**Award Number:** W81XWH-15-1-0258

**TITLE:**
Targeting Trypsin-Inflammation Axis for Pancreatitis Therapy in a Humanized Pancreatitis Model

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**REPORT DATE:**
October 2016

**TYPE OF REPORT:**
Annual

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

**DISTRIBUTION STATEMENT:**
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Title: Targeting Trypsin-Inflammation Axis for Pancreatitis Therapy in a Humanized Pancreatitis Model

Authors: Stephen J Pandol and Aurelia Lugea

Abstract:
Acute pancreatitis especially due to alcohol and smoking goes onto chronic pancreatitis which, in turn, is a risk factor for pancreatic cancer. Because only a relatively small portion of patients with alcohol abuse and smoking develop pancreatitis, it is very likely that there are genetic underlying predisposing factors that have not been discovered that explain why certain individuals develop pancreatitis. A genetic defect in the trypsinsogen gene (PRSS1 gene) causing hereditary pancreatitis is now well established. We developed a transgenic mouse using a Bacterial Artificial Chromosome harboring the full-length human PRSS1 with the key mutation of hereditary pancreatitis (PRSS1R122H). During the funding year we used this novel mouse model to determine whether PRSS1R122H predispose to pancreatitis. Our data so far indicates that mice expressing PRSS1R122H develop a more severe form of pancreatitis than wild type mice controls. We are working now in understanding the mechanisms underlying the observed effects.

Subject Terms:
Pancreatitis, acute pancreatitis, chronic pancreatitis, hereditary pancreatitis, alcohol abuse, smoking, endoplasmic reticulum stress.

Security Classification: U

Limitation of Abstract: UU

Number of Pages: 9

Distribution / Availability Statement: Approved for public release; distribution unlimited.
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4. INTRODUCTION:
Pancreatitis is an inflammatory disease of the pancreas that causes significant morbidity and mortality. Pancreatitis occurs in acute and chronic variants. Acute pancreatitis especially due to alcohol and smoking goes onto chronic pancreatitis which, in turn, has the highest proportional risk for pancreatic cancer of any known environmental risk factor. Because only a relatively small portion of patients with alcohol abuse and smoking develop pancreatitis, it is very likely that there are genetic underlying predisposing factors that have not been discovered that explain why certain individuals develop pancreatitis. A genetic defect in the trypsinogen gene (PRSS1 gene) causing hereditary pancreatitis is now well established. We developed a transgenic mouse using a Bacterial Artificial Chromosome harboring the full-length human PRSS1 with the key mutation of hereditary pancreatitis (PRSS1_R122H). With this novel model, we will test our central hypothesis that physiological/environmental factors interact with PRSS1_R122H to activate the trypsin-endoplasmic reticulum (ER) stress- inflammation axis and cause both acute and chronic pancreatitis and progression to pancreatic cancer. Targeting the ER stress and inflammatory cascade will be beneficial for pancreatitis prevention and therapy. We expect that this study will elucidate fundamental mechanisms of pancreatitis and provide a preclinical platform for testing potential therapies.

5. KEYWORDS:
Pancreatitis, acute pancreatitis, chronic pancreatitis, hereditary pancreatitis, alcohol abuse, smoking, endoplasmic reticulum stress.

6. ACCOMPLISHMENTS:

6.1. Major goals of the project (Cedars-Sinai Medical Center site):
The major goals described in the approved SOW remain the same.

Specific Aim 2: Determine the role of PRSS1_R122H and alcohol/smoking in the development of pancreatitis. 
Subtask 1: Test the hypothesis that mutant PRSS1 expression cooperates with ethanol and smoke extract NNK to induce increased trypsin activity and pancreatic acinar damages.

Subtask 2. Test the hypothesis that PRSS1R122H will sensitize the pancreas to ethanol and NNK-induced pancreatitis.

6.2. Accomplishments under the goals stated in the approved SOW

A. Local IACUC approval. The Cedars-Sinai IACUC protocol (IACUC6303; PI Pandol) was approved on 7/22/2015.

B. ACURO approval. This approval was obtained on September 2015. Continuation renewal was sent on September 12, 2016.

C. Transfer of R122H mice from the animal facilities at Mayo Foundation for Medical Education (PI Baoan Ji) to the animal facilities at Comparative Medicine, Cedars-Sinai Medical Center (PI Stephen J Pandol).
   This task required: (a) Preparation and final approval of an MTA between the two institutions; (b) administrative coordination between the two animal facilities for final approval and transferring of mice to Cedars-Sinai Medical Center; (c) shipping of two male mouse breeders (R122H strain) from Mayo Foundation to Cedars-Sinai Medical Center on 10-27-2015.

D. Mandatory-rederivation of the R122H mice at Cedars-Sinai
   A mandatory rederivation of the transferred R122H mice was performed at the Cedars-Sinai Mouse Genetics Core. The purpose was to ensure Specific Pathogen Free (SPF) status in all experimental animals to be used in the project. The process involves a short quarantine and acclimation period for the imported mouse
breeders, producing embryos free of pathogens, transfer of embryos into clean foster mothers, confirming genotype of new pups using standard genotyping techniques, and weaning and delivering of SPF R122H mice to the Principal Investigator. After rederivation, the PI received from the Mouse Genetic Core 10 founder R122H mice (male and females; 4-weeks-old) on 1/12/2016.

E. Breeding and expansion of the R122H mouse colony:
Period: February 2016-present.
After rederivation, the colony of R122H has been expanded at the animal facilities of the department of Comparative Medicine at Cedars-Sinai Medical Center to obtain sufficient R122H and wild-type mice for experiments. Breeding started on February 2016, and is currently ongoing. Breeding is performed following institutional approved procedures.

F. Specific Aim 2: Determine the role of PRSS1R122H and alcohol/smoking in the development of pancreatitis.

Subtask 1. Test the hypothesis that mutant PRSS1 expression cooperates with ethanol and smoke extract NNK to induce increased trypsin activity and pancreatic acinar damages.
Period: March 2016-present

The activities in this part of the project involve the use of freshly isolated pancreatic acini (clusters of acinar cells) obtained from wild-type (WT) and R122H mouse pancreas. Acini are used within 24 h after isolation and cultured in standard culture conditions.

Initial experiments were designed to characterize the phenotype of R122H pancreatic acini in basal conditions and during pancreatitis stress conditions. Acini isolated from WT and R122H mice were kept untreated or treated for different periods of time with the secretagogue cholecystokinin (CCK) at physiological and supraphysiological concentrations. CCK is a hormone that at physiological concentrations induces the release of digestive enzymes by pancreatic acinar cells. However, when used experimentally at supra-physiological concentrations, CCK induces acinar cell damage and pancreatitis responses including aberrant trypsin activity, autophagy, mitochondria and endoplasmic reticulum (ER) dysfunction, activation of inflammatory signals, and cell death. We observed, that compared to WT cells, R122H cells display in basal conditions similar morphology and protein levels of digestive enzymes including amylase and trypsinogen, and markers of ER and autophagy function (Figure 1). As expected, we found that R122H cells contain high levels of the human mutated trypsinogen form (R122) as well as the trypsin inhibitor SPINK3; these data suggest that the presence of mutated trypsinogen may trigger a protective response in R122H acinar cells to prevent toxic levels of trypsin activity. CCK at high concentrations increased trypsin activity in both WT and R122H cells, but this increase was higher in long-term cultured R122H cells; these results indicate that mutated R122H trypsinogen may be resistant to degradation (as suggested by other authors) and sustain trypsin activity for longer periods of time than wild-type trypsinogen (Figure 2). Compared to WT cells, CCK-treated R122H acinar cells display more rapid decreases in pro-survival signals (Figure 3), supporting the concept that R122H acinar cells are prompted to pancreatitis responses under conditions of stress. During the next funding period, we plan to measure cell death and inflammatory signals in R122H and WT cells in response to high concentrations of CCK and other pancreatitis inputs.
During the funding period, we also initiated studies to measure the effects of alcohol and the smoking compounds NNK (a nicotine derivative) and cigarette smoke extracts (CSE; a condensate of experimental cigarette smoke) on pancreatitis responses in cultured WT and R122H pancreatic acini. These compounds were tested at concentrations estimated to be found in blood and tissues of heavy, longtime drinkers or smokers. Acini were isolated from mouse pancreas and cultured for up to 24 h in the presence of alcohol, NNK or CSE alone or in combination. Since mouse acini can be cultured only for short periods of time (up to 24 h), we also measured the effects of prolong exposure to alcohol and smoking compounds in the rat acinar cell line AR42J cells. As illustrated in Figure 4, ethanol or smoking compounds (CSE) alone had little effect on acinar cell death responses, but this effect was greatly enhanced in cells treated with ethanol and CSE in combination. At the incubation times (up to 72 h) and concentrations tested (100 nM), NNK had little effect on acinar cell death (not shown). The toxic effects of the combined treatment of CSE + ethanol were associated with increased endoplasmic reticulum (ER) stress (Figure 5), reduction in protective cellular stress responses associated with the transcription factor XBP1s (Figure 6A), and oxidative stress (Figure 6B). Moreover, the toxic effects of ethanol+CSE in acinar cells, could be partially reproduced by a pharmacologic agent that abrogates protective ER stress responses elicited by XBP1s (Figure 4B), suggesting that alcohol and smoking can predispose to pancreatitis by similar mechanism.
Ethanol and smoking compounds induce a dysregulated ER stress response. Acinar cells were kept untreated (control; C) or treated for 48 h with (EtOH, 50 mM) or cigarette smoke extract (CSE, 40 mg/ml) alone or in combination. (A) Expression levels of the adaptive ER stress regulator XBP1s and the proapoptotic transcription factor CHOP were measured by qPCR. (B) Expression levels of the oxidative stress responses genes NAD(P)(H:Quinone Oxidoreductase 1 (Nqo1) and Glutamate-Cysteine Ligase (Gclc) were measured by qPCR. Graphs show means ± SEM, n=3; *p<0.05 vs. control.

To determine whether the presence of mutated trypsinogen predisposes to alcohol and smoking-induced toxic effects on acinar cells, wild-type and R122H acini were treated with ethanol and/or smoking compounds (NNK and CSE) as indicated above. These studies are currently ongoing. Initial results indicate that, as found in WT cells, the combined ethanol + CSE treatment increased cell death compared to ethanol+NNK (not shown) or the individual treatments (Figure 7). Moreover, ethanol + CSE toxic effects were greater in R122H cells than in WT cells. During the next funding period, we plan to complete this set of experiments to increase the statistical power of the data, and to elucidate the mechanisms underlying alcohol and smoking-induced cell death in R122H acini. In addition, as stated in the SOW, we plan to measure other pancreatitis responses in acini treated with alcohol and smoking compounds: amylase secretion, trypsin activity and inflammatory signals including ROS levels and NF-kB activation.

Subtask 2. Test the hypothesis that mutant PRSS1 expression cooperates with ethanol and smoke extract NNK to induce increased trypsin activity and pancreatic acinar damages.

Period: March 2016–present.
In this part of the project, we plan to measure pancreatitis responses in WT and R122H mice fed ethanol containing diets and treated with NNK or CSE. To perform a first round of ethanol feeding, we established breeding pairs of mice in July 2016 to obtain 20 WT and R122H mice (male and females). Mice have been already genotyped, and ethanol feeding in this first set of mice will start in October 2016. WT and R122H mice will be fed control or ethanol-containing Lieber-DeCarli diets for 6 weeks, and then subjected to acute cerulein pancreatitis to study the susceptibility of R122H to alcoholic pancreatitis. In subsequent sets of ethanol-fed mice, we will measure the effects of alcohol and smoking on pancreatitis responses. End points to be measured are detailed in the SOW.

6.3. Opportunities for training and professional development
Nothing to report.

6.4. How were the results disseminated to communities of interest?
Nothing to report.

6.5. Plans during the next reporting period to accomplish the goals
Plans detailed in the approved SOW for the next reporting period remain the same.

7. 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.

8. CHANGES/PROBLEMS:
Changes in approach and reasons for change
Nothing to report.
a. Actual or anticipated problems or delays and actions or plans to resolve them
Nothing to report.

b. Changes that had a significant impact on expenditures
Nothing to report.

c. Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to report.

d. Significant changes in use or care of human subjects
Nothing to report.
e. Significant changes in use or care of vertebrate animals.
Nothing to report.

f. Significant changes in use of biohazards and/or select agents
Nothing to report.

9. PRODUCTS:
i. Journal publications.
Nothing to report.

ii. Books or other non-periodical, one-time publications.
Nothing to report.

iii. 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.

10. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS
a. What individuals have worked on the project?
All individuals indicated as personnel in the project submission.

b. Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period? There is a new project funded:
Title: Targeting Protein Kinase D in Alcoholic Pancreatitis

Effort: 10% (PI, Pandol)

Supporting Agency: National Institutes of Health/NIAAA (R01AA024464)

Name & Address of Funding Agency/Grants Officer:
Division of Metabolism and Health Effects
National Institute on Alcohol Abuse and Alcoholism
5635 Fishers Lane, Room 2102, MSC 9304
Bethesda, MD 20892-9304/ Peter Gao, PhD

Performance Period: 09/01/2016 – 08/31/2021

Funding Amount: $225,000 per year

Project Goals: The goals of this project are to characterize the roles of protein kinase D in pancreatitis and to develop new treatments.

Specific Aims:
Aim 1. Identify the dominant PKD isoform in human exocrine pancreas by immunohistochemistry using PKD isoform specific antibodies and in situ hybridization.
Aim 2. Generate validated structural models and testing platforms for the dominant human PKD isoform(s) and use these to screen libraries of ligands to identify new chemical entities for treatment of pancreatitis.
Aim 3. Determine the role of PKD in features of pancreatitis (NF-κB activation, necrosis and intracellular trypsinogen activation) using rodent models, human pancreatic primary acinar cells.

Overlap: There are no administrative, financial or scientific overlaps with the other previous, active or pending grant applications.

11. SPECIAL REPORTING REQUIREMENTS
a. COLLABORATIVE AWARDS:
Nothing to report.

b. QUAD CHARTS:
Nothing to report.

12. APPENDICES:
None