Pancreatology 14 (2014) 100–106

Contents lists available at ScienceDirect

Pancreatology

journal homepage: www.elsevier.com/locate/pan

Original article

Identification of commensal flora-associated antigen as a pathogenetic factor of autoimmune pancreatitis

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ARTICLE INFO

Article history:
Received 7 November 2013
Received in revised form
30 December 2013
Accepted 24 January 2014

Keywords:
Autoimmune pancreatitis (AIP)
Innate immunity
Commensal bacteria
FliC of *Escherichia coli*
Autoantibody

ABSTRACT

Objectives: Autoimmune pancreatitis (AIP) is a chronic fibro-inflammatory disease of the pancreas constituting, in part, a recently defined nosological entity of IgG4-related systemic sclerosing diseases. The pathogenetic factors of AIP have not been fully elucidated. We previously established a mouse model of AIP using chronic exposure to a commensal bacteria, *Escherichia coli*.
Methods: To determine the pathogenetically relevant antigen of *E. coli*, the outer membrane fractions of *E. coli* were subjected to two-dimensional gel electrophoresis followed by immunoblotting against sera from the AIP model. Immunoreactive spots were determined using MALDI TOF/MS and Mascot search. The recombinant protein of the identified antigen was examined for their ability to induce AIP-like disorder in C57BL/6 mice. Furthermore, the antibody titer against that antigen was determined in AIP patients.
Results: One representative spot reacting with sera from *E. coli*-inoculated mice was identified as FliC from *E. coli*, based on the results of TOF/MS. The repeated inoculation of recombinant FliC in C57BL/6 mice induced AIP-like pancreatitis and higher titers of anti-lactoferrin and anti-carbonic anhydrase II. Sera from patients with AIP had the highest antibody titer, while those from patients with pancreatic diseases other than AIP had a higher antibody titer against *E. coli* and FliC, compared with pancreatic disease-free controls.
Conclusions: FliC from *E. coli* may pathogenetically generate an AIP-like inflammation status. A reconsideration of the importance of commensal bacteria as an environmental factor(s) capable of inducing autoimmunity could provide insight to overcoming AIP.

1. Introduction

Autoimmune pancreatitis (AIP) is a chronic fibro-inflammatory disease of the pancreas. Although the frequency of AIP accounts for 2%–4% of all forms of chronic pancreatitis [1], its incidence continues to increase worldwide [2,3]. Even though the cause of AIP is unknown, its autoimmune entity has been suggested [4]. AIP is classified into two distinct types; type 1 is a systemic disease associated with IgG4, whereas type 2 appears to be a pancreas-specific disorder [3,5]. As patients with AIP often exhibit a pancreatic mass or painless obstructive jaundice mimicking pancreatic cancer, a differential diagnosis from pancreatic cancer is particularly important [3,5].

Several serological markers of the disease including autoantibodies have been reported [4,6–10]. Acinar cell-presenting peptide, a homologue of plasminogen-binding protein (PBP) of *Helicobacter pylori*, was suggested to be an autoantigen inducing AIP based on its molecular similarity. However, none of these markers are sufficiently specific to enable a definite diagnosis of AIP [4].

Several diagnostic criteria for AIP have been reported, but their usefulness is still under debate [4]. In 2010, the International Consensus Diagnostic Criteria (ICDC) for AIP were proposed and were published in 2011 [5]. The ICDC adopted five cardinal features of AIP: (i) characteristic imaging findings for the pancreatic

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1424-3903/$ – see front matter
http://dx.doi.org/10.1016/j.pan.2014.01.004

parenchyma and duct, (ii) an increase in serum IgG4, (iii) the involvement of other organs, (iv) characteristic histological findings, and (v) an optional criterion consisting of a response to steroid therapy. Although an elevation in IgG4 that is not proportional to the IgG and/or antinuclear antibody (ANA) titers is commonly associated with AIP, these serological markers are often elevated in patients with pancreatic cancer or other diseases; consequently, an elevation in these serological markers is not sufficient for a diagnosis of AIP unless typical imaging findings are documented [5].

Organ-specific autoimmune diseases are thought to develop through a combination of hereditary and environmental factors that induce adaptive immune responses to self-antigens [11]. Microbial antigens may underlie the pathogenesis of IgG4-related disease [12–14]. Watanabe et al. reported the possible involvement of abnormal innate immune responses against intestinal microflora in the development of AIP by demonstrating that the activation of nucleotide-binding oligomerization domain (NOD)-2 and Toll-like receptor (TLR) ligands enhances IgG4 responses by peripheral blood mononuclear cells (PBMCs) from AIP [14]. We previously reported that repeated inoculation with heat-killed *Escherichia coli* into C57BL/6 mice induced AIP-like pathological alterations accompanied by an elevation in serum IgG and the production of autoantibodies against carbonic anhydrase (CA) II and lactoferrin (LF) [15]. Moreover, the transfer of spleen cells from C57BL/6 mice inoculated with *E. coli* into $RAG2^{-/-}$ mice induced cellular infiltration in the pancreas, especially around the pancreatic ducts, in the recipient $RAG2^{-/-}$ mice. The infiltrated cells in the recipient pancreases were confirmed to be CD3-positive donor-originated cells. According to Witebsky's postulates with modern revision by Rose and Bona [16], the criteria for determining whether a condition may be considered to be autoimmune include (i) indirect evidence based on the reproduction of the autoimmune disease in experimental animals, (ii) direct evidence of the transfer of pathogenic antibodies or pathogenic T cells, and (iii) indirect evidence of the isolation of autoantibodies or autoreactive T cells. Therefore, several lines of evidence have suggested that the AIP-like disorders in our C57BL/6 mice inoculated with a commensal bacteria, *E. coli*, is possibly of autoimmune origin.

In the present study, we examined relevant antigens capable of inducing AIP in our mouse model [15]. Furthermore we would like to propose that by combination with other antibodies for AIP, anti-FliC from *E. coli* could much more increase the diagnostic sensitivity, serologically.

2. Methods

2.1. Preparation of cell surface proteins of E. coli and two-dimensional fluorescence difference gel electrophoresis (2-D DIGE)

Water solubilization of *E. coli* ATCC 25922^{T} (*bfpA*-, *eaeA*-, *st*-, *lt*-, stx_1-, stx_2-, *ial*-, *aggR*-, *daaE*-) surface proteins was performed as previously described [15]. No DNA was detectable in the spectrophotometric analysis at 260 nm, indicating a minimum of cell lysis. Each 200 μg of protein extract from live and heat-treated cells at 80 °C for 30 min was labeled with Cy3 and Cy5, respectively. 2-D DIGE was performed on Ettan IPGphor3 IEF using Immobiline DryStrip pH3-10 (GE Healthcare Bio-Sciences, Little Chalfont, Buckinghamshire, UK), and acrylamide gels. Spot detection and matching were performed using Multi Gauge Software on FLA5100 (Fuji Film, Tokyo, Japan).

2.2. 2-D western blotting

Proteins were resolved in 2-D SDS-PAGE and were transferred to nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was blocked in TBS-TM buffer (10 mM Tris–HCl [pH7.4], 0.9% NaCl, 0.05% Tween 20, 10% skimmed milk) and incubated with sera from experimental AIP mice [15] diluted 1:2000 in TBS-TM. Immunoreactive spots were detected using Immunostar enhanced chemiluminescence kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

2.3. Identification of proteins using MALDI-TOF-MS

For protein identification, 2-D gel was stained with SYPRO-Ruby (Life Technologies, Carlsbad, CA, USA), and spots of interest were excised using EXQuest (Bio-Rad). Proteins were digested with trypsin (Trypsin Gold, Mass Spectrometry Grade; Promega Corporation, Fitchburg, WI, USA), and anlyzed using MALDI-TOF mass spectrometry (AutoflexII; Bruker Daltonics, Billerica, MA, USA) in the positive ion reflector mode and in an m/z range of 600–4000 Da. Mass spectra were calibrated using a peptide calibration standard mono (Bruker Daltonics). The data were investigated based on their peptide mass fingerprint (PMF) using the non-redundant NCB Inr database with a MASCOT search engine (Matrix Science, London, UK) through the BioTools 3.0 interface provided by Bruker Daltonics, within the taxonomy of *E. coli*.

2.4. Recombinant protein expression and purification

Recombinant (r) FliC1-595 protein was expressed with a hexahistidine tag at the N termini using TAGZyme pQE2 vector (Qiagen, Hilden, Germany). The gene fragment was amplified with *E. coli* 25922 genomic DNA as template, *Phusion* polymerase (New England Biolabs, Ipswich, MA, USA), and primers incorporated with SphI and HindIII for ligation at complementary sites of the enzyme-digested vector. Primer sequences are forward primer FliC1 5′-CCCGCATGCATGGCACAAGTCATTAATAC-3′ and reverse primer FliC595 5′-CCCAAGCTTTTAACCCTGCAGCAGAG-3′. The recombinant protein was expressed and purified by Ni^{2+}-affinity chromatography. When necessary, endotoxin was removed from rFliC using Detoxin Gel Endotoxin Removing Columns (TaKaRa Bio Inc., Shiga, Japan), and the endotoxin level was determined using the ToxinSensor Chromogenic Limulus Amebocyte Lysate Endotoxin Assay Kit (Funakoshi Co., Ltd., Tokyo, Japan).

2.5. Mouse model of bacterial component-triggered pancreatitis

C57BL/6 mice were purchased from SLC (Hamamatsu, Japan). The mice were bred in the animal facility at the Department of Microbiology and Immunology, Tokyo Women's Medical University (TWMU). Approximately 6-week-old female mice were used for the experiments; all the experiments were performed in accordance with the guidelines of the Ethics Review Committee for Animal Experiments of TWMU. Ten mice in one group were injected peritoneally with FliC (1–10 μg) in 200 μL of PBS or PBS alone once a week for a total of 8 weeks. The mice were sacrificed at the indicated time after the final inoculation. Preparation of tissues was described previously [15], using monoclonal anti-CD3 antibody (Abcam, Tokyo, Japan), anti-CD45R/B220 antibody (BD Biosciences, CA, USA), and polyclonal anti-mouse IgG1 (Abcam). The serum anti-LF and anti-CA II antibody titers were examined using ELISA and western blot analysis, respectively [15].

2.6. Patients and samples

The serum samples were obtained from 14 patients with AIP (8 men, 6 women; age, 30–75 [median, 61.5] years). The diagnosis of AIP was based on the criteria established by the Japanese Society of Pancreas [5]. The serological features of the AIP patients are