissue from subjects undergoing ocular surgery for PVR reveals the presence of A2-immunoreactive cells that express both macrophage and RPE cell markers within the PVR scar.

2. KEYWORDS.

proliferative vitreal retinopathy
annexin
macrophage
retinal pigmented epithelial cell
dispase
penetrating ocular injury
diabetic retinopathy
epithelial-mesenchymal transition

3. ACCOMPLISHMENTS.

Major accomplishments in the past year are presented below under headings for each Specific Aim.

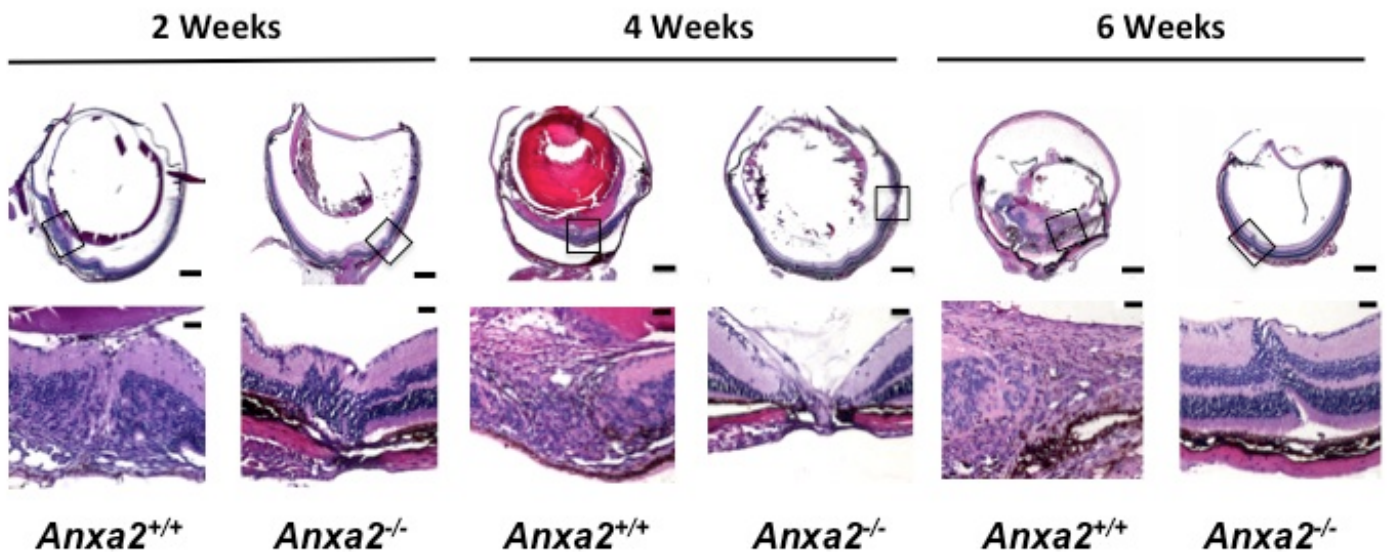
SPECIFIC AIM 1: TO DETERMINE THE ROLE OF ANXA2 IN MURINE PVR.

Task 1: Establishing the model. Under IACUC approval, we have now established a standard murine model of human PVR. Using a single batch of dispase over a range of time points (24 hours - 6 weeks) and series of concentrations (0.001 - 0.3 U/ul), we have established that a dose of 0.3 U/ul provides the best model of disease, and best illustrates the differential response to dispase in *Anxa2*^{-/-} and *Anxa2*^{+/+} mice. RPE migration was observed at all doses, but significant histologic modifications were most apparent at doses between 0.1 and 0.3 U/ul.

Key results:

[1] Overall, we observe no significant difference between the two genotypes at 24 hours after dispase injection. In both *Anxa2*^{-/-} and *Anxa2*^{+/+} mice, a few displaced pigmented cells can be found near the scleral edge within the healing region of the injection site. In addition, a small amount of hemorrhage near the vitreal surface of the retina is also typically present with occasional membranous strands and associated inflammatory cells in both genotypes.

[2] On the other hand, we see striking differences between *Anxa2*^{-/-} and *Anxa2*^{+/+} mice at 2, 4, and 6 weeks after dispase injection (**Figure 1**). By 2 weeks post-dispase, RPE cells, which are easily recognized by their pigmented nature, can be seen streaming along the injection site and residing on the vitreal surface of the retina in the *Anxa2*^{+/+} mouse (**see higher power views**). In the *Anxa2*^{-/-} mouse, however, RPE cells are seen in these locations infrequently, and remain associated with the RPE epithelial layer. By 4 weeks post-dispase, we typically observe a chaotic disruption of the normal cell layers of the retina in the *Anxa2*^{+/+} mouse, with continuing invasion of RPE cells toward the vitreal surface of the retina. These findings are almost never seen in the *Anxa2*^{-/-} retina, although there is sometimes formation of a small, non-pigmented scar at the injection site. At 6 weeks, there is extreme disruption of the normal architecture of the *Anxa2*^{+/+} retina with loss of defined cell layers and retraction of the retina to form a mass containing pigmented cells posterior to the ocular lens. In the *Anxa2*^{-/-} eye, on the other hand, there usually only is slight disruption of the retinal cell layers, but no ectopic presence of RPE cells, and no retraction of the retina. In the *Anxa2*^{-/-} eye, the normal architecture of the retina and its typical distribution over the inner surface of the eye are also preserved.



Size markers for low and high power views indicate 400 μ m and 40 μ m, respectively.

Figure 1. Histology of PVR in *Anxa2*^{-/-} and *Anxa2*^{+/+} mice. Hematoxylin and eosin stained sections through the dispase injection site of eyes from *Anxa2*^{-/-} and *Anxa2*^{+/+} mice at 2, 4, and 6 weeks. Note the minimal histologic disruption in *Anxa2*^{-/-} eyes versus extreme and progressive dysmorphology, with invasion of RPE cells (pigmented) into and beyond the retina in *Anxa2*^{+/+} eyes.

[3] We are now in the process of completing a comprehensive, semi-quantitative histologic analysis of hematoxylin and eosin-stained sections of dispase eyes. For semi-quantitative scoring, we have devised an overall PVR Histology Index (PHI) (**Figure 2**). The PHI is based on the degree of retinal damage (detachment and disruption of normal cell layers), as well as formation of an epiretinal membrane or scar with cellular invasion. Our preliminary data suggest that retinal detachment, disruption of the retinal architecture, and formation of an epiretinal membrane or scar are significantly more prominent in the wild type versus ANXA2-deficient mouse.

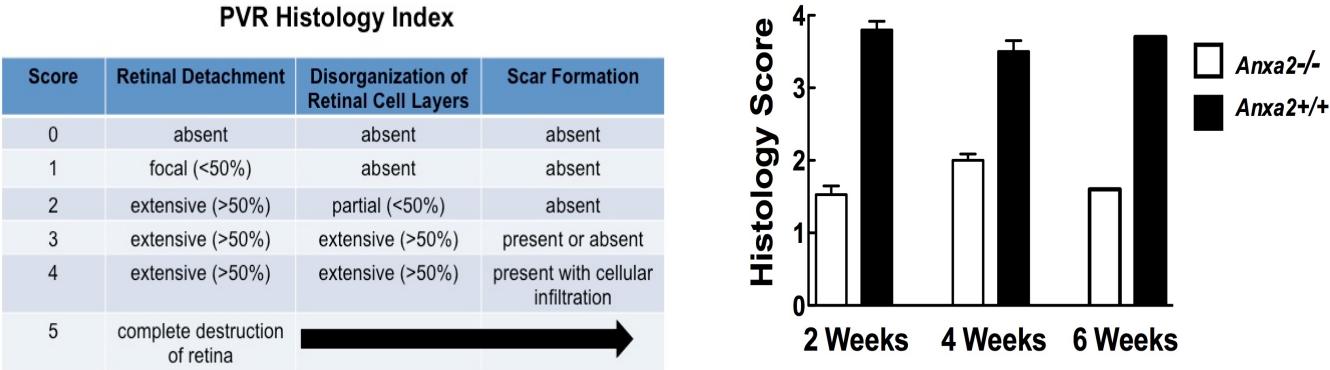


Figure 2. Analysis of ocular histology in the dispase model of PVR. After dispase injection, hematoxylin- and eosin-stained sections from a total of 53 mice were analyzed. Sections from six groups of mice (n=7-11 mice per group) representing *Anxa2*^{-/-} or *Anxa2*^{+/+} mice at 2, 4, or 6 weeks were scored using a standard algorithm (table above) were evaluated by 3 trained observers in a double-blind fashion. Inter-observer variability for the 6 groups averaged 10.4 ± 3.1%. The results indicate a highly significant increase in severity, as judged by histology score, in *Anxa2*^{+/+} vs. *Anxa2*^{-/-} mice (bar graph).

We are now working to expand this analysis to include measures of RPE cell migration through and beyond the retina within dispase-treated eyes.

Together, these data indicate that the PVR response in the mouse is greatly attenuated in the absence of annexin A2.

Task 2: Macrophage depletion and tissue specific knockout. We have completed the characterization of macrophage specific knockout mice, and have performed a preliminary experiment comparing the response to dispase in *Anxa2*^{ff}/*LysM*:*Cre* mice (macrophage-specific knockouts), as well as *Anxa2*^{ff} mice (controls) at 4 weeks. Our initial data indicate a significantly more severe response in *Anxa2*^{ff}/*LysM*:*Cre* mice in terms of increased epiretinal membrane formation and disruption of the overall ocular architecture. We are now in the process of planning a more extensive experiment utilizing these mice at a series of time points.

Task 3: Cytokine and growth factor profiling. Plasma and vitreous fluid from *Anxa2*^{-/-} and *Anxa2*^{+/+} mice treated with intraocular dispase will be profiled for cytokine and growth factors in year 3.

Task 4: ANXA2 blockade in murine PVR. In Year 3, we will test the effects of anti-ANXA2 antibodies, peptides, and possibly small molecule inhibitors on the response to dispase injection in *Anxa2*^{+/+} mice.

SPECIFIC AIM 2: TO DEFINE ANXA2-DEPENDENT MACROPHAGE-RPE CELL INTERACTIONS IN MURINE PVR.

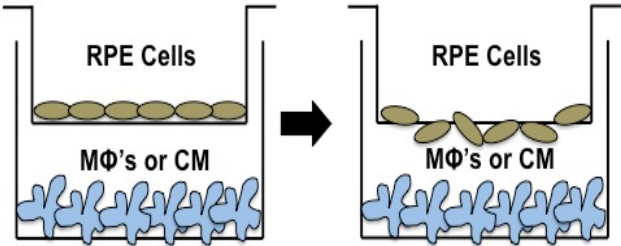
Task 1: Cell harvest and culture of genotype-specific murine cells.

Harvest of macrophages. We have now established a reliable method for the harvest and propagation of bone marrow-derived macrophages from both *Anxa2*^{-/-} and *Anxa2*^{+/+} mice. Based upon expression of the F4/80 macrophage marker, we routinely achieve ~99% purity, and have been using these cells in co-culture cell migration assays (see below).

Harvest and culture of RPE cells. We have developed a reproducible method for the harvest and culture of mouse RPE cells. To isolate RPE cells, the cornea, vitreous, and retinas were removed from enucleated mouse eyes. The resulting shells (eye cups) are flattened and incubated with fresh, sterile 0.25% trypsin (pH 8.0, 1h, 37°C) with occasional trituration. Released sheets of RPE cells are collected and retrypsinized, and the digestion halted with serum-containing RPE medium. After washing by centrifugation, the cells are plated on chamber slides for immunohistochemistry, or on transwell filters for migration assays (37°C, 5% CO₂). Cells are fed on day 5, and, thereafter, 3 times per week with RPE medium. Using this procedure, we typically obtain ~98% purity over 10 days in culture, based on the presence of pigmented granules in the cells, and their expression of RPE65, a specific RPE cell marker. We have also verified abundant expression of ANXA2 in wild type murine RPE cells.

Task 2: Genotype-specific co-culture experiments. We have now completed a series of co-culture migration studies using murine bone marrow-derived macrophages and RPE cells. Either *Anxa2*^{+/+} or *Anxa2*^{-/-} RPE cells were seeded in transwell assemblies on laminin-coated, 2-micron pore filters in upper chambers positioned above a lower well containing RPE medium, macrophage medium, or macrophage conditioned medium, with or without *Anxa2*^{+/+} or *Anxa2*^{-/-} macrophages (**Figure 3A**). After removal of residual cells from the upper side of the filter, the underside was stained with crystal violet, and RPE cell migration to the underside assessed at 24h using NIH Elements image software. Of interest, migration of RPE cells appears to depend upon both the presence of *Anxa2*^{+/+} macrophages in the lower chamber, and the expression of *Anxa2*^{+/+} in RPE cells in the upper chamber. Importantly, there was almost no migration of *Anxa2*^{-/-} RPE cells under any condition (**Figure 3B**).

A.



B.

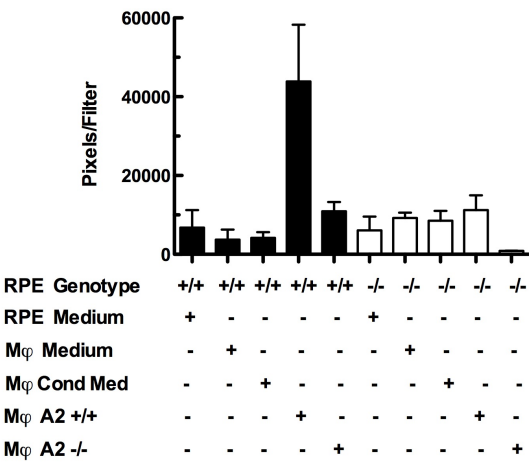


Figure 3. RPE cell macrophage-induced directed migration. **A.** *Anxa2*^{+/+} or *Anxa2*^{-/-} RPE cells were cultured atop transwell filters positioned above macrophage medium with or without macrophages of either genotype as indicated. **B.** Migration of RPE cells to the underside of the filter was quantified using standard image analysis software.

*These results suggest that *Anxa2*^{-/-} RPE cells have a very limited capacity to undergo migration in response to macrophages or their secretion products. In addition, migration of *Anxa2*^{+/+} RPE to the lower chamber was much more robust when stimulated by *Anxa2*^{+/+} macrophages as opposed to their conditioned medium, suggestion that either cell-cell contact or the presence of a labile secreted product is required.*

Task 3: Cytokine and growth factor analyses. In the next project period, we will profile cytokines and growth factors present in the post-culture medium of macrophages cultured in the presence and absence of genotype-specific RPE cells.

Task 4: Blockade of ANXA2-dependent cytokines. In Year 3, we will test the effects of anti-ANXA2 antibodies, peptides, and possibly small molecule inhibitors on the migration response of RPE cells to *Anxa2*^{+/+} macrophages.

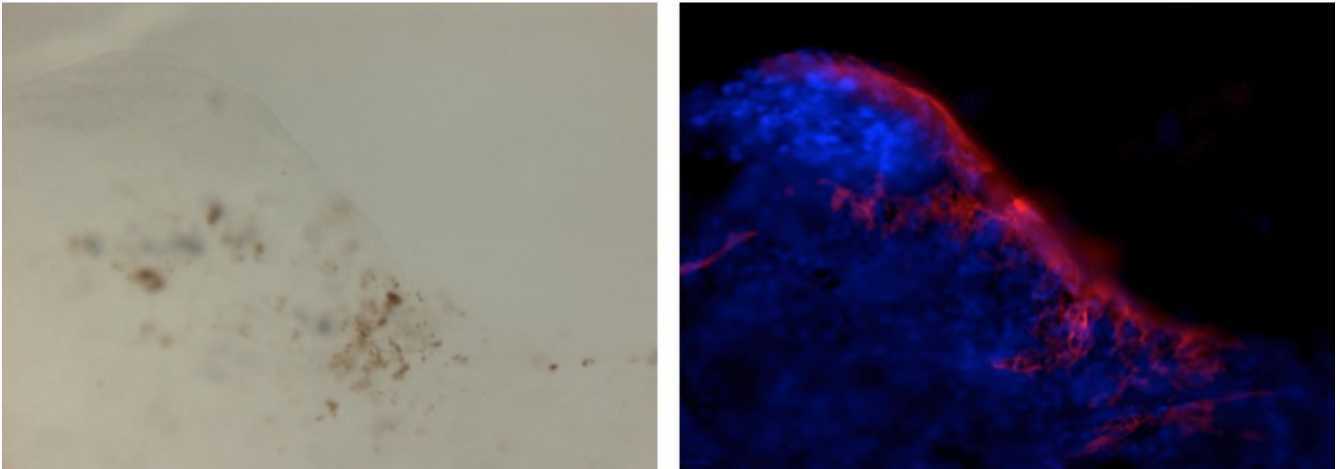
SPECIFIC AIM 3: ANXA2 IN HUMAN PVR.

Task 1: IRB protocol preparation and approval. Completed.

Task 2: Sample procurement. Through our Department of Ophthalmology colleagues, we have now received a total of three PVR samples from consenting human subjects. We have accelerated efforts to obtain additional samples.

Task 3: Sample analysis. Epiretinal PVR membranes were harvested in the Operating Room of the New York-Presbyterian Hospital by Dr. Szilard Kiss, a co-investigator on this protocol. Samples were placed on sterile MF-Millipore filters, and collected immediately by a member of Dr. Hajjar's research group. PVR membrane-containing filters were placed in tissue culture dishes, and the membrane fixed with 2% paraformaldehyde (10 min, 21°C). The membrane was then rinsed three times with 1X PBS containing Ca⁺⁺ and Mg⁺⁺ and stored at 4°C for up to 22 days. For immunofluorescence staining, autofluorescence was quenched with NH₄Cl, and sections were then blocked with normal donkey serum. Staining with primary and secondary antibodies was at 4°C overnight and 30 min at 21°C, respectively. Rabbit anti-annexin A2, followed by Cy3-labeled donkey anti-rabbit, was used. Sections were counterstained with DAPI to visualize cell nuclei. Sections were imaged using Nikon 80i microscope at 200x power (**Figure 4**).

Figure 4 (below). Cells within human PVR membranes express annexin A2. Brightfield (**left**) and immunofluorescence (**right**) images of human PVR membrane removed at surgery. The brightfield image shows the presence of pigmented cells, most likely RPE cells, or a derivative thereof. The immunofluorescence image shows the cellular nature of the membrane by DAPI staining, and also reveals cell-associated anti-annexin A2 immunoreactive material (**red**), at the vitreal border of the membrane.



Further preliminary immunofluorescence staining of human PVR samples reveal cellular co-association of the human macrophage marker, CD68, with anti-annexin A2 (**Figure 6A**) and co-association of the RPE cell marker RPE65 with anti-annexin A2 (**Figure 6B**). Confocal images will be analyzed quantitatively to determine whether marker co-staining represents co-expression within the same cell.

Figure 6A:

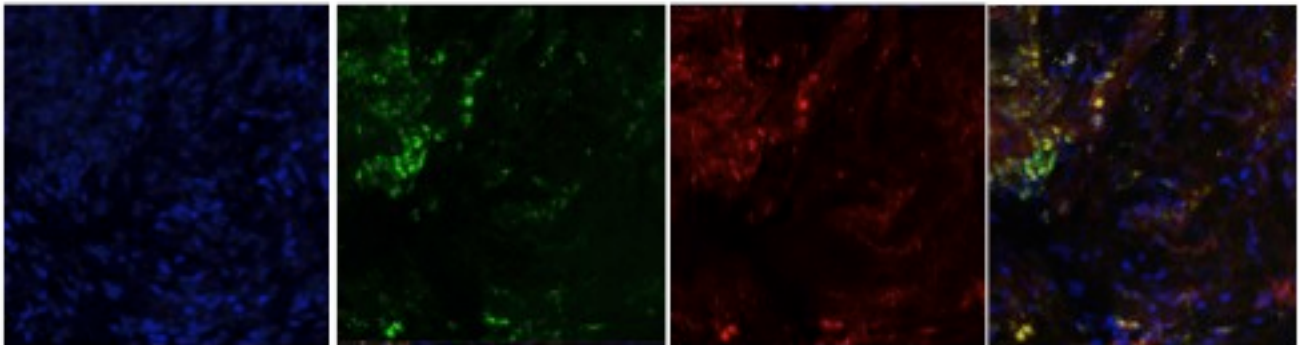


Figure 6B:

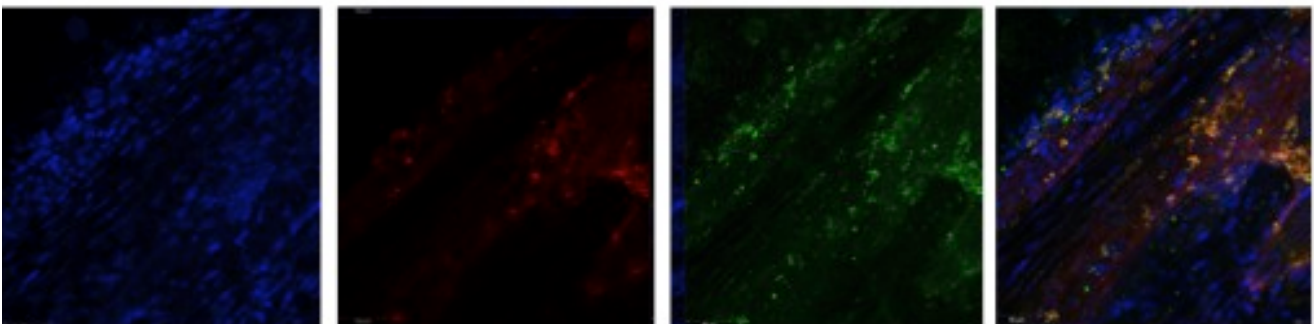


Figure 6 (above). ANXA2 and CD68 co-staining in human surgical PVR samples. Samples were obtained fresh from the operating room, fixed in 2% paraformaldehyde, embedded in paraffin, and sectioned. Sections were quenched with NH_4Cl , and then blocked with normal donkey serum. **A.** Section stained with DAPI (**blue**), anti-annexin A2 (**red**), and anti-CD68 (**green**). **B.** Section stained with DAPI (**blue**), anti-annexin A2 (**red**), and anti-RPE65 (**green**).

Together, these data suggest that both ANXA2-positive macrophages and ANXA2-positive RPE cells are present within epiretinal membranes of human subjects with proliferative vitreal retinopathy.

Task 4: Human co-culture. We have begun working on isolating human RPE cells from cadaveric eyes provided by the Eye Bank of New York.

Opportunities for training and professional development: This grant has provided a unique training opportunity for Dr. Nadia Hedhli. After two years, she has developed strong expertise in execution of the dispase PVR model, in macrophage and RPE cell isolation, and in interpretation of retinal histology in the mouse and human.

Dissemination of results to communities of interest: No public reports yet.

Plans for next reporting period:

Specific Aim 1: Under this objective and over the next year, we will complete our histologic analysis of ocular tissue from dispase-treated *Anxa2*^{+/+} or *Anxa2*^{-/-} mice. We will complete experiments on the response to dispase induced PVR in macrophage-specific ANXA2-deficient mice. We will complete plasma and vitreal cytokine and growth factor profiling in dispase-treated mice. We will conduct annexin A2-blockade experiments in the dispase mouse model in *Anxa2*^{+/+} mice.

Specific Aim 2: Under this aim and over the next year, we will profile cytokines and growth factors present in the post-culture medium of macrophages cultured in the presence and absence of genotype-specific RPE cells. We will also test the effects of anti-ANXA2 antibodies, peptides, and possibly small molecule inhibitors on the migration response of RPE cells to *Anxa2*^{+/+} macrophages.

Specific Aim 3: Under Specific Aim 3, we continue to procure and archive additional matched retinal and blood samples from human patients with PVR. We will continue our analysis of marker expression for RPE and macrophage cells, and will complete our analysis of plasma cytokines in these subjects.

4. IMPACT.

On the principal discipline of the project: Our data reveal that ANXA2 is required for the full expression of PVR in the murine dispase model. We are developing an improved

mechanistic understanding of how ANXA2 contributes to PVR. We hope to define this mechanism at the molecular level, thus providing a conceptual basis for novel preventative treatments for PVR through blockade of ANXA2.

On other disciplines: Nothing to report

On technology transfer: Nothing to report

On society beyond science and technology: Nothing to report

5. CHANGES/PROBLEMS.

Changes in approach and reasons for change: none

Actual or anticipated problems or delays and actions or plans to resolve them:

In the first half of the year, recruitment of human subjects has proven to be somewhat slower than anticipated. Several months ago, however, met with our colleagues in the Department of Ophthalmology and instituted a weekly reminder system for identifying eligible patients and obtaining consent. This has resulted in an increased rate of sample procurement.

Changes that had significant effect on expenditures: none

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents: none

6. PRODUCTS.

Journal publications, conference papers, and presentations: nothing to report

Website or other internet site: nothing to report

Technologies or techniques: nothing to report

Inventions, patent applications, and/or licenses: nothing to report

Other products: nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS.

Name	Katherine A. Hajjar, MD
Project role	PI
Researcher identifier	
Nearest person month	2
Contribution	Oversight of all aspects of the project
Funding support	This grant

Name	Szilard Kiss, MD
Project role	Co-PI
Researcher identifier	
Nearest person month	1
Contribution	Establishment of human protocol; human sample procurement
Funding support	This grant

Name	Nadia Hedhli, PhD
Project role	Postdoctoral associate
Researcher identifier	
Nearest person month	12
Contribution	Establishment of RPE/macrophage co-culture
Funding support	This grant

Name	Dena Almeida
Project role	Technician
Researcher identifier	
Nearest person month	2
Contribution	Establishment of dispase model, cell and tissue staining
Funding support	This grant

Has there been a change in the active other support of the PI/PD or senior/key personnel since the last reporting period? Nothing to report

What other organizations are involved as partners? Nothing to report

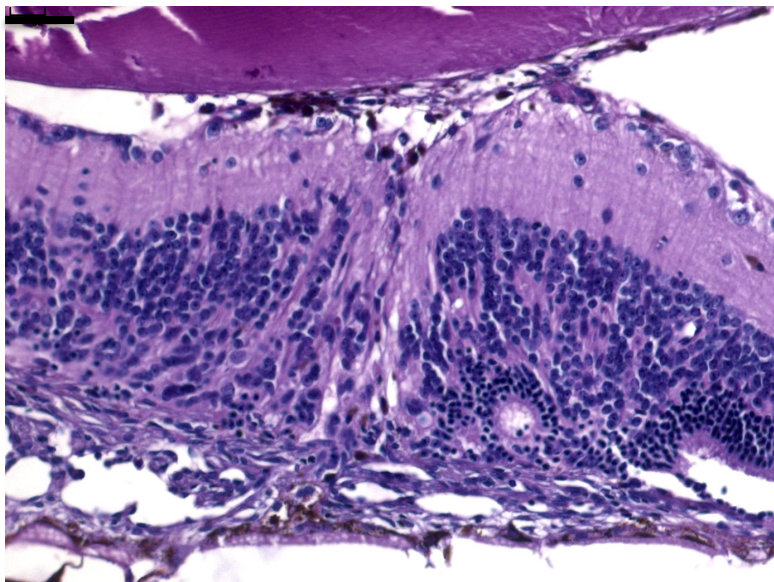
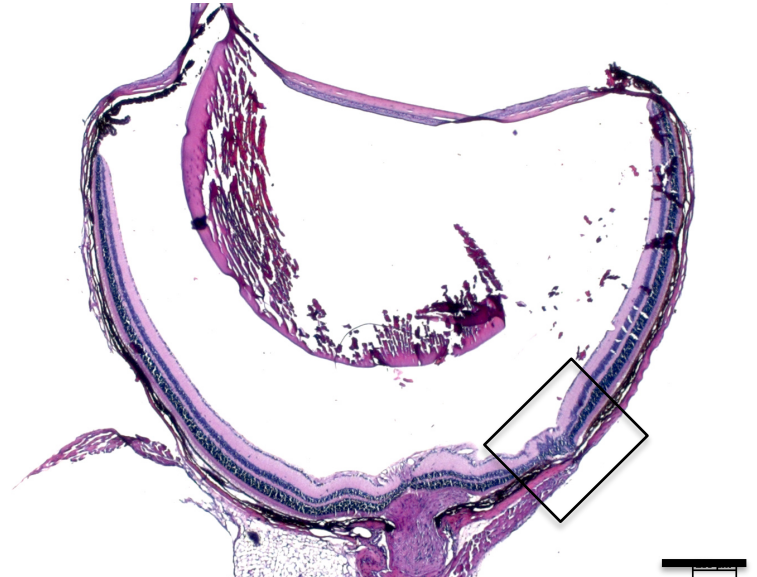
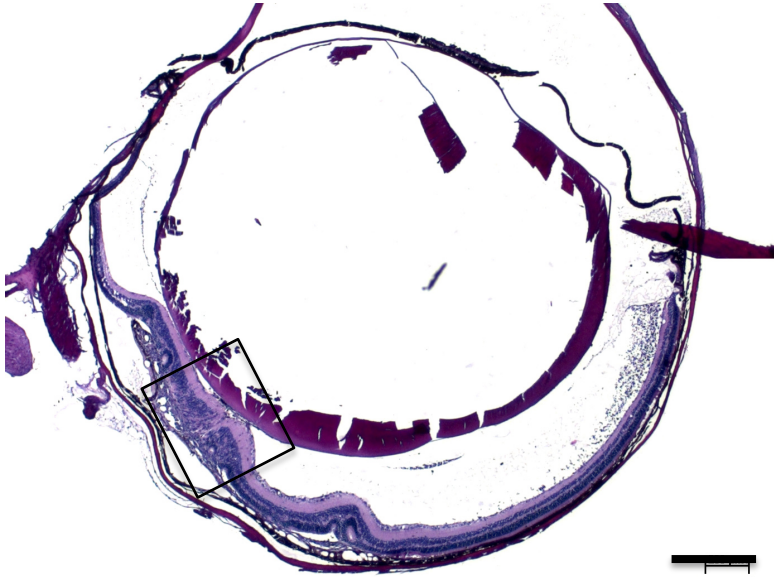
8. SPECIAL REPORTING REQUIREMENTS

Please find attached Quad Chart

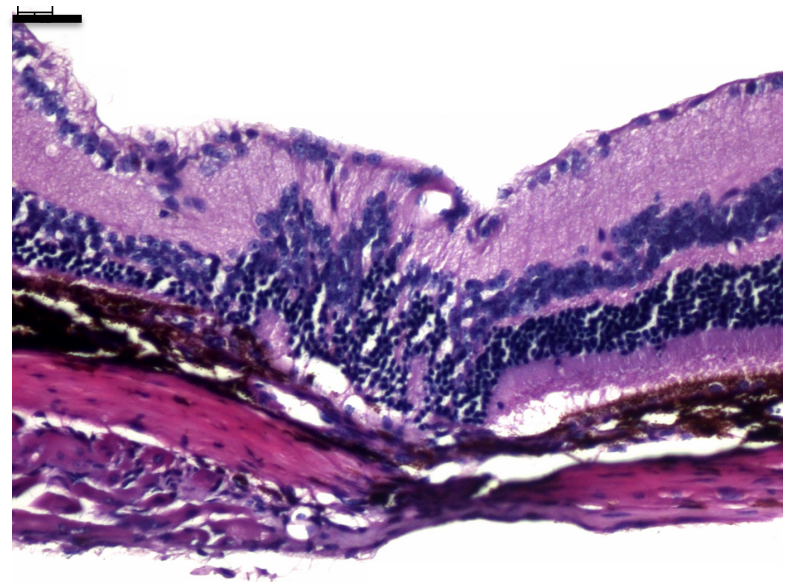
9. APPENDICES

Enlarged images: PVR at 2, 4, and 6 weeks in *Anxa2*^{+/+} and *Anxa2*^{-/-} mice.

PVR 2wks 0.3U/ μ l

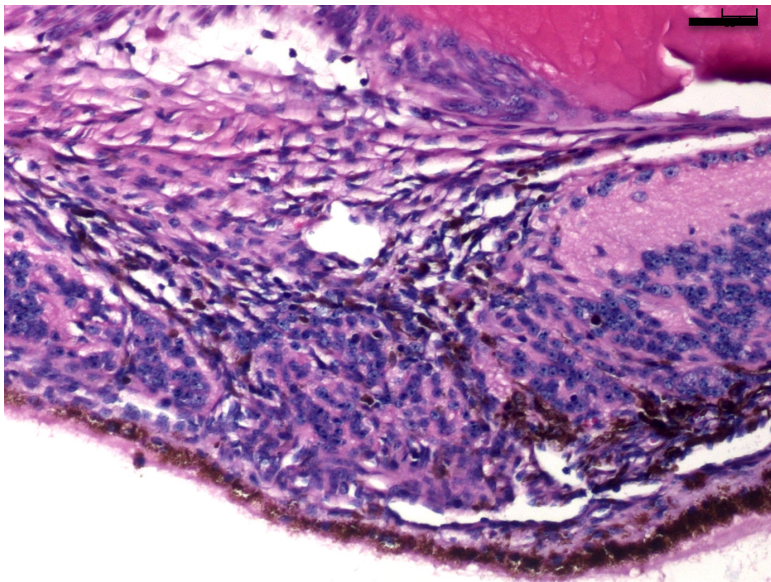
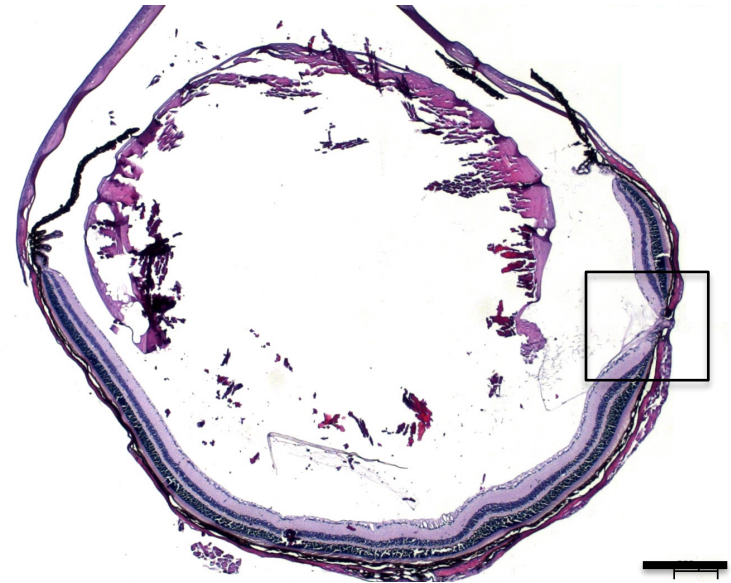
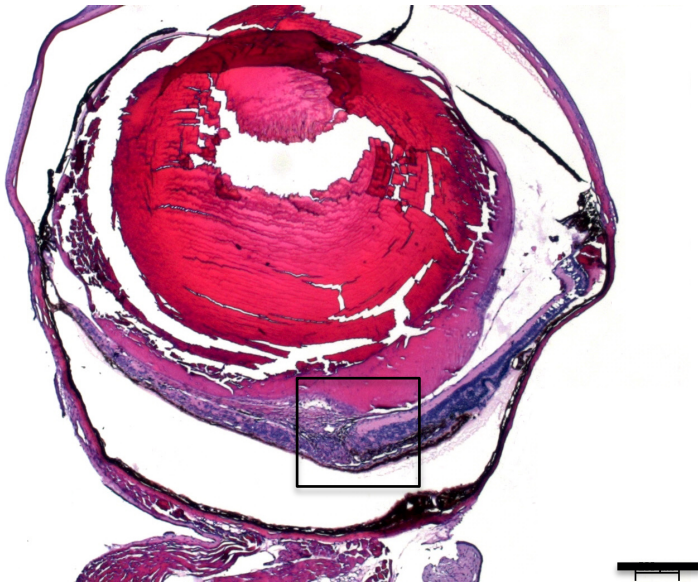


Anxa2^{+/+}

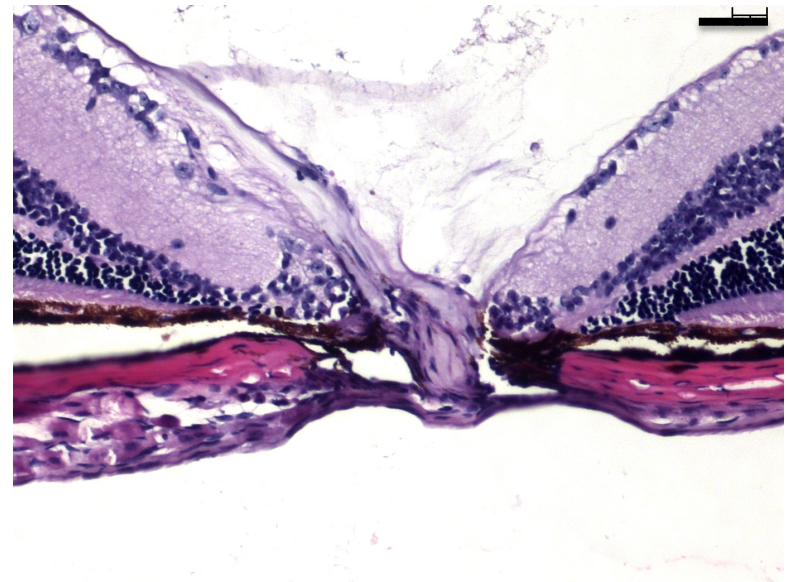


Anxa2^{-/-}

PVR 4wks 0.3U/ μ l

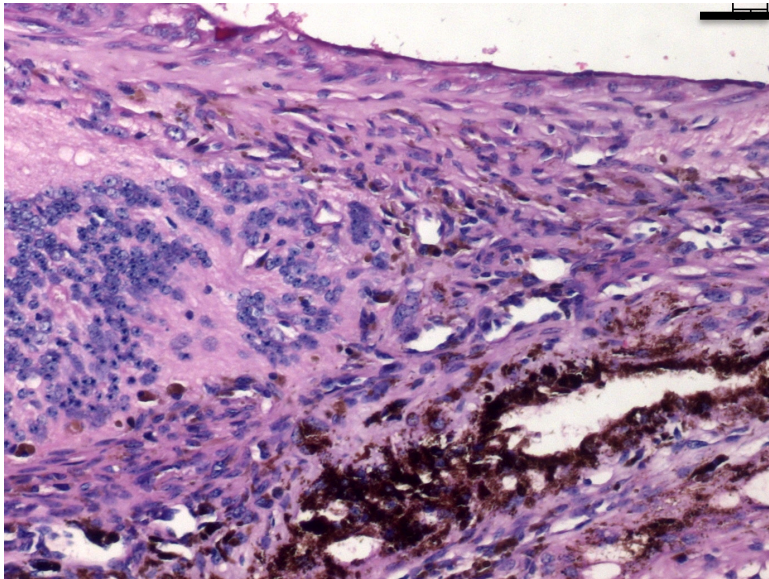
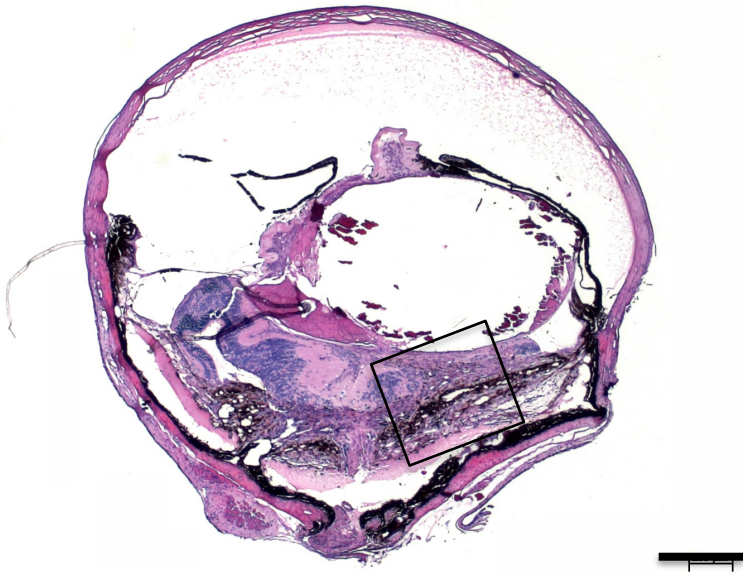


Anxa2^{+/+}

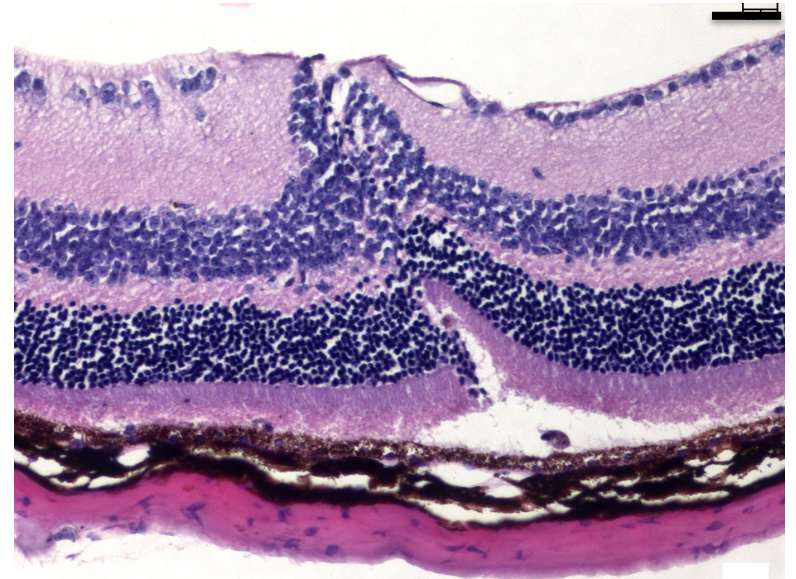
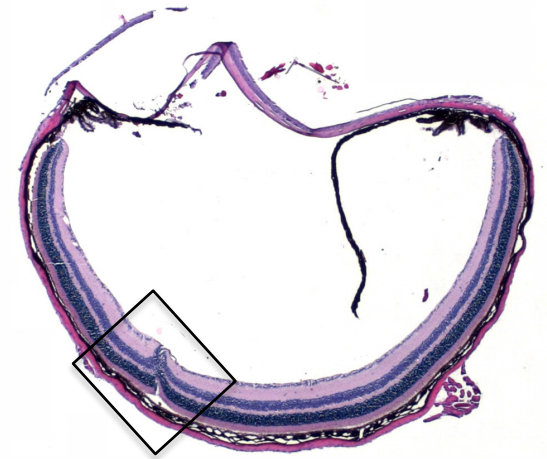


Anxa2^{-/-}

PVR 6wks 0.3U/ μ l



Anxa2^{+/+}



Anxa2^{-/-}

Annexin A2 and Proliferative Vitreoretinopathy

Log No. MR130194



PI Katherine A. Hajjar, MD

Org: Weill Cornell Medical College

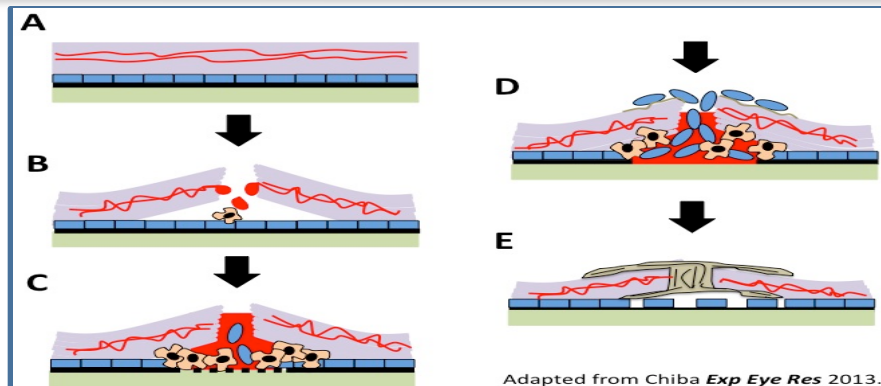
Award Amount: \$1,000,000

Study/Product Aim(s)

- To analyze the functional role of annexin A2 and related molecules in a mouse model of proliferative vitreoretinopathy (PVR).
- To specify PVR-related, annexin A2-dependent interactions between RPE cells and macrophages.
- To define the role of the annexin A2 system in the pathogenesis and progression of human PVR.

Approach

This project will address the hypothesis that, in PVR, early recruitment and activity of macrophages to sites of retinal injury depends upon their expression of annexin A2. We postulate that macrophages produce proteases, growth factors, and signaling molecules that transform quiescent RPE cells into motile, fibrogenic cells that engender pre- and epiretinal scar formation, leading to further retinal damage and loss of vision.



Hypothesis: Upon retinal injury, macrophage (orange) expression of annexin A2 leads to RPE cell (blue) activation, migration, and epi/preretinal membrane scars.

We have recently established that macrophage recruitment to the hypoxic mouse retina is greatly reduced in the annexin A2-deficient mouse.

Timeline and Cost

Activities	CY	14	15	16	17
Prepare and submit application					
Aim1: Mouse model completed					
Aim 2: Co-culture studies underway					
Aim 3: Human subject studies underway					
Estimated Budget (\$K)		\$000	\$333	\$333	\$334

Goals/Milestones (Example)

CY13 Goal – Submit pre-application

☒ Completed

CY14 Goals – Submit full application and initiate project

☒ Establish PVR model in *AnxA2^{-/-}*, *S100A10^{-/-}* and *S100A4^{-/-}* mice

☒ Establish macrophage-RPE co-culture systems

☒ Initiate collection of human PVR samples

CY15 Goal – Continue experiments related to Aims 1-3

☒ Study PVR macrophage-specific A2 knockouts

☒ Continue macrophage and RPE signaling experiments

☐ Initiate human RPE cell-macrophage experiments

CY16 Goal – Complete experiments and submit manuscripts

☐ Initiate A2 blockade experiments in mice

☐ Complete in vitro A2 blockade experiments

☐ Complete human vitreal profiling

Comments/Challenges/Issues/Concerns

Budget Expenditure to Date

Projected Expenditure: \$1,000,000

Actual Expenditure: \$0

Updated: 36/30/15