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14. ABSTRACT

The proposed project relates to the FY17 PRMRP topic area on pancreatitis. The project will explore a previously undescribed mechanism of acute pancreatitis in which pathological hemodynamic changes in the pancreas could induce acute pancreatitis responses. Two Aims are proposed. Aim 1 will determine the mechanism by which pathological hemodynamic changes cause acute pancreatitis. In Aim 2, we will determine the role of lymphangiogenesis in the development and/or the resolution of acute pancreatitis. During the reporting period, we have established tissue clearing method necessary for 3-dimentional imaging of lymphatic vessels in the pancreas. The method will be useful for analysis of not only lymphatic vessels, but also other cells in the pancreas. We also showed that macrophages promote pancreatic lymphangiogenesis in mice with portal hypertension.

15. SUBJECT TERMS

Acute pancreatitis, lymphatic system, lymphangiogenesis, etiology, blood flow, experimental model, risk factors, macrophages, T-cells, pancreatic stellate cells, inflammation, edema, VEGF-C

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1. INTRODUCTION:

The proposed project relates to the FY17 PRMRP topic area on pancreatitis. The causes of acute pancreatitis are not fully elucidated. Further, the role of the lymphatic system is little understood in acute pancreatitis in particular and in the study of the pancreas in general. The development of simple and reproducible experimental models of acute pancreatitis that are relevant to human disease are urgently needed. This project addresses these critical problems with innovative ideas. First, the project will define a new etiology of acute pancreatitis and explore a previously undescribed mechanism in which pathological blood flow changes in the pancreas could induce acute pancreatitis. Second, it will examine the role of the pancreatic lymphatic system in acute pancreatitis, representing the first step toward understanding biology of the pancreatic lymphatic system. Third, a new and simple experimental model of acute pancreatitis, which can also be used for the study of the pancreatic lymphatic system, will be established. Addressing a new etiology and a new area of study, the project will significantly contribute to our understanding of the etiology and mechanism of acute pancreatitis and could lead to the identification of new risk factors as well as new therapeutic strategies for this disease.

2. KEYWORDS: *(limit to 20 words).*

Acute pancreatitis, lymphatic system, lymphangiogenesis, etiology, blood flow, experimental model, risk factors, macrophages, T-cells, pancreatic stellate cells, inflammation, edema, VEGF-C

3. ACCOMPLISHMENTS:

- **What were the major goals (Specific Aims) of the project?**

Specific Aims 1: Determine the mechanism by which pathological hemodynamic changes cause acute pancreatitis.

1. Determine the role of mechano- signaling in the development of acute pancreatitis (AP) (Exp 1-1). In progress
2. Determine the role of immune cells in the development of AP (Exp 1-2 to 1-4). In progress
3. Assess a sensitizing effect of pancreatic hemodynamic changes on AP (Exp 1-5). In progress

Specific Aim 2: Determine the role of lymphangiogenesis in the development and/or the resolution of acute pancreatitis.

4. Establish a 3-D imaging method for lymphatic vessels in the pancreas (Exp 2-1). 50%
5. Determine the role of lymphangiogenesis in the development of AP (Exp 2-2 & 2-3). 50%
6. Meeting presentation and manuscript preparation. 30%

- **What was accomplished under these goals (Specific Aims)?**

Major accomplishments are made particularly in Specific Aim 2. We are also actively performing experiments proposed in Specific Aim 1. Below are summary of major activities, specific objectives and significant results/key outcomes in each Aim (Goal).

Specific Aims 1: Determine the mechanism by which pathological hemodynamic changes cause acute pancreatitis (AP).

The objective of this Aim is to elucidate the role of mechano-sensing of pancreatic vasculature in the initiation of acute pancreatitis and to determine the role of immune cells in this process. We are in the preparation of setting up breeding mice (VE-cad-TMD heterozygous mice), which will be provided by our collaborator, Dr. Martin Schwartz of Yale University. To determine the role of macrophages in the development of AP, we have confirmed sufficient depletion of macrophages in pancreas by an intraperitoneal injection of clodronate liposome in two days. We will perform partial portal vein ligation (PPVL) surgery on mice with macrophage depletion and determine effects of macrophage depletion on AP.

Specific Aim 2: Determine the role of lymphangiogenesis in the development and/or the resolution of acute pancreatitis (AP).

The objective of this Aim is to determine whether lymphangiogenesis facilitates or mitigates AP. First, we proposed to establish a 3-D imaging method for lymphatic vessels in the pancreas using podoplanin (pdpn)-GFP reporter mice [B6;D2-Tg(Pdpn, -EGFP)16Dobb/J, JAX Stock#:028357, The Jackson Laboratory, Bar Harber, ME] (Exp 2-1). The pdpn-GFP mice allow us to visualize lymphatic vessels in the pancreas. We are currently waiting for cryo-recovery of these mice. Once it is recovered, we will establish breeding colonies to obtain sufficient number of mice. In the meantime, we have tested several tissue clearing methods essential for 3-D visualization and identified the most effective method to visualize pancreatic vasculature and lymphatic vessels. Figure 1 shows lymphatic vessels (red) immunolabeled with Lyve-1 (a lymphatic vessel marker) and all vasculatures (green, VE-cadherin-positive endothelial cell). The clearing reagent is available through Sunjin Lab Optical Clearing Innovation (Cedarlane Corporation, Burlington, NC). This is exciting because this method will allow us and other

researchers to visualize pancreatic lymphatic vessels three dimensionally. Once we generate sufficient numbers of pdpn-GFP reporter mice, we will visualize lymphatic vessels using these mice and tissue clearing method we established here.

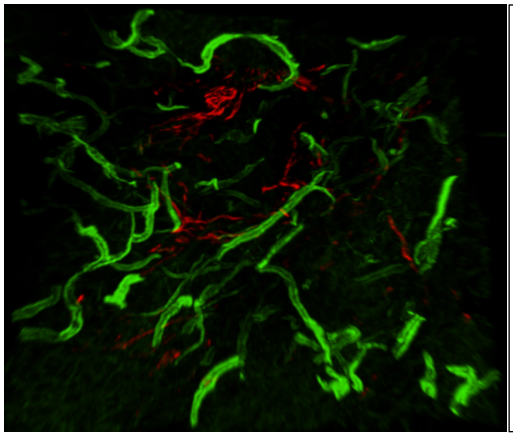

Figure 1 3-D image of lymphatic vessels (red) in relation to endothelial cells (green) in the pancreas. Sunjin Lab Optical Clearing Innovation (Cedarlane Corporation, Burlington, NC) was used for tissue clearing. Cdh5-cre mTmG reporter mice, which express GFP in endothelial cells, was used to visualize all endothelial cell in the pancreas. Lyve-1 was used for immunolabeling lymphatic endothelial cells.

We also proposed to determine the role of macrophages in pancreatic lymphangiogenesis in portal hypertension. Clodronate liposome was injected two days before PPVL surgery to deplete macrophages. Pancreas samples were isolated 10 days after PPVL surgery. There was a significant reduction in lymphangiogenesis in PPVL group, compared to those with macrophage depletion, suggesting that macrophages facilitate pancreatic lymphangiogenesis in portal hypertension (Figure 2). Using the same methods, we will evaluate determine effects of macrophage depletion on AP and pancreatic fibrosis (Specific Aim 1 above).

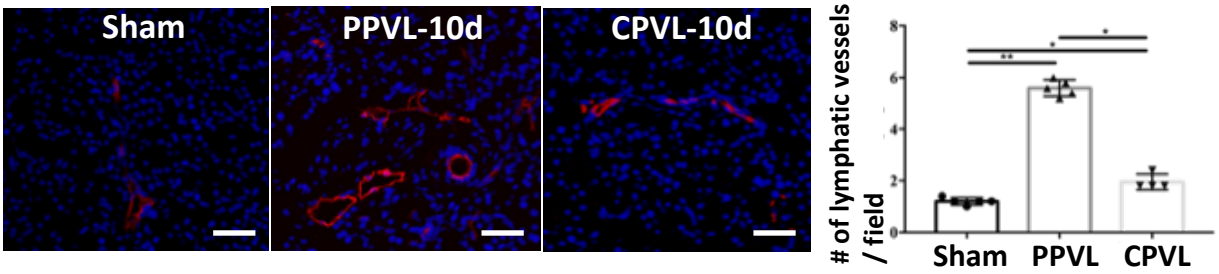

Figure 2 Macrophage depletion significantly decreased lymphatic vessels in PPVL mice. Podoplanin: red, a lymphatic endothelial cell marker. Nucleus: blue, DAPI. N=4-5 per group. PPVL: partial portal vein ligation. CPVL: PPVL rats given clodronate liposome to deplete macrophages. * $p<0.05$, ** $p<0.01$. Scale bar: 25μm.

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

Two lab members, Sanchuan Lai (a MD/PhD student) and Taiichi Wakiya (a visiting Assistant Professor), presented their works related to this project at the annual meeting of American Pancreatic Association (October 31 – November 3, 2018, in Miami, FL). Attending this meeting provided them to present their progress and receive useful comments to improve their work. In addition, Sanchuan Lai received a travel award, which is very much encouraging to a young investigator to pursue their career in biomedical sciences. Below are their abstract titles.

1. Sanchuan Lai, Masatake Tanaka, Teuro Utsumi, Jingwei Mao, Fred Gorelick, and Yasuko Iwakiri. Portal vein hypertension induces lymphangiogenesis in the pancreas through macrophage-derived VEGF-C production.

2. Taiichi Wakiya, Jingwei Mao, Teruo Utsumi, Masatake Tanaka, Fred Gorelick, and Yasuko Iwakiri. A mouse model of interstitial pancreatic edema induced by partial portal vein ligation.

- **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?**

Specific Aims 1: Determine the mechanism by which pathological hemodynamic changes cause acute pancreatitis (AP).

1. Determine the role of mechano- signaling in the development of AP (Exp 1-1).
   Plan: We will generate a sufficient number of VE-cad-TMD mice and their WT control, perform PPVL surgery and determine effects of mechano-sensing defect on AP and pancreatic fibrosis.
2. Determine the role of immune cells in the development of AP (Exp 1-2 to 1-4).
   Plan: First, we assess effects of macrophage depletion on AP and pancreatic fibrosis induced by PPVL. Samples have been obtained from the study in Aim 2 (Figure 1 was generated using these samples). Second, we will perform PPVL surgery on immune deficient mice (nude mice) and assess AP and pancreatic fibrosis. Third, we will isolate macrophages and T-cells from pancreas of mice given PPVL or sham operation and perform RNA sequencing to determine genes responsible for the development of AP and pancreatic fibrosis.
3. Assess a sensitizing effect of pancreatic hemodynamic changes on AP (Exp 1-5).
   Plan: We will administer cerulein intraperitoneally to mice with sham or PPVL surgery and AP and pancreatic fibrosis.

Specific Aim 2: Determine the role of lymphangiogenesis in the development and/or the resolution of acute pancreatitis (AP).

4. Establish a 3-D imaging method for lymphatic vessels in the pancreas (Exp 2-1).
   Plan: We will establish mouse colonies of pdpn-GFP mice to generate a sufficient number of mice for 3-D imaging of lymphatic vessels in the pancreas using tissue clearing method that has been established during the first year period.
5. Determine the role of lymphangiogenesis in the development of AP (Exp 2-2 & 2-3).
   Plan: We will perform PPVL surgery on mice with control or adnoviral-sVEGFR3 delivery to block lymphangiogenesis, then assess AP and pancreatic fibrosis.

I would like to thank the Department of Defense to support our research on pancreatitis. This grant provides us to pursue our research on pancreatitis which was new to us. In the next funding period, we will try to present our progress again at the American Pancreatic Association meeting and start preparing an R01 type grant application using results generated from the proposed study.

4. IMPACT:

- **What was the impact on the development of the principal discipline(s) of the project?**
  We have established tissue clearing method necessary for 3-D imaging of lymphatic vessels in the pancreas. The method can be used for visualizing not only lymphatic vessels, but also other cells in the pancreas. We also showed that portal venous congestion, commonly seen in portal hypertension, a serious complication of liver disease, can lead to lymphatic edema, lymphangiogenesis and acute pancreatitis. This is the first demonstration that portal hypertension alone can lead to acute pancreatitis.

- **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?**
  The project provided opportunities to two young physician scientists to study acute pancreatitis and present their work at a national meeting. Given a decrease in the number of physician scientists, this experience is precious and encouraging for them.

5. CHANGES/PROBLEMS:

Nothing to report.

6. PRODUCTS:

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

1. **Journal publications.**

   Nothing to report.

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

   Nothing to report.

3. **Other publications, conference papers, and presentations.**

   Two lab members, Sanchuan Lai (a MD/PhD student) and Taiichi wakiya (a visiting Assistant Professor), presented at the annual meeting of American Pancreatic Association (October 31 – November 3, 2018, in Miami FL). Below are their abstract titles. Please also refer to APPENDICES for abstracts.

   1. Sanchuan Lai, Masatake Tanaka, Teuro Utsumi, Jingwei Mao, Fred Gorelick, and Yasuko Iwakiri. Portal vein hypertension induces lymphangiogenesis in the pancreas through macrophage-derived VEGF-C production.

   2. Taiichi Wakiya, Jingwei Mao, Teruo Utsumi, Masatake Tanaka, Fred Gorelick, and Yasuko Iwakiri. A mouse model of interstitial pancreatic edema induced by partial portal vein ligation.

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

  Nothing to report.

- **Technologies or techniques**

  We have established a tissue clearing method necessary for 3-D imaging of lymphatic vessels in the pancreas. The method can be used for visualizing not only lymphatic vessels, but also other cells in the pancreas. We will share this technique in our future publication.

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

  Nothing to report

- **Other Products**

  This project generated a new animal model of acute pancreatitis, namely partial portal vein ligation (PPVL).

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Name:	*Sanchuan Lai*
Project Role:	*Postgraduate fellow*
Researcher Identifier (e.g. ORCID ID):	*None*
Nearest person month worked:	*19 months*
Contribution to Project:	*Dr. Lai has performed work in the mechanism of pancreatic lymphangiogenesis in portal hypertension.*
Funding Support:	*China Scholarship Council*

Name:	*Taiichi Wakiya*
Project Role:	*Visiting Assistant Professor*
Researcher Identifier (e.g. ORCID ID):	*None*
Nearest person month worked:	*21 months*
Contribution to Project:	*Dr. Wakiya has performed work on the mechanism of acute pancreatitis in mice given partial portal vein ligation and cerulein.*
Funding Support:	*The Fund from Hirosaki University School of Medicine, Japan.*

- **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 change.

- **What other organizations were involved as partners?**

  Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS:**

  Nothing to report.

- **QUAD CHARTS:**

  Nothing to report

9. APPENDICES:

Two meeting abstracts for the following titles are attached. These were presented at the annual meeting of American Pancreatic Association (October 31 – November 3, 2018, in Miami FL).

1. Sanchuan Lai, Masatake Tanaka, Teuro Utsumi, Jingwei Mao, Fred Gorelick, and Yasuko Iwakiri. Portal vein hypertension induces lymphangiogenesis in the pancreas through macrophage-derived VEGF-C production.

2. Taiichi Wakiya, Jingwei Mao, Teruo Utsumi, Masatake Tanaka, Fred Gorelick, and Yasuko Iwakiri. A mouse model of interstitial pancreatic edema induced by partial portal vein ligation.

Portal vein hypertension induces lymphangiogenesis in the pancreas through macrophage-derived VEGF-C production

Sanchuan Lai[1], Masatake Tanaka[1], Teruo Utsumi[1,2], Jingwei Mao[1], Fred Gorelick[1,2], and Yasuko Iwakiri[1]

1. Section of Digestive Diseases, Yale School of Medicine, New Haven, CT, USA
2. VA Connecticut Healthcare System, West Haven, CT, USA

Background
Lymphatic vessels are a primary pathway for drainage of excessive interstitial fluid and infiltrating immune cells in pathological conditions. Little is known about the formation of new lymphatic vessels (lymphangiogenesis) in the pancreas. The aim of this study was to determine 3D structure of lymphatic vessels and the mechanism of lymphangiogenesis in the pancreas of rats with increased portal vein pressures.

Method
Partial portal vein ligation (PPVL) surgery was performed in rats to induce portal vein hypertension. Pancreases were collected 3 and 10 days after PPVL as well as from rats with sham operation. Lymphatic vessels (LVs) and lymphangiogenesis were determined by LYVE-1/podoplanin and podoplanin/PCNA co-immunolabeling, respectively. 3D images of LVs were visualized using scanning confocal microscopy.

Results
Severe pancreatic edema was observed at 3 days after PPVL, but was cleared by 10 days. Lyve-1^+/podoplanin$^+$ LVs significantly increased at 3 days (3.6-folds, $p<0.01$) and continued to increase at 10 days after PPVL (4.5 folds, $p<0.01$) compared to those of sham rats. The number of CD68-positive macrophages increased significantly at 3 days (3.3-fold, $p<0.01$) but decreased by 10 days (1.6-fold, $p<0.05$) compared to that of sham rats. 3D imaging showed newly developed LVs along with pancreatic blood vessels. Macrophage depletion by clodronate liposomes significantly decreased LVs (2.5-fold, $p<0.01$) in the pancreas of rats with 10-day PPVL. Treatment of 10-day PPVL rats with a neutralizing antibody to VEGF-C, the most potent lymphangiogenesis inducer, significantly decreased pancreatic lymphangiogenesis (2.0-fold, $p<0.05$) compared to 10-day PPVL rats given control IgG. Given that macrophages are known as a source of VEGF-C, macrophages contribute to pancreatic lymphangiogenesis induced by portal vein hypertension through VEGF-C production.

Conclusion
Portal vein hypertension by PPVL induces edema and lymphangiogenesis in the pancreas, suggesting a link between edema formation and lymphangiogenesis. Macrophage-derived VEGF-C mediates pancreatic lymphangiogenesis.

295 words/300 words limit

A new etiology of acute pancreatitis: The role of portal vein congestion and pancreatic edema

Taiichi Wakiya[1, 2], Jingwei Mao[1], Teruo Utsumi[1,3], Masatake Tanaka1, Fred Gorelick[1,3], and Yasuko Iwakiri[1]

3. Section of Digestive Diseases, Yale School of Medicine, New Haven, CT, USA
4. Department of Gastroenterological Surgery, Hirosaki University Graduate School of Medicine, Hirosaki, Aomori, JAPAN
5. VA Connecticut Healthcare System, West Haven, CT, USA

Background: The etiology and pathogenesis of acute pancreatitis (AP) remain to be fully elucidated. Venous congestion can lead to edema, which in turn can cause inflammation and injury through a process known as edemagenic stress. Because pancreatic venous flow enters the portal vein, we hypothesized that portal vein congestion would lead to pancreatic edema and acute pancreatitis.

Method: Partial portal vein ligation (PPVL) surgery was performed in mice. Pancreases were collected 1 and 3 days after PPVL to assess edema formation and AP. To characterize AP induced by PPVL, the conventional model of AP, cerulein injection (six hourly intraperitoneal injections, 50 μg/kg) was performed in a different set of mice.

Results: PPVL mice developed significant pancreatic edema after 1 day; the severity score was much higher than cerulein-treated mice ($p<0.001$) that only induced mild edema. Mice with PPVL and mice with cerulein exhibited similar levels of pancreatic inflammation but different time courses of immune cell infiltration. In cerulein-injected mice, the infiltration of macrophages and neutrophils was simultaneous. In PPVL mice, macrophages increased infiltration 1 day after PPVL, followed by neutrophil infiltration at 3 days. Patterns of acinar cell injury also differed between two groups. Though pyknotic nuclei levels in acinar cells were similar, vacuolization increased 4.5-fold in cerulein-injected mice ($p<0.001$) but not in PPVL mice. Cleaved caspase-3 (apoptosis marker)-positive acinar cells were increased 10-fold in mice with 1-day PPVL ($p<0.001$) and nearly 15-fold in mice with 3-day PPVL ($p<0.001$), while cerulein-injected mice tended to show increased LC3B (autophagy marker)-positive acinar cells (7.5-fold). These observations suggest that patterns of acinar cell damage between PPVL and cerulein-induced AP are different.

Conclusion: We find that portal vein congestion leads to pancreatic edema and AP in PPVL mice with features that differ from cerulein-induced pancreatitis.

291 words/300 words limit