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TITLE: Elucidating the Mechanism of Gain of Toxic Function From Mutant C1 Inhibitor Proteins in Hereditary Angioedema

PRINCIPAL INVESTIGATOR: Dr. Bruce Zuraw, M.D.

CONTRACTING: Veterans Medical Research Foundation
ORGANIZATION San Diego, CA 92161-0002

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Dr. Bruce Zuraw, M.D.

E-Mail: bzuraw@ucsd.edu

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Veterans Medical Research Foundation
3350 La Jolla Village Drive, MC-151A
San Diego, CA 92161-0002

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14. ABSTRACT
HAE is autosomal dominant. Cells, heterozygous for the SERPING1 mutation, express both mutant and WT C1INH proteins. HAE is clearly a loss-of-function disease. Plasma functional C1INH levels in symptomatic HAE patients, however, are very low - far less than the predicted 50%. We have shown that this is due, at least in part, to an additional acquired GOTF defect caused by the mutant protein that interferes with the secretion of WT C1INH. Our overall hypothesis is that mutant C1INH proteins exert a variable GOTF phenotype that inhibit secretion of WT C1INH protein and worsen disease severity. This hypothesis suggests that abrogating the GOTF should result in clinical benefit. Two aims will address complementary sub-hypotheses. Aim #1 will assess the mechanisms of the GOTF with a hypothesis that misfolding of mutant C1INH protein in the ER causes impairment of WT C1INH secretion. Elucidating the mechanism of the mutant C1INH-induced GOTF opens the possibility for new therapeutic approaches designed to abrogate the GOTF. Aim #2 will use a synthetic biology approach to assess the structural determinants of mutant C1INH-induced GOTF with a hypothesis that unique structural characteristics of C1INH make it more susceptible to GOTF than other serpins.

15. SUBJECT TERMS
Hereditary angioedema, C1 inhibitor, serpin, mutation, secretion, aggregates, misfolding

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1. INTRODUCTION:

HAE is autosomal dominant. Cells, heterozygous for the SERPING1 mutation, express both mutant and WT C1INH proteins. HAE is clearly a loss-of-function disease. Plasma functional C1INH levels in symptomatic HAE patients, however, are very low - far less than the predicted 50%. We have shown that this is due, at least in part, to an additional acquired GOTF defect caused by the mutant protein that interferes with the secretion of WT C1INH. Our overall hypothesis is that mutant C1INH proteins exert a variable GOTF phenotype that inhibit secretion of WT C1INH protein and worsen disease severity. This hypothesis suggests that abrogating the GOTF should result in clinical benefit. Two aims will address complementary sub-hypotheses. Aim #1 will assess the mechanisms of the GOTF with a hypothesis that misfolding of mutant C1INH protein in the ER causes impairment of WT C1INH secretion. Elucidating the mechanism of the mutant C1INH-induced GOTF opens the possibility for new therapeutic approaches designed to abrogate the GOTF. Aim #2 will use a synthetic biology approach to assess the structural determinants of mutant C1INH-induced GOTF with a hypothesis that unique structural characteristics of C1INH make it more susceptible to GOTF than other serpins.

2. KEYWORDS:

Hereditary angioedema, C1 inhibitor, serpin, mutation, secretion, aggregates, misfolding

3. ACCOMPLISHMENTS:

What were the major goals of the project?

1) To define the mechanism how mutant C1INH proteins inhibit secretion of normal C1INH.
   - a) To identify the role of C1INH polymerization in the inhibition of secretion of normal C1INH (months 1-20; 50% completed)
   - b) To identify the role of ER stress in the inhibition of secretion of normal C1INH (months 12-32; 5% completed)
   - c) To identify whether abrogating the GOTF phenotype decreases evidence of C1INH polymerization or ER stress (months 24-36; 0% completed)

2) To analyze the structural requirements for the mutant C1INH protein-induced GOTF.
   - a) To assess the ability of alpha$_1$-AT mutations engineered into C1INH to induce a GOTF (months 1-24; 25% completed)
   - b) To assess the GOTF in chimeric C1INH/alpha$_1$-AT proteins with C1INH or alpha$_1$-AT mutations to induce a GOTF (months 6-36; 10% completed)

What was accomplished under these goals?

We performed mutagenesis and sequencing to make and confirm the expression plasmids that needed for the project. We successfully made the following plasmids:

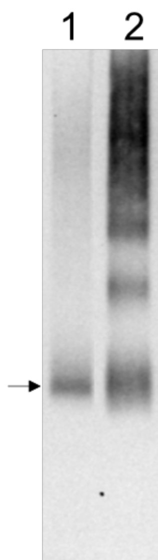
1) HAE C1INH mutant plasmids:
   - C1INH G162R

- C1INH C183Y
- C1INH V196D
- C1INH A436T

2) alpha1-AT mutant plasmids:
- α_1-AT E342K (Z)
- α_1AT H334D (King's)
- α_1-AT S53F (Silyama)
- α_1AT c.1086dupC (Saar)
- α_1AT c.953_954delTC (NHK)

3) C1INH plasmids with alpha1-AT equivalent mutations
- C1INH E429K
- C1INH H421D
- C1INH S148F
- C1INH W02R
- C1INH α_1-ATfsX14
- C1INH α_1-ATfsX17

We encountered substantial unexpected difficulties optimizing native gels. Initially, we could not detect the C1INH protein, and then tended to observe mostly protein smears. By systematically addressing the buffers, lysis conditions, and running conditions we have largely solved this problem. As shown in the gel below, we can now detect C1INH aggregates in mutant C1INH transfected cells but not in WT C1INH transfected cells.

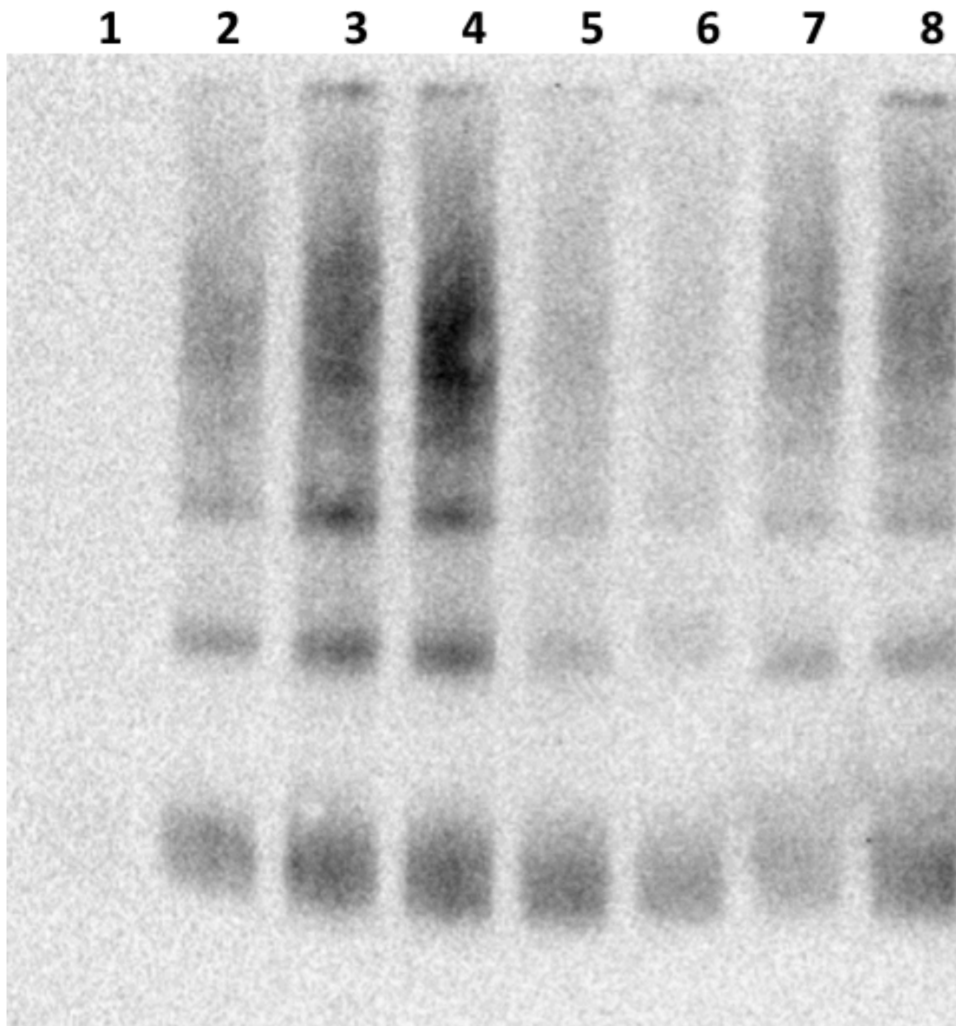


Lane 1: WT C1INH only
Lane 2: Mutant C1INH

The arrow shows monomeric C1INH. The mutant C1INH lane shows a variety of higher molecular weight aggregates in addition to the monomer.

We then examined the consequences of transfecting COS-7 cells with 7 different mutant C1INH proteins as well as WT C1INH. As shown in the gel below, there the band for the WT C1INH was extremely faint, as the vast majority was secreted. Among the 7 mutant proteins, there was

variable amounts of C1INH that accumulated within the cell. In all cases, 10 ug of lysate was loaded.

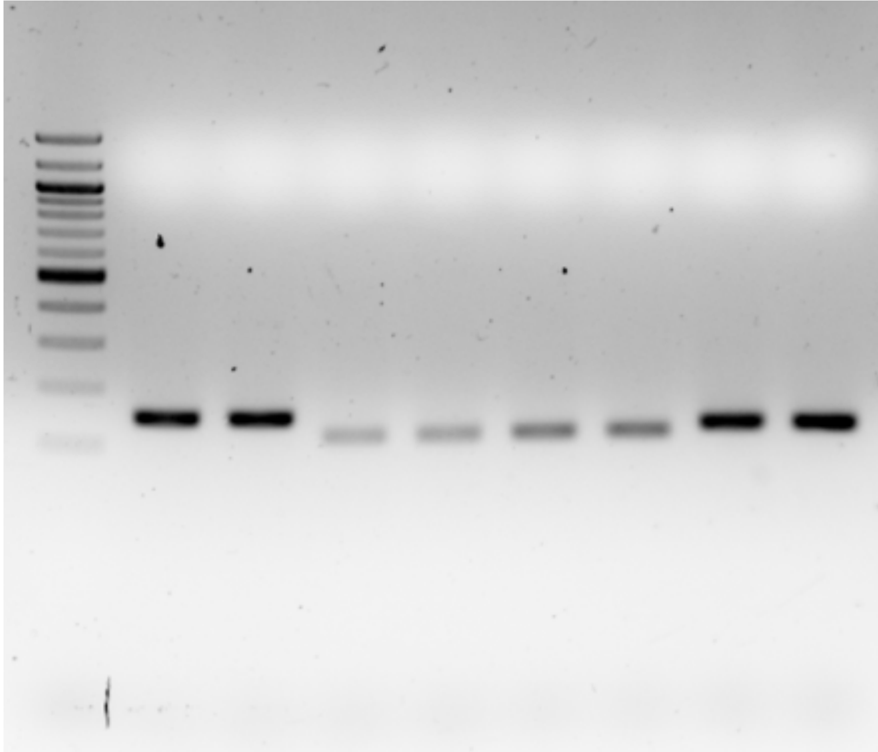


Sample

Lane 1: WT C1INH
Lane 2: Mutant C1INH G162R (2694G-A)
Lane 3: Mutant C1INH G162R (4351 G-A)
Lane 4: Mutant C1INH V196D
Lane 5: Mutant C1INH C183Y
Lane 6: Mutant C1INH E429K
Lane 7: Mutant C1INH H421D
Lane 8: Mutant C1INH S148F

Lanes 3-5 are naturally occurring HAE mutations, Interestingly lanes 6-8 show $alpha_1$-antitrypsin mutations equivalents that were introduced into C1INH. Unexpectedly, introducing the α_1-AT E342K Z mutation equivalent into C1INH appeared to cause the least amount of mutant protein intracellular retention and oligomerization relative to the other HAE and α_1-AT deficiency mutations.

We are also beginning to examine evidence of mutant C1INH protein-induced ER stress. We have designed primers to amplify mRNA for selected endoplasmic reticulum markers of ER stress. The gel below shows that our PCR successfully amplifies the mRNA.



Lane 1: Size markers
Lanes 2-3: BiP (GRP78)
Lanes 4-5: XBP sliced varient 1
Lanes 6-7: Derlin 2
Lanes 8-9: Calnexin

We have also begun to isolate monocytes from human blood samples using anti-CD14 magnetic beads to obtain relatively pure monocyte preparations. The purity is estimated to be >90% by visual examination of the cultured cells, however we have not started to quantitate this yet by flow cytometry. This is being done as part of a separate protocol, and we have not yet enrolled any subjects in this study. The technical aspects of monocyte isolation will be used for the current project once we are ready to begin using primary human cells.

What opportunities for training and professional development has the project provided?
Nothing to Report.

How were the results disseminated to communities of interest?
Dr. Zuraw organized and ran a NIH sponsored international R13 conference grant entitled "Expanding the Boundaries of HAE Knowledge" of which he was PI. This included a session on

serpinopathies. This conference and session in particular increased awareness and knowledge of mutant C1INH misfolding among HAE investigators around the world.

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

In the next period, we will measure polymerization of C1INH when cells are co-transfected with mutant-C1INH plus WT C1INH. We are growing HepG2 cells in addition to COS-7 cells so that we can also perform these experiments in the human hepatoma cells. The use of HepG2 hepatoma cells as an alternative continuous cell line was included and discussed in the original application. Unlike COS-7 cells, HepG2 cells make endogenous wild-type C1INH and we can vary the rate of synthesis using cytokines. We will also assess ER stress in cells that are transfected with varying combinations of plasmids. We hope to soon begin to study primary cells from HAE patients.

4. IMPACT:

This project has not yet had a significant impact

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

Nothing to Report.

What was the impact on other disciplines?

Nothing to Report.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Nothing to Report.

CHANGES/PROBLEMS:

Nothing to Report.

Changes in approach and reasons for change

There are no anticipated modifications of the plans relative to the SOW.

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

We experienced difficulty getting consistent results when examining intracellular C1INH polymerization in transfected COS-7 cells using native gels. We slowly made progress in solving this problem and can now move forward.

Changes that had a significant impact on expenditures

We have not been able to identify a suitable candidate yet to serve as a research associate for this project. We are continuing to look for the right person.

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

No changes. The most recemnt IRB approval was 09/10/2015.

5. PRODUCTS:

Nothing to Report.

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

Presented information about the project to physician investigators at the HAE Japan meeting in Tokyo, 11-JAN-2015. The citation is as follows:

Zuraw, B.L. HAE: A Scientific Overview and Prospects for Clinical Practice. Presented at the HAE Japan Scientific Meeting, Tokyo, Japan, January 11, 2015.

- **Inventions, patent applications, and/or licenses**

None.

- **Other Products**

None

6. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Bruce Zuraw
Project Role: Principal Investigator
Research Identifier: bzuraw
Nearest person month worked: 3
Contribution to project: supervised project and other staff
Funding Support: None

Name: Marc Riedl
Project Role: Co-Investigator
Research Identifier: mriedl
Nearest person month worked: 1
Contribution to project: Building database of HAE patients who can be contacted for the study
Funding Support: None

Name: Jack Herschbach
Project Role: Research Technician
Research Identifier:
Nearest person month worked: 12
Contribution to project: constructed all plasmids and performed mutagenesis
Funding Support: None

Name: Shamir Kansakar
Project Role: Research Technician
Research Identifier:
Nearest person month worked: 12
Contribution to project: Performed native gels and working on stress response
Funding Support: None

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

Nothing to Report.

What other organizations were involved as partners?

Nothing to Report.

7. SPECIAL REPORTING REQUIREMENTS: None

8. APPENDICES:

None