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CONTRACTING ORGANIZATION: Yale University, New Haven, CT

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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 with a immune-labeling technique 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 are a key player for acute pancreatitis and pancreatic lymphangiogenesis in mice with portal hypertension.

15. SUBJECT TERMS
None listed.

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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:

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?**

Study goals:

1) To determine pathological hemodynamic changes in the pancreas as a new etiology of acute pancreatitis, and to understand the biology of the pancreatic lymphatic system in acute pancreatitis.
2) To establish the partial portal vein ligation (PPVL) model as a new and simple experimental model of acute pancreatitis and pancreatic lymphangiogenesis.
3) To establish a 3-dimensional (3D) imaging method to visualize pancreatic lymphatic vessels.

Specific aims:

1. Determine the mechanism by which pathological hemodynamic changes cause acute pancreatitis.
2. Determine the role of lymphangiogenesis in the development and/or the resolution of acute pancreatitis.

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

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

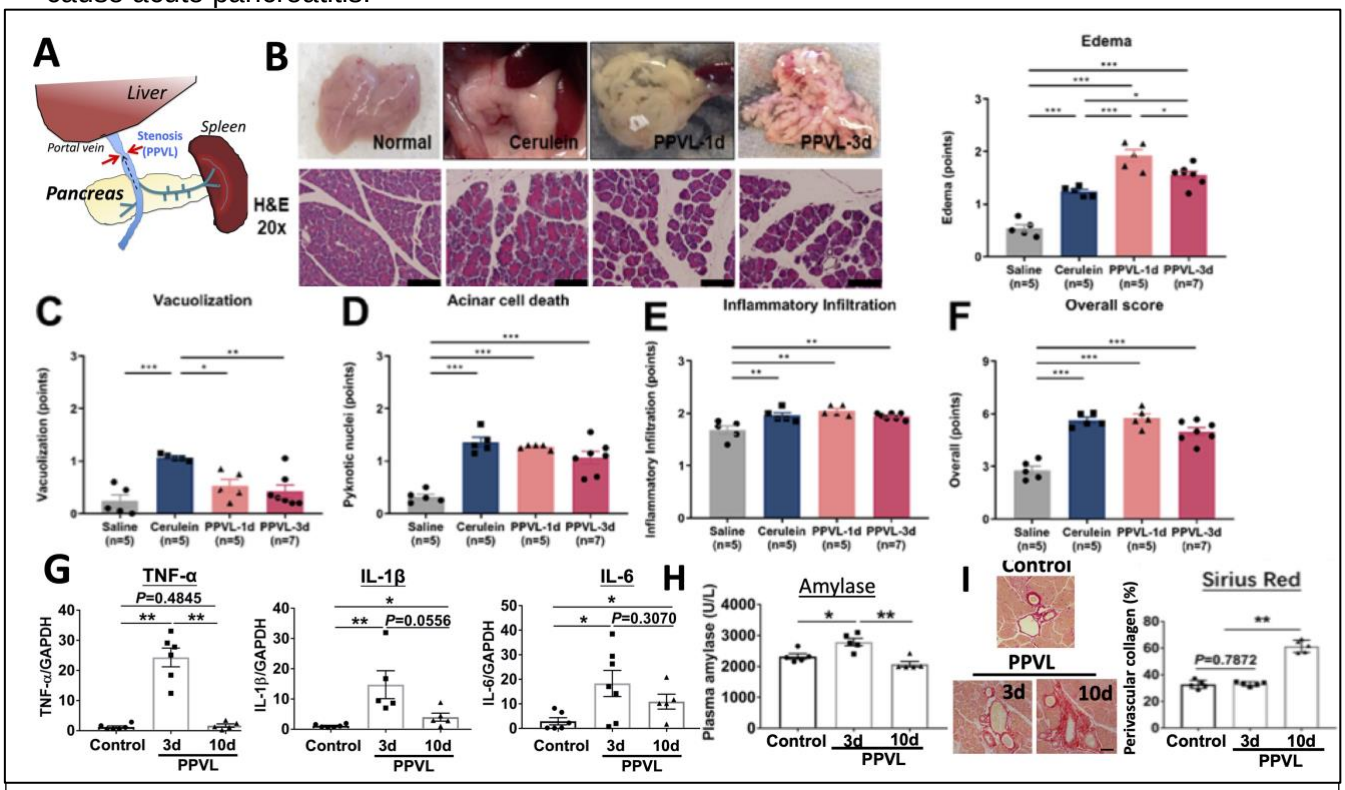

Fig 1. Portal venous congestion leads to acute pancreatitis in mice. A. Partial portal vein ligation (PPVL). **B.** Pancreas isolated from sham, 1 and 3 days after PPVL, and the cerulein model as a reference. Scale bar: 100μm. Evaluation of edema. **C.** Vacuolization. **D.** Acinar cell death. **E.** Inflammatory infiltration. **F.** Overall score of C, D and E. **G.** Inflammatory cytokine expression. **H.** Amylase. **I.** Sirius red staining for assessment of collagen deposition. Scale bar: 200μm. **p*<0.05, ***p*<0.01, ****p*<0.005.

We have demonstrated that portal venous congestion, induced by partial portal vein ligation (PPVL) surgery (Fig 1A), leads to pancreatic edema (Fig 1B), inflammation (Figs 1C, D, E, F and G), injury (Fig 1H) and excess collagen deposition (Fig 1I), which are all typical of acute pancreatitis. These changes are comparable with those induced by cerulein injection, a conventional model of acute pancreatitis. Thus, pathological hemodynamic changes in the pancreas caused by portal venous congestion represent a new etiology of acute pancreatitis. Other conditions, such as transarterial chemoemborization for hepatocellular carcinoma[1] and hypercoagulation states (acquired or inherited), may cause similar hemodynamic changes in the pancreas, indicating the broad implications of this study. The PPVL procedure, which has been used for the study of portal hypertension, can be used as a new experimental model for acute pancreatitis induced by pathological hemodynamic changes.

We have also demonstrated that macrophages facilitate lymphangiogenesis in mice with portal venous congestion (Fig 2). Macrophages infiltrated the pancreas as early as 1 day after PPVL surgery (Fig 2A), when pancreatic edema was present (Fig 1B). The presence of macrophages remained high at 3 days after PPVL, but returned to basal levels by 10 days. Pancreatic lymphangiogenesis occurred at 3 days after PPVL (Fig 2B), following edema and macrophage infiltration. Macrophage depletion by chrodronate liposomes significantly reduced PPVL-induced pancreatic lymphangiogenesis in rats (Fig 2C). Further, macrophages isolated from the pancreas of 3-day PPVL rats showed VEGF-C expression, the most potent lymphangiogenic factor (Fig 3). Collectively, these observations indicate that macrophages promote pancreatic lymphangiogenesis in the setting of portal venous congestion.

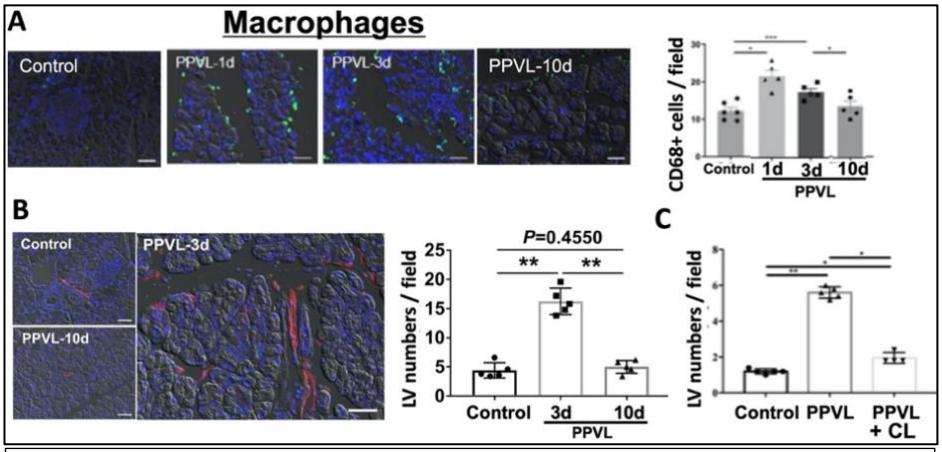

Fig 2. Macrophages promote pancreatic lymphangiogenesis. A. Macrophage infiltration in the pancreas isolated from sham, 1, 3 and 10-day PPVL mice. Green: CD68 (macrophage), Blue: DAPI (nucleus). Scale bar: 10μm. *p<0.05, **p<0.01. **B.** Lymphatic vessels (LV). Red: podoplanin (a lymphatic endothelial cell marker). Blue: DAPI. Scale bar: 25μm. *p<0.05, **p<0.01. **C.** Macrophage depletion decreases lymphatic vessel (LV) numbers. CL: Clodronate liposome. *p<0.05, **p<0.01.

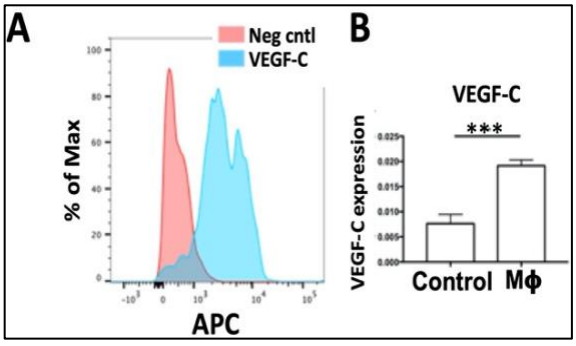

Fig 3. Macrophages isolated from the pancreas of PPVL rats express VEGF-C. A. Pancreatic macrophage suspensions were gated on VEGF-C+**.** Histograms showing an expression profile of VEGF-C+ (Blue line) macrophages. **B.** Total RNA was isolated from macrophages from the pancreas of 3-day PPVL rats and analyzed for VEGF-C mRNA. The results were normalized to GAPDH expression. Data were presented as mean+/-SE. ***p<0.005.

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

The objective of this Aim is to determine whether lymphangiogenesis facilitates or mitigates acute pancreatitis. To accomplish this objective, first, we proposed to develop a protocol for efficient tissue clearing and immunolabeling of pancreatic lymphatic vessels to visualize them in 3 dimension (Fig 4). We have established a protocol for visualizing pancreatic lymphatic vessels three-dimensionally. Fig 4 shows lymphatic vessels (red) immunolabeled with Lyve-1 (a lymphatic vessel marker) and all vasculatures (green, VE-cadherin-positive endothelial cell).

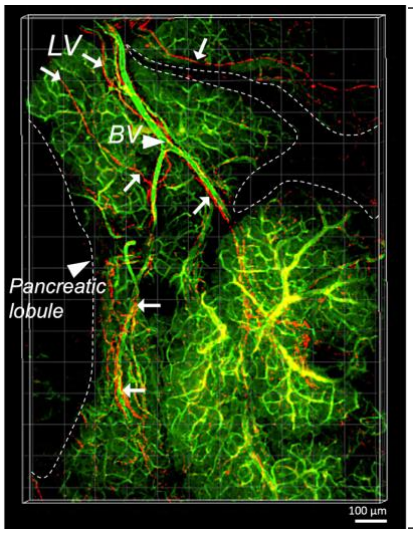

Figure 4. 3D image of lymphatic vessels (LV; red; arrows) in relation to all vasculatures (BV; green; arrow heads) in the pancreas.

SunJin Lab Optical Clearing solution (Cedarlane Corporation, Burlington, NC) was used for tissue clearing. Cdh5-cre mTmG reporter mice, which express GFP in endothelial cells, were used to visualize all endothelial cells (all vasculatures) in the pancreas. Lyve-1 (red) was used for immunolabeling lymphatic endothelial cells.

Next, we proposed to determine the effect of inhibition of lymphangiogenesis on pancreatitis. Pancreatic lymphatic vessels may play a critical role in clearing edema. Blocking VEGF-C/VEGFR3 signaling reduced lymphangiogenesis and increased edema in the pancreas (Fig 5). Given that sustained edema could lead to severe tissue injury, pancreatic lymphangiogenesis may be critical for resolving pancreatitis by clearing of edema, inflammatory cytokines and tissue waste materials.

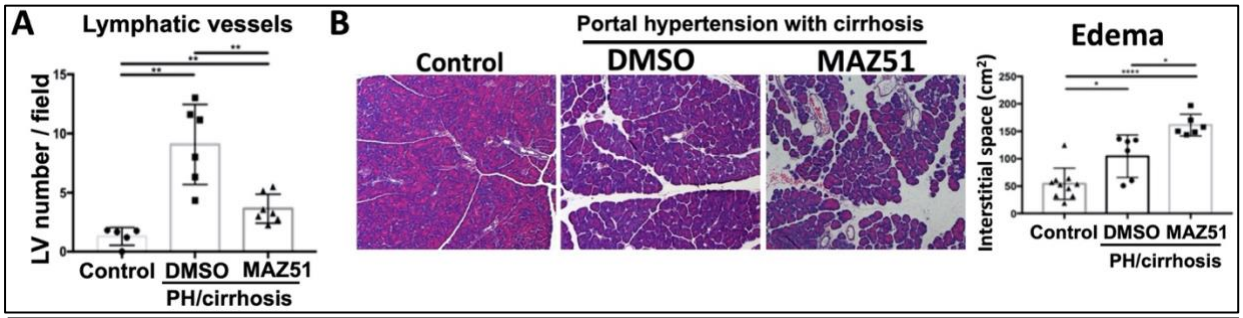

Figure 5. A decrease in lymphatic vessels increases pancreatic edema in cirrhosis with portal hypertension. Rats with liver cirrhosis and portal venous congestion were injected with MAZ51, a VEGFR-3 kinase inhibitor for 3 times a week for 3 weeks. **A.** Lymphatic vessel (LV) numbers in the rat pancreas. Pancreas sections were co-stained with LYVE-1 and podoplanin antibodies to identify lymphatic vessels. ***p*<0.01. **B.** H&E staining was used to evaluate the interstitial space of the pancreas (an indicator of edema). **p*<0.05, *****p*<0.001.

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

  Nothing to report.

- **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 (Final report)

I would like to thank the Department of Defense for supporting our research on acute pancreatitis, which was new to us when we first started this project. This grant provided us to generate numerous preliminary data and tools to study the role of lymphatics in acute pancreatitis. Using data generated from this grant, we will submit a manuscript this summer and apply R01 application later in 2022.

4. IMPACT:

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

  We have established tissue clearing method and immune-labeling protocol 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. Pathological hemodynamic changes in the pancreas caused by portal venous congestion represent a new etiology of acute pancreatitis. Other conditions, such as transarterial chemoemborization for hepatocellular carcinoma and hypercoagulation states (acquired or inherited), may cause similar hemodynamic changes in the pancreas, indicating broader implications of this study.

- **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.

5. CHANGES/PROBLEMS:

Due to the COVID19 pandemic, the hiring process for a research personnel for this study has been delayed significantly. This was a serious issue for this project. We also had limited access to some instruments and a slower service in core facilities. Since our lab's situation is getting closer to that before COVID-19 pandemic and we will have new lab members soon this year, we will continue to work on this project and submit a manuscript this summer.

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 with immune-labeling techniques 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.*

Name:	Jain Jeong
Project Role:	*Postdoc associate*
Researcher Identifier (e.g. ORCID ID):	*None*
Nearest person month worked:	8 months
Contribution to Project:	*Dr. Jeong has worked on the experimental protocol to establish immune-labeling and 3-D imaging methods for pancreatic tissues and analysis of lymphangiogenesis and edema*
Funding Support:	DOD and NIAAA

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

  During the entire reporting period, we received the following two grants. In addition, the PI has been promoted to a Professor of Medicine since the last reporting period.

R56 DK121511 (Iwakiri) 09/17/19 – 09/16/20 1.2 Calendar
NIH/NIDDK
Lymphatics in the liver
This study investigates the mechanism of hepatic lymphangiogenesis focusing on sympathetic nerve system in the liver and determines the role of lymphatics in the pathogenesis of liver disease. There is no overlap with the current DOD grant.

1 R01 DK130362-01 (Iwakiri) 07/01/2021 – 06/30/2024 2.4 Calendar
NIH/NIDDK
Endotheliopathy and liver injury in COVID-19
This study investigates the mechanism of endotheliopathy and liver injury observed in patients with COVID-19

- **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]

1. Section of Digestive Diseases, Yale School of Medicine, New Haven, CT, USA
2. Department of Gastroenterological Surgery, Hirosaki University Graduate School of Medicine, Hirosaki, Aomori, JAPAN
3. 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