

AWARD NUMBER: W81XWH-14-1-0507

TITLE: Annexin A2 in Proliferative Vitreoretinopathy

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REPORT DATE: October-2015

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE October 2015		2. REPORT TYPE Annual		3. DATES COVERED 30 Sep 1014 - 29 Sep 2015	
4. TITLE AND SUBTITLE Annexin A2 in Proliferative Vitreoretinopathy				5a. CONTRACT NUMBER W81XWH-14-1-0507	
				5b. GRANT NUMBER n/a	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Katherine A. Hajjar, MD E-Mail: khajjar@med.cornell.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Weill Medical College of Cornell University 407 East 61 st St, Rm 106 1 st Floor New York, New York 10065-4805				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Proliferative vitreoretinopathy (PVR) is a potentially blinding disease that occurs in almost one-half of military personnel who suffer 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. We are examining the potential role of the calcium-regulated, phospholipid-binding protein, annexin A2 (A2) in PVR. In a robust model of PVR, we find that mice lacking A2 have a greatly diminished scarring response to intra-ocular injury. At early time points, we see a lack of migration of RPE cells into the vitreous space in the A2-deficient mouse, and, later time points, the degree of scarring and retinal detachment appear to greatly diminished in these animals. We have developed a method for the isolation of mouse RPE cells, and are studying their directed migration <i>in vitro</i> in response to macrophage-specific signals. In addition, we have approvals in place and have set up procedures for examining expression of A2 and related molecules in PVR membranes from human patients with PVR due to retinal surgery. Going forward, we plan to expand on these studies by identifying the macrophage-dependent molecular cues that direct RPE migration, and define the specific role played by A2 in this process.					
15. SUBJECT TERMS					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	12	19b. TELEPHONE NUMBER (include area code)

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1. INTRODUCTION.

Proliferative vitreoretinopathy (PVR) is a potentially blinding disease 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. We discovered that when we induce an eye injury in mice that lack a protein called annexin A2 (ANXA2), there is almost no PVR scar formation. ANXA2 is a calcium-dependent, phospholipid-binding protein that mediates membrane remodeling events. With our collaborators, we showed recently that this protein supports repair of the lysosome's limiting membrane in macrophages, thus preventing activation of the inflammasome and release of inflammatory cytokines, such as, interleukin-1 β . The current project is aimed at determining whether loss of ANXA2 prevents PVR scar formation, and, if so, the mechanism by which it carries out this action. New data obtained in the past year appear to reinforce our original hypothesis that ANXA2 is required for the full expression of PVR. In the dispase model, we see at 4 weeks that RPE migration from the scleral to vitreal side of the retina occurs only in *Anxa2*^{+/+} mice and not in *Anxa2*^{-/-} mice. In addition, we have developed methods for the harvest of RPE cells from the mouse retina, and have characterized these cells from both *Anxa2*^{+/+} and *Anxa2*^{-/-} mice in preparation for migration studies. Finally, all components are in place to begin archiving human PVR tissue for analysis of A2 expression and blood cytokines.

2. KEYWORDS.

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

3. ACCOMPLISHMENTS.

Major activities, specific objectives, and significant results

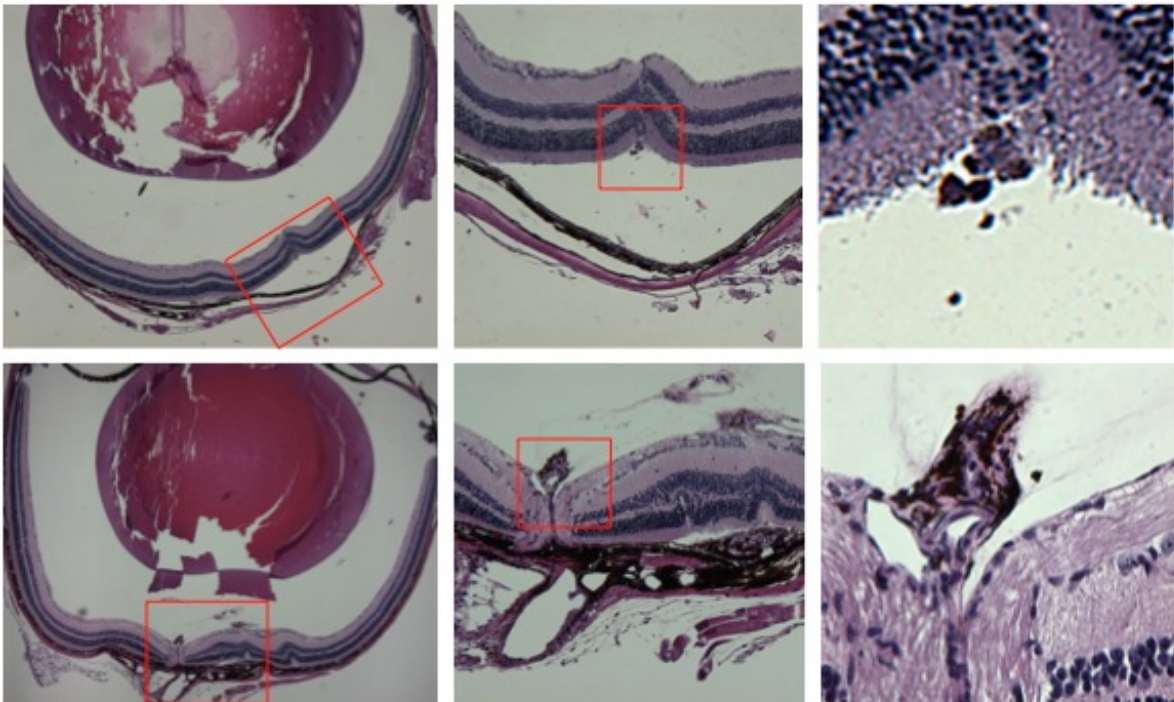
Specific Aim 1: To determine the role of ANXA2 in murine PVR.

Task 1: Establishing the model. To initiate this aim, we obtained IACUC approval for the proposed model system. Approval from the DOD required more time than anticipated, and resulted in a delay in initiating the experiments. DOD approval was obtained on February 11, 2015, slightly over 4 months from the initiation of the project. Nevertheless, we have made progress toward establishing a detailed PVR model. We have completed time course studies at 24 hours, 2 weeks, and 4 weeks, and will initiate the 6-week time point in October of 2015. For each time point tested, we have also tested three dispase doses (0.1, 0.2, and 0.3 U/ul).

Significant preliminary data indicate the following:

- [1]** We find no clear difference between *Anxa2*^{+/+} and *Anxa2*^{-/-} eyes at 24 hours following either dispase or PBS injection. In all both cases, a few pigmented cells can be found within the healing region of the injection site. Small amounts of hemorrhage near the surface of the retina are also typically evident with occasional membranous strands and associated inflammatory cells.
- [2]** At 2 weeks, we can see apparent invasion of pigmented cells within the healing retinal scar and into the retina and vitreous in dispase-injected *Anxa2*^{+/+} eyes. Hemorrhage is also typically present, and membranous strands are more numerous. Importantly, we have not so far seen invasion of pigmented cells beyond the retina into the vitreous space in *Anxa2*^{-/-} eyes. In addition, we have not seen invasion of pigmented cells in either genotype following injection of PBS.
- [3]** All three doses of dispase appear to be effective; 0.2 U/ul seems as effective as 0.3 U/ul, and slightly more effective than 0.1 U/ul, as judged by mobilization of pigmented cells beyond the RPE/choroid layers. We plan to use a dose of 0.3 U/ul in future experiments.
- [4]** At 4 weeks, RPE cells begin to migrate to the vitreal side of the retina in dispase-treated *Anxa2*^{+/+} eyes, but not in dispase-treated *Anxa2*^{-/-} eyes (**Figure 1**).

***Anxa2*^{-/-} 905 Dispase 0.3 U/ul**



***Anxa2*^{+/+} 41 Dispase 0.3 U/ul**

Figure 1 (above): RPE cells fail to enter the retinal wound at 4 weeks following dispase (0.3 U/ul) injection, and remain at the scleral border of the retina in *Anxa2*^{+/+} eyes. In *Anxa2*^{-/-} dispase-injected eyes, on the other hand, RPE cells have traversed the renal wound and form a frond-like structure on the vitreal side of the retain. This structure is associated with a nearby early membrane that contains pigmented cells, presumptively RPE cells.

[5] We have optimized conditions for immunofluorescence staining of paraffin-embedded eye sections using anti-ANXA2, anti-CD68, and anti-RPE65.

One issue we have encountered is variability in the manufacturer's lots of dispase. Our 4-week animals, which were treated with a new batch of dispase, displayed less intra-ocular pathology than expected. With this issue in mind, we now plan to analyze the potency of dispase, using an assay kit supplied by Worthington, to standardize the doses of the agent used prior to injection. Once this is completed, we will launch a definitive experiment in which all animals are injected at the same time with the same batch and dose of dispase.

Task 2: Macrophage depletion and tissue specific knockout. We have completed final characterization of macrophage specific knockout mice, and have enlarged our colony. We will be subjecting these animals to dispase induced PVR, once we are confident that the model has been reproducibly established.

Task 3: Cytokine and growth factor profiling. We will conduct these analyses once we are confident that the model has been reproducibly established.

Specific Aim 2: To define ANXA2-dpendent macrophage-RPE cell interactions in murine PVR.

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

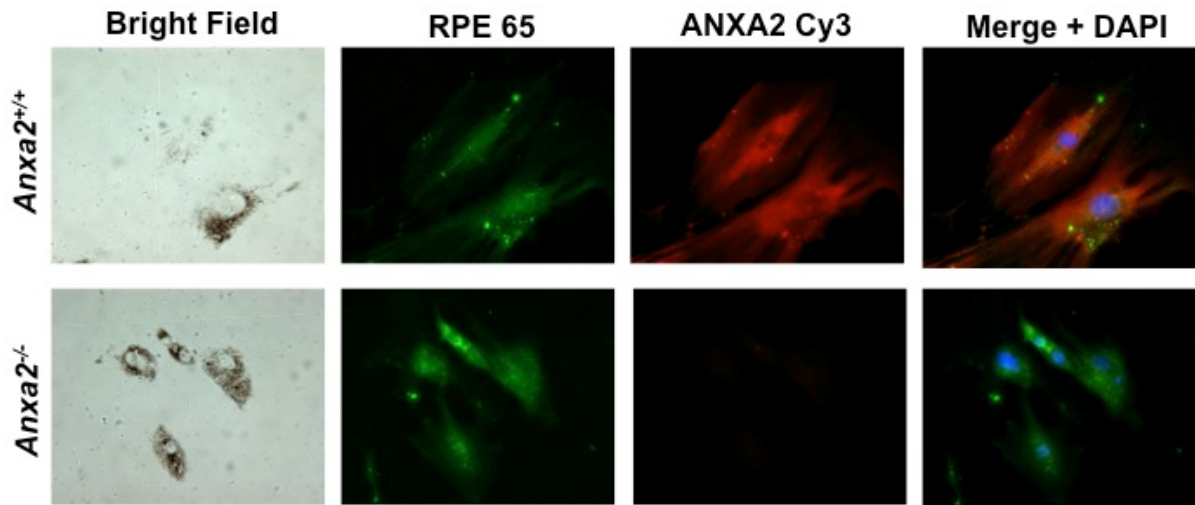
Harvest of macrophages. We have established reliable methods for the harvest and propagation of macrophages from both bone marrow and the peritoneal cavity from both *Anxa2*^{-/-} and *Anxa2*^{+/+} mice. For bone marrow derived macrophages, we routinely achieve 99% purity.

Harvest and culture of RPE cells. We have demonstrated that we can maintain the RPE cells at 98% purity in culture for at least 10 days. We have also verified abundant expression of ANXA2 in wild type murine RPE cells (**Figure 2**), and have also begun studying macrophage-induced RPE migration in cross-genotype experiments.

Briefly, we removed the cornea, vitreous, and retina from enucleated eyes. The resulting shells (eye cups) were flattened and incubated with fresh, sterile 0.25% trypsin (pH 8.0, 1h, 37° C) with occasional trituration. Released sheets of RPE cells are retrypsinized and the digestion halted with complete RPE medium. After washing by centrifugation and resuspension, the cells are plated on chamber slides for

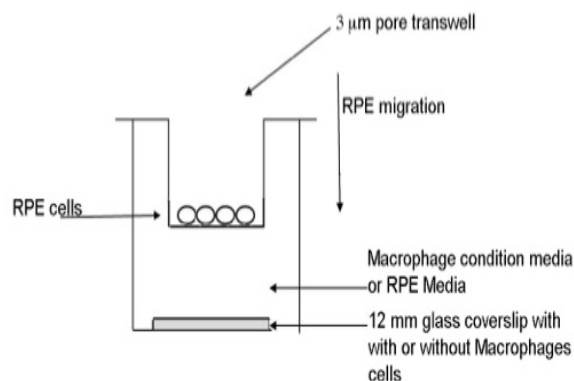
immunohistochemistry, or on transwell filters for migration studies (37° C, 5% CO₂). Cells are fed on day 5, and, thereafter, 3 times per week with RPE medium.

Figure 2. Retinal Pigmented Epithelial Cells. Murine Cells were isolated as described in the text above, and maintained for 10 days in culture. Bright field microscopy reveals expected complement of pigment granules. Essentially all cells stain for RPE 65, a standard marker for this cell type. Cells from *Anxa2*^{+/+} animals stain, as expected, for ANXA2, whereas *Anxa2*^{-/-} cells do not. DAPI is utilized to visualize nuclei and estimate cell number in the final merged images.

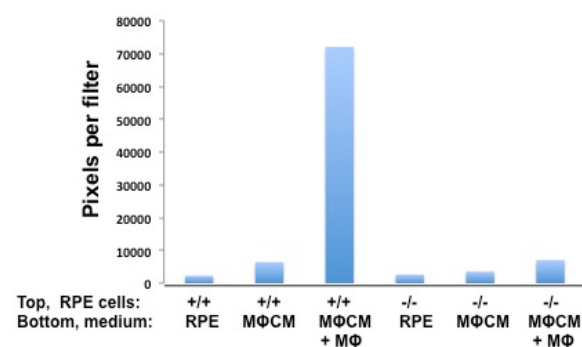


Task 2: Genotype-specific co-culture experiments. We have performed an initial series of co-culture migration studies. Either *Anxa2*^{+/+} or *Anxa2*^{-/-} RPE cells were seeded atop a laminin-coated, 2-micron pore filter on the upper chamber of a transwell plate. The bottom well contained either RPE medium (control), macrophage conditioned medium, or macrophage conditioned medium plus macrophages (**Figure 3A, below**). RPE cell migration was assessed at 24h via crystal violet staining of the under side of the filter, after removing all remaining cells from the upper side. As shown in **Figure 3B**, migration of RPE cells appears to be dependent upon [1] the presence of macrophages in the lower chamber, and [2] the expression of ANXA2 in the migrating RPE cells. Importantly, there was almost no migration of *Anxa2*^{-/-} RPE cells.

A.



B.



In addition, we imaged cells present on the coverslip placed at the bottom of the lower chamber. Macrophages and RPE cells were identified with markers F4/80, and RPE-65, respectively. The results indicate again that migration of RPE cells to the lower chamber was much more robust in ANXA2-expressing cells (**Figure 4A and B**, below).

Figure 4A:

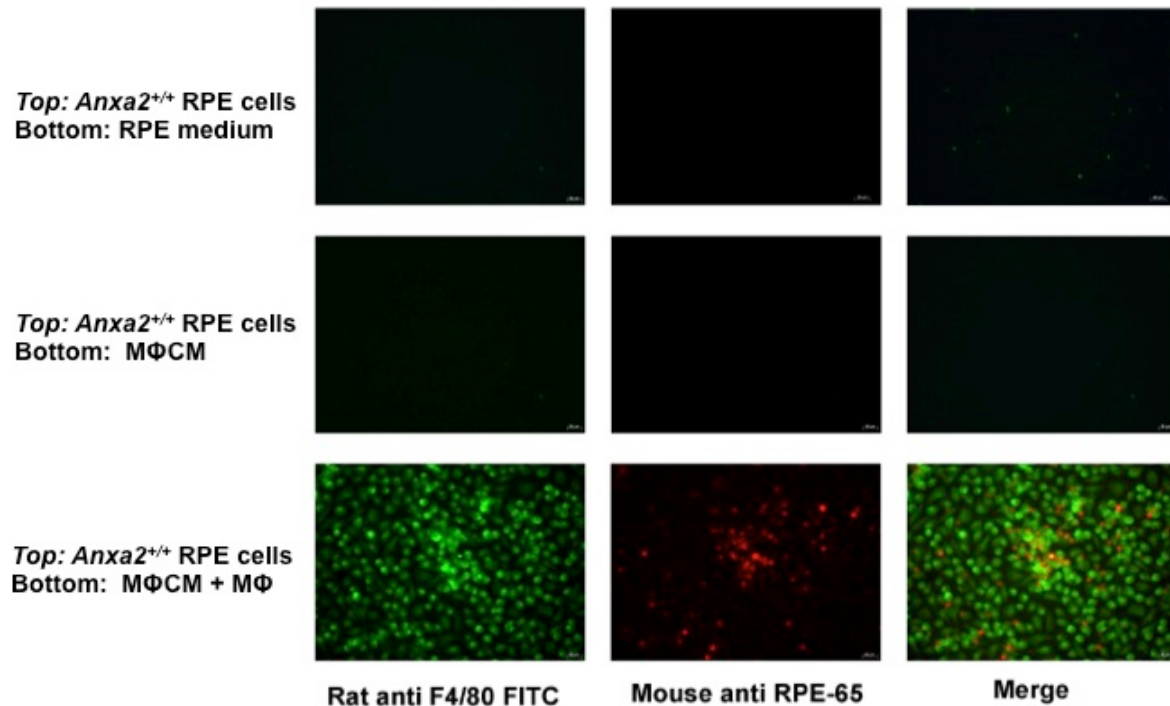
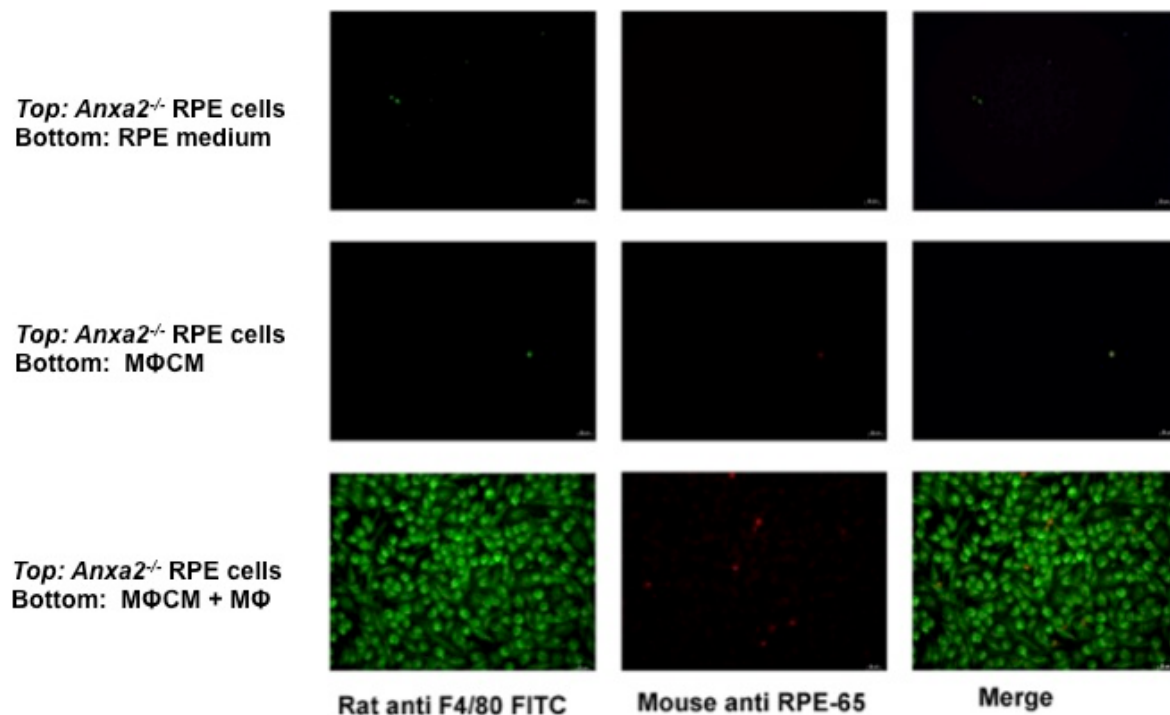


Figure 4B:



Task 3: Cytokine and growth factor analyses. These will be initiated once we are confident that we have a reliable co-culture, migration assay.

Specific Aim 3: ANXA2 in human PVR.

Task 1: IRB protocol preparation and approval. Our human subjects protocol was approved by our IRB in November of 2014, but not by the DOD until June of 2015. This led to an unforeseen postponement in the start of studies on human PVR.

Task 2: Sample procurement. All procedures are now in place and we expect to begin receiving samples of PVR membranes as well as blood in the next several days to weeks.

Opportunities for training and professional development: This grant has provided a unique training opportunity for Dr. Nadia Hedhli. She is developing expertise in macrophage and RPE cell isolation, and in interpretation of histologic sections through the injured retina. In the coming year, she will expand this skill set to include evaluation of human surgical PVR samples from patients with diabetic retinopathy and a history of prior retinal surgery.

Dissemination of results to communities of interest: Nothing to report.

Plans for next reporting period.

Specific Aim 1: Under this objective, we will perform dispass assays to compare lots of the agent. We will complete and analyze eyes from the 6-wk time point, and will set up experiments in macrophage-specific ANXA2-deficient mice. We will initiate analyses of plasma and retinal cytokines, and initiate ANXA2 blockade studies.

Specific Aim 2: We will continue to perform macrophage and RPE cell co-culture experiments, and analyze the effect of genotype of each cell type in RPE cell migration. We will profile cytokines in post-culture medium, and initiate ANXA2 blockade studies.

Specific Aim 3: We will procure and archive retinal and matched blood samples from human patients with PVR. We will establish staining protocols for these samples and begin analyses of plasma cytokines and other factors.

4. IMPACT.

On the principal discipline of the project: So far, this project appears to indicate that ANXA2 is required for the full expression of PVR in the murine dispass model. We are developing a better mechanistic understanding of how ANXA2 contributes to PVR. If this hypothesis continues to be supported as the project continues, we may be able to establish the conceptual basis for a novel preventative treatment 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:

Delays in DOD approval of animal and human subject portions of the research significantly postponed initiation of these portions of the project.

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. Hajar, 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

Quad Chart – please see attached updated quad chart

9. APPENDICES

none

Annexin A2 and Proliferative Vitreoretinopathy

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PI Katherine A. Hajjar, MD

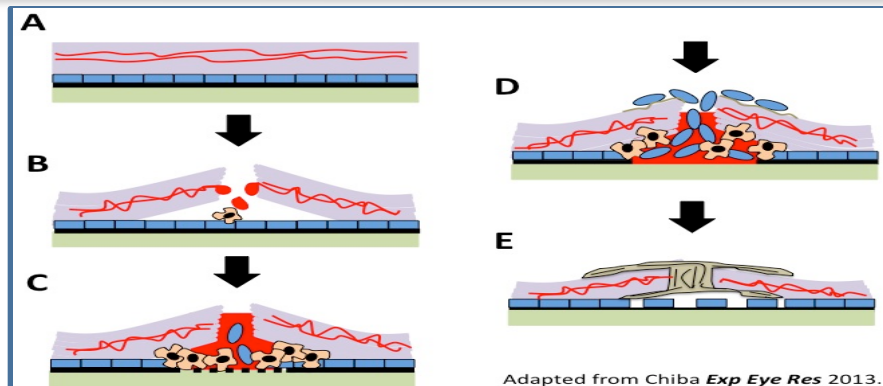
Org: Weill Cornell Medical College Award Amount: \$1,000,000

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: 4 wk evaluation completed					
Aim 2: RPE MΦ co-culture begun					
Aim 3: Human subject accrual begun					
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