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TITLE: Novel Collagen-Based Inflammatory Mediators of Cardiomyopathy

PRINCIPAL INVESTIGATOR: Randy Cowling

CONTRACTING ORGANIZATION: University of California at San Diego
La Jolla, CA

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Reductions in plasma/tissue ascorbate levels due to environmental conditions and/or diet could cause fibroblasts to secrete procollagens as atypical hyperglycosylated forms. Resultant inflammatory events could cause ischemic heart disease and/or aortic stenosis, which can lead to dilated and hypertrophic cardiomyopathies. We proposed to test this hypothesis using cell culture experiments and mouse models. In the in vivo mouse work, we produced our experimental mice and completed the proposed ascorbate treatment/starvation regimens but were unable to find cardiovascular effects via echocardiography or aortic staining. The lack of an effect may have been caused by low levels of ascorbate in the food during the "ascorbate off" cycle. In the in vitro work, we were unsuccessful purifying hyperglycosylated procollagen but did identify O-glycosylated procollagens by Click-iT chemistry, and determined that only PLOD2 knockdown led to noticeable changes in electrophoretic mobility indicative of post-translational procollagen modifications.

15. SUBJECT TERMS

None listed.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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Unclassified	Unclassified	Unclassified	Unclassified	10	19b. TELEPHONE NUMBER *(include area code)*

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1. INTRODUCTION:

We have hypothesized that temporary reductions in plasma/tissue ascorbate levels in humans due to environmental conditions and/or diet cause procollagens to be secreted from fibroblasts as atypical hyperglycosylated forms in tissues with active procollagen turnover (e.g., vessels and valves), which can generate immune responses. These immune responses could initiate inflammatory events for the development of ischemic heart disease and/or aortic stenosis, which can lead to dilated and hypertrophic cardiomyopathies. We have proposed to test this hypothesis by using in vitro cell culture experiments with adult rat cardiac fibroblasts and in vivo mouse models.

2. KEYWORDS:

inflammation, procollagen, vitamin C, ascorbic acid, posttranslational modification

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Major Task 1: Characterize in vivo cardiac effects of fluctuating ascorbate levels in Gulo null mice
Subtask 1: Obtain frozen embryos and re-establish Gulo knockout mouse line – *completed 12/2020*
Subtask 2: Generate breeding colony and experimental mice – *breeding colony established 4/2021*
– all experimental mice generated by 12/2021
Subtask 3: Treat experimental mice generated in subtask 2 with fluctuating ascorbate in drinking water, performing follow-up Doppler echos every 4 months for 12 months total – *started 8/21, completed 2/2023*
Subtask 4: Assess histological alterations in cardiac tissue, cardiac valves, and aorta of mice examined in subtask 3 at death or euthanasia (12 months maximum) – *only aorta analyzed histologically (valve issues assessed by Doppler echo), completed 3/2023*
Major Task 2: Determine whether hyperglycosylated procollagens I and III induce the release of proinflammatory mediators in vitro from bone marrow-derived macrophages and cultured aortic endothelial cells
Subtask 1: Purify both non-glycosylated and hyperglycosylated procollagens from cultured rat cardiac fibroblasts – *not able to complete (see details)*
Subtask 2: Isolate aortic endothelial cells as well as bone marrow-derived macrophages from C57BL/6J mice and treat with procollagens from subtask 1 – *not able to complete*
Subtask 3: Perform RNA-Seq from cellular RNA from subtask 2 – *not able to complete*
Subtask 4: Verify candidates identified in subtask 3 by RT-qPCR and/or western blot – *not able to complete*
Major Task 3: Determine the effect on observed procollagen lysyl modifications by knockdown of PLOD1, PLOD2, PLOD3, GLT25D1, and GLT25D2
Subtask 1: Identify, synthesize, and select best performing DsiRNAs using rat cardiac fibroblasts – *completed 9/2021*
Subtask 2: Determine effect of knockdown of each protein on SDS-PAGE migration of intracellular procollagens I and III – *completed 1/2022*

What was accomplished under these goals?

Specific Aim 1: To assess the effect of long-term fluctuations in ascorbate consumption on cardiac, vascular, and valvular structure/function in mutant mice that cannot synthesize ascorbate (Gulo null mice).

Gulo heterozygotes were bred in order to obtain experimental mice (both homozygote nulls and heterozygotes). All experimental mice were generated by 12/2021. The experimental timeline of fluctuating ascorbate treatment began in 8/2021 and was completed by 2/2023. The experimental groups were as follows: Group 1, Gulo +/-, constant high ascorbate diet (4 males, 4 females); Group 2, Gulo -/-, constant high ascorbate diet (4 males, 4 females); Group 3, Gulo -/-, cycling high ascorbate diet (4 males, 5 females); Group 4, Gulo -/-, cycling low ascorbate diet (5 males, 5 females). Follow-up echocardiography was performed (both standard M-mode and aortic Doppler) every 4 months from approximately 4–12 months of age. Analysis of the echo data indicated no statistically significant effect of ascorbate cycling with increasing age of the mice when compared with that of the control mice (Groups 1 and 2). This was true for both the M-mode (LVIDd, LVIDs, %FS, IVSd, LVPWd, LVIDd/LVPWd, LVIDd/BW, LVM(d), and LVM/BW) and aortic Doppler (AV Peak-HR, AoV VTI, AoV VTI Mean PG, AoV VTI Max PG, and AV Peak Velocity) values, and all values were within normal ranges for mice. Post-necropsy analysis of mouse aortas by Sudan IV staining found no atherosclerotic lesions (Fig. 1). A possible explanation for these negative findings is that the standard mouse diet that contained no added ascorbate may have contained residual ascorbate from its plant-based content; this "contamination" may have varied among batches. However, as the feed supplier did not test for ascorbate content (it is considered nonessential in rodent diet), this remains supposition.

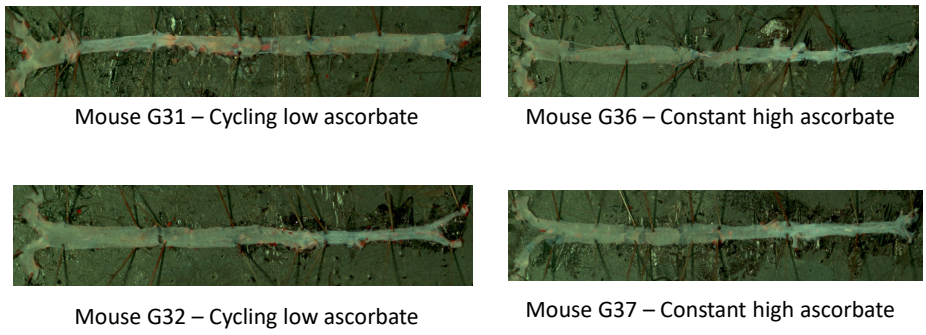

Figure 1 – Sudan IV-stained aortas of experimental mice – no atherosclerotic lesions are visible

Specific Aim 2: To determine whether hyperglycosylated procollagens I and III, which are formed in fibroblasts upon ascorbate withdrawal, are proinflammatory in in vitro assays.

We had decided to purify hyperglycosylated procollagens from the medium of cultured adult rat cardiac fibroblasts using preparative SDS-PAGE rather than immunoprecipitation, as the quality of the polyclonal antibody that we had used in the past had diminished in recent lots. Because of its large apparent molecular weight, this modified procollagen material moves very slowly during electrophoresis. We attempted five times to purify this material (with electrophoresis times often exceeding 12 h) but were never able to obtain Col1a1 immunoreactive fractions, despite the presence of a large band of Coomassie blue-stained protein in the tube gel (see Fig. 2) that contained procollagen isoforms (as determined by mass spectrometry). It became apparent to us that this material could not be purified by SDS-PAGE in practice.

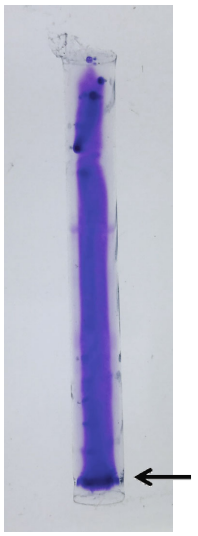

Figure 2 – Coomassie blue-stained protein band near bottom of preparative SDS-PAGE tube gel after ~10 h of electrophoresis

In order to observe this secreted material, we incubated adult rat cardiac fibroblasts with GalNAz (*N*-azidoacetylgalactosamine-tetraacylated), a cell-permeable *N*-acetylgalactosamine mimetic that specifically labels mucin-type *O*-linked glycoproteins that can be detected using the Click-iT reaction with biotin alkyne and then western blotting with streptavidin-HRP. Using that system, we were able to detect a ≥250 kDa band of secreted protein from the fibroblasts. Interestingly, the

intensity of this band increased when PLOD1 was knocked down in the cells and its migration increased (its apparent molecular weight decreased) when PLOD2 was knocked down (Fig. 3). The secretion of this material increased significantly (~2×) when the fibroblasts were starved of ascorbate (Fig. 4). Preliminary mass spectrometry identification of this material (after purification on alkyne-agarose beads) indicated that the most abundant proteins were procollagens 1a2, 1a1, and 4a2 (in that order). These findings were made later in the grant and we were not able to purify and test the effect of sufficient quantities of the material on aortic endothelial cells and bone marrow-derived macrophages. However, during the GalNAz labeling experiments, we did notice that conditions that led to increased secretion of this hyperglycosylated procollagen material (i.e., PLOD1 knockdown) led to enhanced migration of the fibroblasts, while PLOD2 co-knockdown attenuated this increased migration (Fig. 5). This evidence suggests that secretion of this hyperglycosylated procollagen material does have physiological effects on the cells.

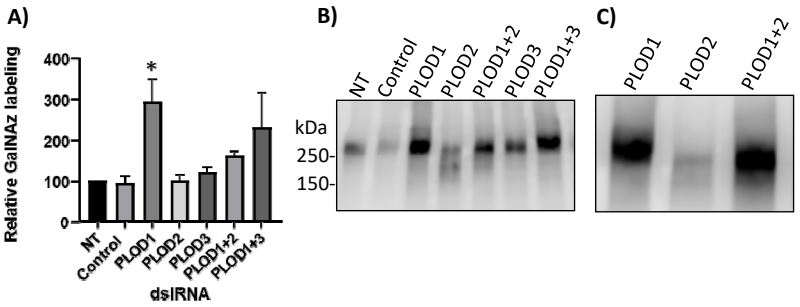

Figure 3 – Secreted GalNAz-labeled protein and effect of PLOD family knockdown
A) Quantitative plot, B) representative blot, and C) clearer image of the SDS-PAGE migratory effects of PLOD2 knockdown
Means + SEM plotted, * p<0.05 vs. NT post-hoc ANOVA – NT, not transfected

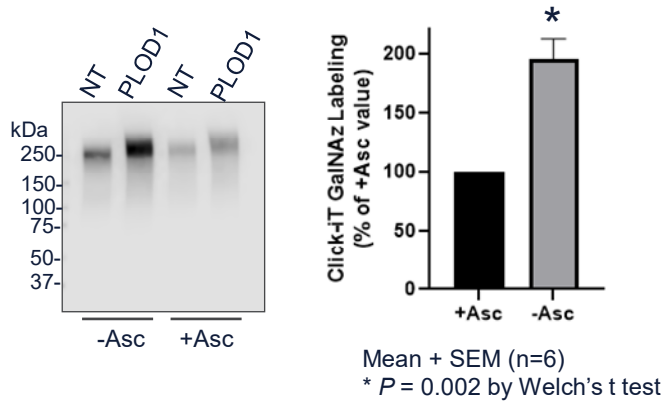

Figure 4 – Increased GalNAz incorporation into secreted protein(s) in the absence of ascorbate (Asc); NT, not transfected; PLOD1, PLOD1 knockdown

Specific Aim 3: To assess the contribution of lysyl hydroxylases and hydroxylysyl glycosyltransferases to procollagen modification in ascorbate-starved cultured fibroblasts.

It was determined that adult rat cardiac fibroblasts express PLOD1 after switching to another supplier's antibody. We then tested the effect of knockdown of PLOD1, PLOD2, PLOD3, and Colgalt1 on the electrophoretic mobility of intracellular procollagens 1a1, 1a2, and 3a1 in adult rat cardiac fibroblasts in the absence of ascorbate. Only PLOD2 knockdown produced any noticeable and consistent change in procollagen electrophoretic mobility, specifically increased mobility indicative of reduced post-translational modification (Fig. 6).

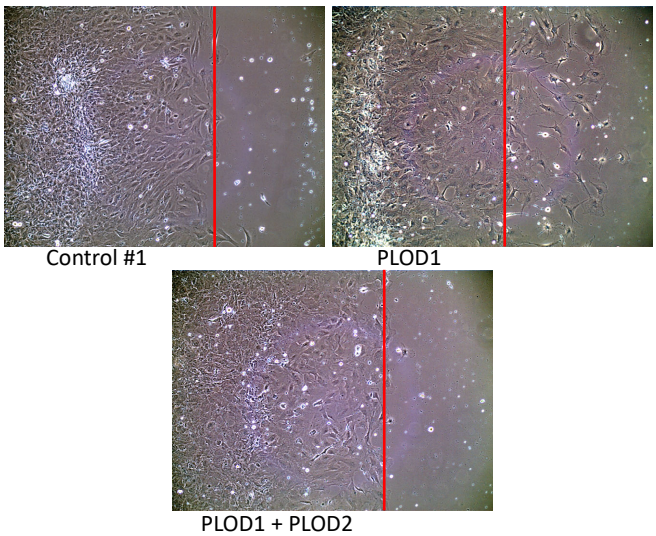

Figure 5 – Effect of PLOD knockdown on rat fibroblast migration
- scratch wound assay – phase contrast images (40×) - red line is drawn the same distance from the scratch front
- dsiRNA used is indicated below each image – representative of three independent experiments

What opportunities for training and professional development has the project provided?

Two UCSD undergraduate volunteers were trained by Randy Cowling and/or Kayleigh Marsh (our laboratory technician) in the techniques and theory of this grant. Specifically, Ashwin Kumar was mentored in the knockdown procedures used in Aim 3 from April 2021 until September 2021, and Grace Meister was mentored in the protein purification techniques used in Aim 2 from April 2021 until May 2022. This training earned them independent research credit in their undergraduate degrees. In addition, another UCSD undergraduate volunteer, Esha Mahajan, was mentored in the cell culture and western blotting techniques as well as mouse colony maintenance from the fall of 2021 until the fall of 2022.

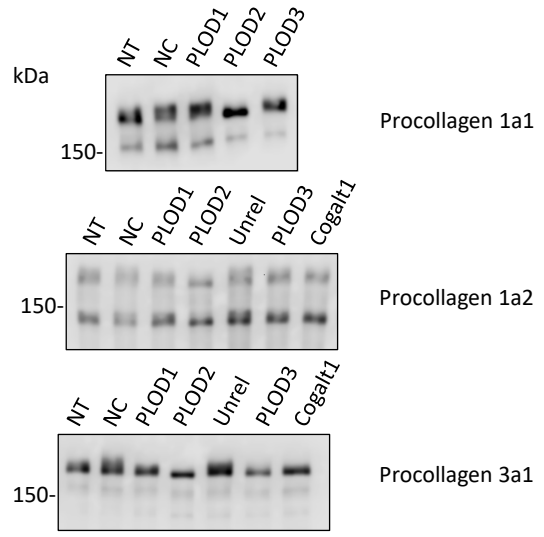

Figure 6 – Effect of PLOD knockdown on intracellular procollagen isoform SDS-PAGE migration – NT, not transfected; NC, negative control dsiRNA; Unrel, unrelated dsiRNA

How were the results disseminated to communities of interest?

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to report

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Nothing to report as we currently do not know the significance of the modified procollagens in the cardiovascular field.

What was the impact on other disciplines?

Impact on cancer research – Given the known positive association between PLOD2 levels and metastasis of many cancers, our finding that PLOD2 appears to post-translationally modify procollagens whose secretion increases the migration of cells is of interest to the field of cancer research.

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Because of our inability to purify hyperglycosylated procollagens and identify them with our current stock of antibodies, we decided to use the Click-iT system to identify them and determine that their secretion was PLOD-dependent. This is described in more detail in Specific Aim 2 of section 3.

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

Nothing to report

Changes that had a significant impact on expenditures

COVID-dependent delays in hiring personnel and carrying out experiments led to the one-year no-cost extension.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use or care of vertebrate animals

To simplify animal maintenance, we switched from providing ascorbate in drinking water to providing it in the mouse feed. This required a protocol amendment that did not need IACUC approval because it involved a change in oral dosing of a non-hazardous substance.

Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS:

- **Publications, conference papers, and presentations**

  Journal publications

  Nothing to report

  Books or other non-periodical, one-time publications.

  Nothing to report

Other publications, conference papers and presentations.

Nothing to report

- **Website(s) or other Internet site(s)**

  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

What individuals have worked on the project?

Name: Randy Cowling
Project Role: Principal Investigator
Researcher Identifier (e.g. ORCID ID): ORCID ID 0000-0001-5815-1512
Nearest person month worked: 2.4

Contribution to Project: Dr. Cowling was responsible for overall administration and direction of the project. He oversaw all experimentation and was actively involved in data analysis, interpretation, and troubleshooting. He also performed experimentation as needed.

Name: Kayleigh Marsh
Project Role: Laboratory Technician
Nearest person month worked: 4

Contribution to Project: Kayleigh was involved in all technical aspects of the project, including in vitro experiments and mouse treatments.

Name: Kimberly Weldy
Project Role: Laboratory Technician
Nearest person month worked: 2.4

Contribution to Project: Kim was involved in maintenance of the mouse colony.

Name: Ashwin Kumar
Project Role: Undergraduate Student
Nearest person month worked: 1

Contribution to Project: Ashwin carried out experiments related to Aim 3 (dsiRNA-mediated knockdown of procollagen modifying enzymes in cardiac fibroblasts).

Name: Grace Meister
Project Role: Undergraduate Student
Nearest person month worked: 2

Contribution to Project: Grace carried out experiments related to Aim 2 (purification and identification of hyperglycosylated procollagens).

Name: Esha Mahajan
Project Role: Undergraduate Student
Nearest person month worked: 2

Contribution to Project: Esha carried out experiments related to Aims 2 and 3 as well as mouse colony maintenance.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

R. Cowling
1/1/2023 – 6/30/2023
Lexeo Therapeutics, Inc. 3.0 calendar month salary
Project Title: Preclinical Study of Gene Therapy for TNNI3
This industry-sponsored research project is looking at the preclinical efficacy of AAV-mediated TNNI3 gene therapy in a mouse model of TNNI3-mediated cardiomyopathy. This milestone-oriented project is completely separate from the project in question.
Role: PI

What other organizations were involved as partners?

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

N/A

QUAD CHARTS:

N/A

9. APPENDICES:

Nothing attached