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Research Article

The Relationship between Pancreatic Fibrosis and Pancreatic Cell Apoptosis Induced by Fas/Fas Ligand Pathways in *Aly/Aly* Mice

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1. Abstract

1.1. Objective: In this study, we examined the relationship between the expression of Fas/Fas ligand apoptotic signaling pathway and pancreatic fibrosis in autoimmune chronic pancreatitis of *aly/aly* mice.

1.2. Methods: The histopathological changes of inflammatory cell infiltration in pancreatitis *aly/aly* mice were observed. Immunohistochemistry was used to detect the expression of CD4, CD8, CD45R/B220, F4, Fas/Fas ligand, collagen I, TGF-β and α-SMA in pancreatic tissues.

1.3. Results: Inflammatory cell infiltration, such as T and B lymphocytes, as well as macro phages, were seen in pancreatic tissues of 5-week-old *aly/aly* mice, but the degree and area of inflammatory cell infiltration gradually increased with age. Fas/Fas ligand, collagen I, TGF-β and α-SMA were expressed in the inflammatory infiltration area of the pancreas, and some areas showed necrosis and fibrosis. The infiltration of T and B lymphocytes and macrophages was associated with the occurrence of autoimmune pancreatitis in *aly/aly* mice.

1.4. Conclusion: The activation and expression of Fas/Fas ligand signaling pathway may induce apoptosis of pancreatic acinar cells and inflammatory cells, resulting in pancreatic tissue necrosis and fibrosis.

2. Keywords: *Aly/Aly* mouse; Chronic pancreatitis; Fas/Fas ligand; Pancreatic fibrosis

3. Introduction

Chronic pancreatitis is a progressive and irreversible inflammatory and fibrotic disease [1]. Histologic features of chronic pancreatitis include chronic inflammation, fibrosis, acinar cell atrophy and stenotic ducts, in which the pancreatic acinar and islet cells destructed, the generated extracellular matrix replaced of normal tissue and made pancreas dysfunctionultimately [2, 3]. Apoptosis, as a more active form of cell death, is regulated by the apoptosis signaling pathway [4], but the relationship between pancreatic cell apoptosis and pancreatic fibrosis is not clear. Alymphoplasia/alymphoplasia (*aly/aly*) mice are autosomal recessive mutants of the C57BL/6J (H-2b) strain that possess a point mutation in the gene encoding NF-κB-inducing kinase (NIK), and are characterized by a complete lack of lymph nodes and Peyer's patches [5].The spleen of *aly/aly* mutants is devoid of well-defined lymphoid follicles, its white pulp is atrophic, and the thymus does not show clear cortical-medullary demarcation. The pathological pancreatic signs of *aly/aly* mice start as early as 3 weeks of age, with various populations of leukocytes involved in the inflammation response of the pancreas, eventually leading to fibrous necrosis of the exocrine pancreatic [6,7]. In the present study, we used *aly/aly* mice to elucidate the relationship between the activation of Fas/Fas ligand signaling pathway of the pancreatic acinar cells with the pancreatic tissue inflammation

and fibrosis during the occurrence and development of autoimmune pancreatitis.

4. Materials and Methods

4.1. Animals and Tissue Preparation

Male and female *aly/aly* mice were purchased from CLEA Japan, Inc. (Japan), bred and maintained in our animal facility in Tokyo Medical University under pathogen-free conditions, and treated in accordance with the guidelines of Tokyo Medical University for animal experimentation. Tissue preparation for immunohistochemistry was performed as previously described [7]. Briefly, 5-, 10-, 15-, 20-, 30-, 40- and 50-week-old *aly/aly* mice (n = 3-5 at each time point, irrespective of sex) were first anesthetized with ether. After the animals were completely anesthetized, the abdominal cavity was opened, and a catheter was inserted retrograde into the abdominal aorta immediately above its bifurcation into the common iliac arteries. Perfusion was initiated with normal saline containing heparin (10 IU/mL), and thereafter with 0.01 M PBS (pH 7.4) containing 4% paraformaldehyde (PFA); then the pancreatic tissue was stripped and immersed/fixed in 4% PFA liquid overnight at 4°C. The pancreas by gradient alcohol dehydration, transparent, xylene soaking paraffin embedding, maintained 5 μm thick slices.

4.2. Antibodies

The primary antibodies used were rat anti-mouse CD4 antibody (monoclonal, no. 550280; BD Pharmingen), rat anti-mouse CD8a(Ly-2) antibody (monoclonal, no. 550281; BD Pharmingen), rat anti-mouse CD45R/B220 antibody (monoclonal, no. 550286; BD Pharmingen), rat anti-mouse F4/80 antibody (monoclonal, no. ab6640; Abcam), rat anti-mouse Fas (X-20) and Fas ligand (Kay-10) antibody (monoclonal, no. sc-1024 and sc-19988, respectively; Santa Cruz biotechnology), rat anti-mouse Collagen α-I antibody (no. ab6640;Sigma), rat anti-mouse TGF-β antibody (no. sc-146;Sigma), and rat anti-mouse α-SMA antibody (no. A5228; Sigma); all antibodies were diluted according to each antibody manufacturer. The secondary antibody used was anti-rat IgG biotinylated antibody (AK 5004; Vectastain), diluted at 1:200.

4.3. HE and Immunohistochemical Staining

The sections were stained with Hematoxylin-Eosin (HE) and immuno staining was performed according to our previous study [7]. Briefly, the sections were immersed in Block Ace (Dainippon Pharmaceutical Co., Japan) containing 0.3% (v/v) hydrogen peroxide for 10 minutes to block endogenous peroxidase activity. After rinsing in 0.01M PBS, the sections were blocked with 1.5% normal rabbit serum for 20 minutes to reduce nonspecific staining, and incubated with the primary antibody for 2 h in a humidified chamber, and then with the secondary antibody for 30 minutes. Subsequently, the avidin-biotin complex technique (AB Complex/HRP; Vector Laboratories, Inc., Burlingame, CA, USA) was performed by incubating the sections with ABC complexes for 30 minutes, and then sections were stained with 3-3-diaminobenzidine and 0.005% H_2O_2. The sections were counterstained with Harris hematoxylin for 2-5 minutes, dehydrated in a graded ethanol series and xylene, and then cover slipped with Entellan new (Merck, Germany).All procedures in this section were performed at room temperature. After sealing the dye with plastic, sections were observed and analyzed using an Olympus microscope (Bx51). In addition, 0.05 M Tris-BSA buffers were used instead of primary antibody as a negative control.

5. Results

5.1 The Pathological Characteristics of Autoimmune Pancreatitis in *Aly/Aly* mice

In 5-week-old *aly/aly* mice, there were many neutrophil infiltrations in the interlobular areas of the pancreas (Figure 1A), and in 10-week-old *aly/aly* mice, a large number of lymphocyte in filtrations in the local acini was found in the pancreatic tissues (Figure 1B). In 30-week-old *aly/aly* mice, infiltration of lymphoid cells was found around the blood vessels and glandular tube in the pancreatic tissue; acinar necrosis and fibrosis were also observed (Figure 1C). Diffuse fibrosis, contractive islets and hyper plastic small pancreatic ducts were found in the pancreatic tissues of 50-week old *aly/aly* mice (Figure 1D).

CD4-, CD8-, and B220-positive cells were detected in pancreatic tissues of *aly/aly* mice. In 30-week-old mice, the number of CD4-positive cells was significantly higher than the two other cell types; in addition, F4/80-positive macrophages were seen in the same area (Figure 2). In the fibrosis area of the pancreas of 50-week-old *aly/aly* mice, CD4-, CD8-, B220- and F4/80-positive cells were significantly decreased, but the number of F4/80-positive macro phages was significantly higher than that of T and B cells (data not shown).

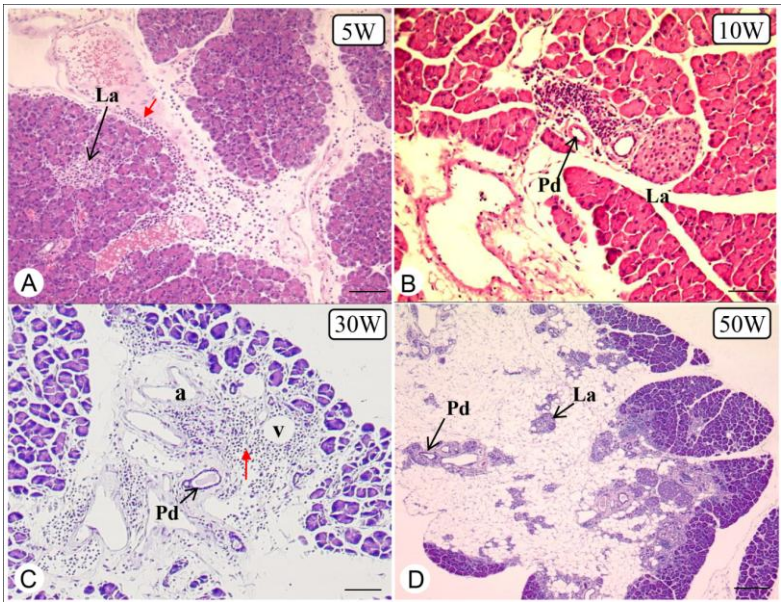

Figure 1: Histopathological analysis of pancreatitis development in *aly/aly* mice at 5-, 10-, 30- and 50-weeks of age. Red arrows indicate lymphoid cell infiltration; a, artery; pd, pancreatic duct; La, islet of Langerhans; v, vein. Scale bars: 200 μm.

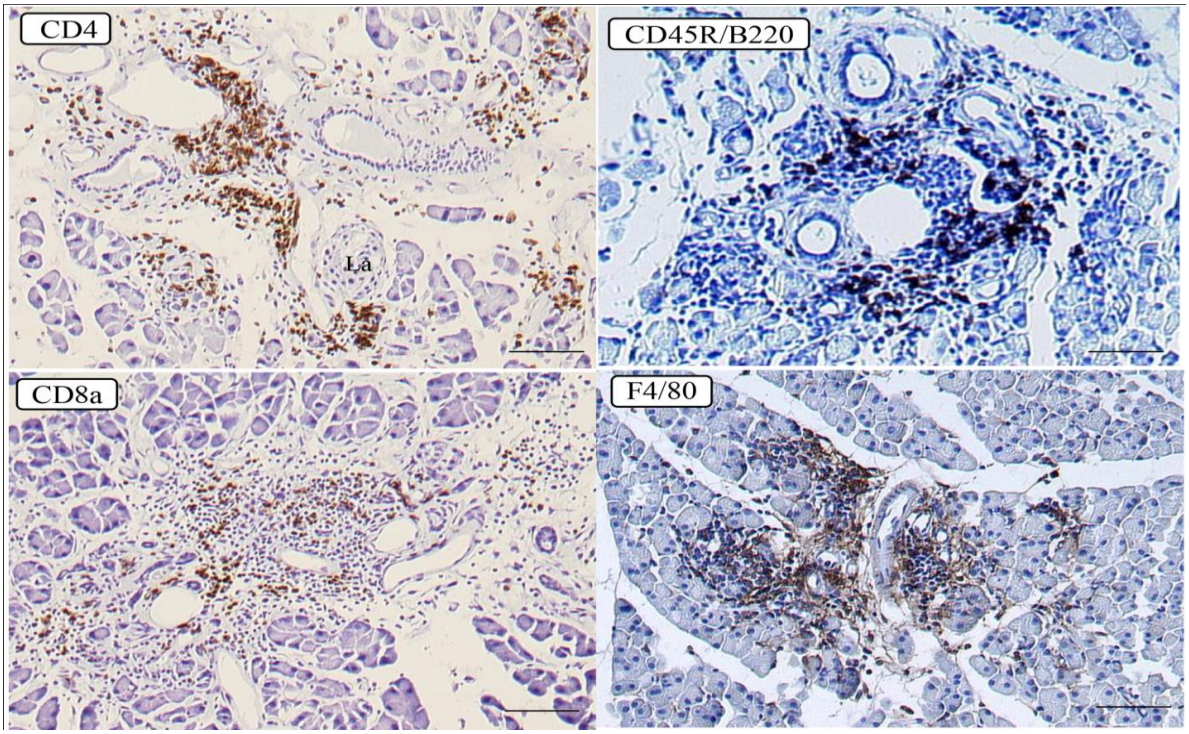

Figure 2: The expression of CD4+, CD8+, CD45R/B220+ and F4/80+ cells in pancreatic tissues of 30-week-old *aly/aly* mice. Scale bars: 200μm.

5.2. Activation and Expression of Fas and Fas Ligands in *Aly/Aly* Mice with Autoimmune Pancreatitis

A large number of Fas-positive cells were found in the inflammation infiltration area of the pancreas of 20-week-old *aly/aly* mice (Figure 3A). In addition, Fas ligand-positive cells were also found in the inflammatory infiltration area of 20-week-old mice, and the number was higher than that of Fas ligand-positive cells in 30-week-old mice (Figure 3B and 3C).

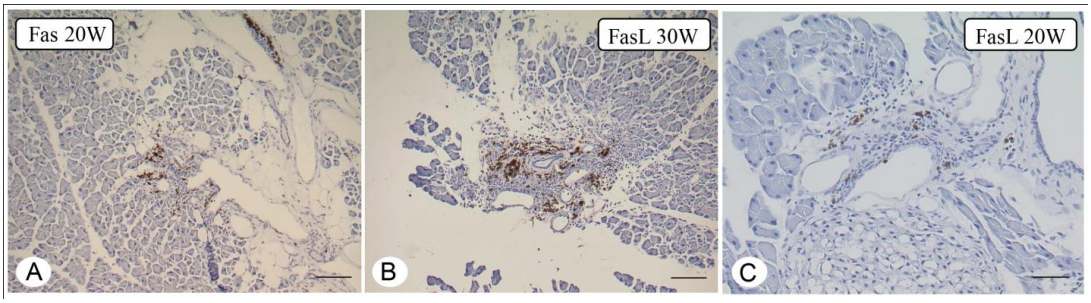

Figure 3: The expression of Fas and Fas ligand in the pancreas of *aly/aly* mice. Scale bars: 200μm (A and B) and 100μm (C).

5.3. The Expression of A-SMA, Collagen I and TGF-B in Pancreatic Tissues of *Aly/Aly* mice with Autoimmune Pancreatitis

With the development of autoimmune pancreatitis, acinar atrophy, necrosis and pimelosis were found in infiltration areas of pancreatic tissues in *aly/aly* mice. α-SMA was expressed in the pancreas ductal wall of the interstitial tissue during the early period of chronic pancreatitis, collagen I was distributed in a wide star-like manner in the inflammatory infiltration area and distributed diffusely in acinar cells appearing serious necrosis region, and TGF-β was markedly distributed in the inflammatory infiltration area of pancreatic tissues in *aly/aly* mice (Figure 4).

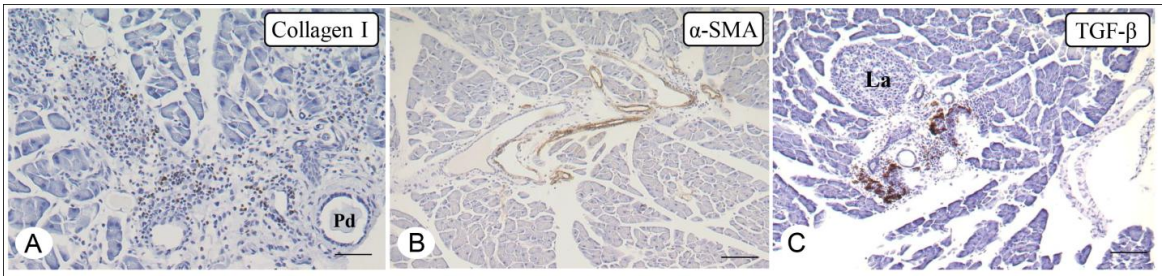

Figure 4: The expression of collagen I, α-SMA and TGF-β inpancreas of 20-week-old *aly/aly* mice. Scale bars: 100μm (A) and 200μm (B and C).

6. Discussion

Chronic pancreatitis is characterized by chronic inflammatory lesions of the pancreatic tissue, which can lead to irreversible parenchyma atrophy and even fibrosis [2, 3].The pathological occurrence of chronic pancreatitis follows the progression of necrosis and fibrosis [8]. Autoimmune chronic pancreatitis is mainly caused by auto antigens that activate immune cells. Over activated cells infiltrate pancreatic tissues and accumulate in acinar stroma, releasing inflammatory factors, which then leads to apoptosis and atrophy of local acinar cells. Fibrosis was found to occur around the pancreatic duct and interlobular tissue, whereby pancreatic exocrine function was impaired and eventually islet endocrine function was affected [9]. *Aly/aly* mice are produced by the mutation of the C57BL/6J mouse gene. Our previous study in *aly/aly* mice showed that the exocrine glands, especially the pancreas, had inflammatory cell infiltration during the early stage, which gradually progressed to chronic inflammation of the pancreas, and resulted in a large area of pancreatic tissue necrosis [6]. In the present study, we found that the occurrence of chronic pancreatitis in *aly/aly* mice was a gradual process. In the pancreas of 30-week-old *aly/aly* mice, a large number of inflammatory cells were distributed around the ducts and blood vessels, and apoptosis and necrosis appeared in the pancreatic acini in the area of inflammatory cell infiltration. By the age of 50 weeks, large areas of adipose fibrosis were found in the pancreas of most mice. The inflammatory cells that infiltrated pancreatic tissue were identified by immuno staining to be predominantly T and B lymphocytes as well as macrophages.

Fas binding to its ligand (Fas ligand) or Fas antibody can induce apoptosis of Fas-positive cells, which is one of the main pathways of apoptosis [10, 11]. A previous study using an acute pancreatitis model in rats confirmed the expression of Fas/Fas ligand in acinar cells, and that the expression of Fas ligand increased with the aggravation of inflammatory injury [12]. In the present study, we found that the expression of Fas/Fas Ligand cells was also located in the inflammatory infiltration area during the occurrence of pancreatitis in *aly/aly* mice. These results showed that Fas- and Fas ligand-positive cells were localized in the infiltrating area of inflammatory cells, suggesting that the apoptosis activation pathway of Fas and Fas ligands may be induced by infiltrating inflammatory cells or inflammatory factors released by Fas/Fas ligands.

Activated T and B lymphocytes and macrophages secrete a large number of inflammatory cytokines, such as interleukin-1 (IL-1), interferon (γ-IFN) and tumor necrosis factor-α (TNF-α), which promote the further development of inflammation [13, 14]. TNF-α is an important mediator of acute pancreatitis. High concentrations of TNF-α were found in peripheral blood and pancreatic tissue of patients with early acute pancreatitis [15]. Furthermore, in the early stage of chronic pancreatitis, TNF-α was expressed in pancreatic interlobular cells and inflammatory cells [16]. Under normal conditions, type IX collagen is expressed in pancreatic tissues. When pancreatic cells are stimulated by TNF-α, they express α-SMA and promote the rapid synthesis of type I collagen, resulting in pancreatic fibrosis [15, 17]. In our study, we found that, in the early stage of pancreatitis in *aly/aly* mice, type I collagen was widely distributed in the area of inflammatory cell infiltration and α-SMA was positive in the wall of the glandular duct in the area of pancreatic inflammatory cell infiltration. Therefore, α-SMA and collagen I may play an important role in the occurrence and development of pancreatic fibrosis in *aly/aly* mice. TGF-β belongs to a group of super family molecules that regulate cell growth and differentiation, and is mainly produced by lymphocytes and mono cytes. TGF-β is closely related to the occurrence of pancreatic fibrosis. TGF-β has been confirmed to be a key factor in the regulation of cytokine networks in pancreatic fibrosis, and is expected to be a suitable target of anti-fibrosis therapy [18, 19]. Our study confirmed that, during the occurrence of chronic pancreatitis in *aly/aly* mice, the expression of TGF-β in the inflammatory cell infiltration area of pancreatic interstitial tissue was strongly positive, and this positive expression of TGF-β was consistent with the inflammatory infiltration and tissue fibrosis in pancreatic tissues. In summary, T and B lymphocytes and macrophages are involved in the inflammatory infiltration of pancreatic tissues during the pathogenesis of autoimmune pancreatitis in *aly/aly* mice. The activation of Fas/Fas ligand signaling pathway induces apoptosis and inflammation of pancreatic acini, which leads to changes in fiber and fat within pancreatic tissues of *aly/aly* mice, and destroys the pancreatic tissue structure.

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