INTERNAL MEDICINE

□ ORIGINAL ARTICLE □

Allergen-specific IgE Antibody Serologic Assays in Patients with Autoimmune Pancreatitis

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Abstract

Objective To clarify the allergic manifestations in patients with autoimmune pancreatitis (AIP).

Methods We assessed 67 AIP patients, before they received steroid therapy, for a past history of allergic disease, the peripheral eosinophil count (n=62) and the serum IgE level (n=53). Allergen-specific IgE antibody serologic assays were performed in 15 patients.

Results A positive past history and/or the presence of active allergic disease were found in 24 AIP patients (36%), including 15 patients with acute allergic rhinitis and eight patients with bronchial asthma. Peripheral eosinophilia and elevation of the serum IgE level were detected in 16% (10/62) and 60% (32/53) of the patients, respectively. Allergen-specific IgE antibody serologic assays were positive in 13 patients (87%). There were no differences between the assay-positive and -negative patients regarding the clinical profiles.

Conclusion In conclusion, 87% of the 15 AIP patients tested had positive allergen-specific IgE antibody serologic assays. Allergic mechanisms may be related to the occurrence of AIP.

Key words: autoimmune pancreatitis, IgE

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Introduction

Autoimmune pancreatitis (AIP) is characterized radiologically by enlargement of the pancreas and narrowing of the main pancreatic duct, serologically by elevation of the serum gammaglobulin, IgG or IgG4 levels and the presence of autoantibodies and histologically by abundant infiltration of IgG4-positive plasma cells and lymphocytes with dense fibrosis of the pancreas and is frequently associated with sclerosis of other organs and steroid responsiveness (1, 2). Based on its systemic manifestations, AIP is currently recognized to be a pancreatic lesion of an IgG4-related disease (3, 4). Based on its serological findings and steroid responsiveness, an autoimmune etiology is presumed to be the pathogenic mechanism of AIP; however, the target antigens for AIP have not yet been identified.

Recently, evidence of allergic manifestations in AIP patients has been accumulating. In our previous study, 20 of 45 (44%) AIP patients had a history of allergic disease (5). Sah et al. also reported that allergic disorders were detected in 12 of 78 (15%) AIP patients (6). Most allergic manifestations, including bronchial asthma, acute allergic rhinitis, hay fever and atopic dermatitis, are caused by type 1 hypersensitivity reactions. Detecting the presence of an allergen-specific IgE antibody is most important for diagnosing type 1 hypersensitivity reactions. In our previous study, elevated serum IgE levels were detected in 12 of 45 (34%) AIP patients (5), and Hirano et al. reported that the serum IgE levels were elevated in 36 of 42 (86%) AIP patients (7). However, the significance of these findings is unclear. To clarify the associated allergic manifestations, we performed allergen-specific IgE antibody serologic assays in AIP patients.

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Materials and Methods

The subjects of this study included 67 type 1 AIP patients (54 men and 13 women, median age: 64 years) who were diagnosed according to the international consensus diagnostic criteria for AIP (2), enlargement of the pancreas and narrowing of the main pancreatic duct (n=67), elevation of the serum IgG4 level (n=52), the presence of histologically proven lymphoplasmacytic sclerosing pancreatitis (n=13) and responsiveness to steroid therapy (n=50). A positive past history and the extent of morbidity from allergic diseases, including bronchial asthma, acute allergic rhinitis, hay fever and atopic dermatitis, were ascertained using a review of the patients' clinical records and definitive clinician diagnoses. The serum IgG4 (<135 mg/dL) and IgE (<250 IU/mL) levels and peripheral eosinophil count (<600 cells/mm^3 (8)) were determined in 67, 53 and 62 patients, respectively. Allergen-specific IgE antibody serologic assays were performed in 15 patients [radioallergosorbent test (RAST), n=7 and multiple antigen simultaneous test (MAST), n=8] before administering steroid therapy in a prospective manner beginning in 2008. Levels of IgE greater than 0.34 UA/mL according to RAST and a result of more than class 1 according to MAST were considered positive findings. The assay-positive and -negative AIP patients were compared with regard to various clinical features, such as allergic disease, symptoms, other organ involvement and serological findings. The two groups were compared using the chi-square and Fisher's exact tests.

Results

Active allergic disease and/or a past history of allergic disease were found in 24 (36%) AIP patients, as follows: hay fever with acute allergic rhinitis, 15 patients (22%); bronchial asthma, 8 patients (12%); drug allergy, 4 patients (6%); atopic dermatitis, 2 patients (3%); and mackerel allergy, house dust allergy and hamster allergy, 1 patient each (1.5%). Peripheral eosinophilia and elevation of the serum IgE level were detected in 16% (10/62) and 60% (32/53) of the patients, respectively. Six (19%) of 31 patients with elevation of the serum IgE level had eosinophilia, while three (14%) of 21 patients without an elevated IgE level had eosinophilia (p=0.92).

Allergen-specific IgE antibody serologic assays were positive in 13 of the 15 (87%) patients tested (RAST, 6 of 7 and MAST, 7 of 8). The causative agents were cedar (n=8), Japanese cypress (n=4), ticks (n=3), shrimp (n=3), crab (n=3), orchard grass (n=3), house dust (n=2), moths (n=2), *Ambrosia artemisiifolia* (n=2), Candida (n=2), wheat (n=2), *Aspergillus* (n=2), guinea pig (n=1), mugwort (n=1), peanut (n=1), buckwheat (n=1), sweet vernal grass (n=1), timothy (n=1), *Betula platyphylla* (n=1) and *Penicillium* (n=1). Among 15 patients tested with allergen-specific IgE antibody serologic assays, seven had allergic diseases [hay fever (n=4), bronchial asthma (3), drug allergy (1) and mackerel allergy (1)]. All seven patients were positive for allergen-specific IgE antibody assays.

Table. Differences in Clinical and Serological Features between Allergen-specific IgE Antibody Serologic Assay-positive and -negative AIP Patients

	Assay-positive (n=13)	Assay-negative (n=2)	p values
Age (average, years)	62.5	74.5	0.67
Male/female	7/6	1/1	
Steroid responsiveness	13/13	2/2	
Recurrence of AIP	0	0	
Allergic disease	6	1	0.50
Obstructive jaundice	3	0	0.44
Acute pancreatitis	1	0	0.68
Other organ involvement	9	2	0.95
Elevation of serum IgG	7	0	0.50
Elevation of serum IgG4	9	1	0.78
Elevation of serum IgE	8	1	0.64
Eosinophilia	5	0	0.78

There were no significant differences between the assay-positive and -negative patient groups in age, gender, steroid responsiveness, recurrence of AIP, allergic disease, obstructive jaundice, acute pancreatitis, other organ involvement, elevation of the serum IgG, IgG4 or IgE levels or eosinophilia (Table).

Discussion

Recently, evidence of allergic manifestations in AIP patients has been accumulating. It has been reported that, in the setting of AIP, allergic diseases are observed in 15% (12/78) (6), 17% (7/42) (7), 41% (10/24) (9) and 44% (20/45) (5) of patients, peripheral blood eosinophilia is observed in 8% (1/13) (9), 11% (5/45) (5) and 28% (22/78) (6) of patients, elevation of the serum IgE levels is observed in 34% (12/35) (5) and 86% (36/42) (7) of patients and marked eosinophil infiltration in the pancreas is detected in 67% (12/18) (6) and 88% (21/24) of patients (9). It has been demonstrated that the Th2 and regulatory cytokine expressions are upregulated in the affected tissues of AIP patients and that regulatory T-cells are involved in the in-situ production of IL-10 and TGF-β, which is followed by an IgG4 class switch and fibroplasia (10). The immune reactions that are predominantly mediated by Th2 and regulatory T-cells are closely involved in the pathogenesis of allergic disorders, such as bronchial asthma and atopic dermatitis (11).

In this study, allergic disease, peripheral eosinophilia and elevation of the serum IgE level were detected in 36%, 16% and 60% of AIP patients, respectively. The major allergic manifestation was allergic rhinitis (63%) followed by bronchial asthma (33%) in our series, while allergic rhinitis (43%) and bronchial asthma (43%) were observed in another Japanese series (7). However, bronchial asthma is predominant in the USA, with reported rates ranging from 75% (9/12) (6) to 80% (8/10) (9). Such differences may be attrib-

utable to racial or geographic factors.

Allergen-specific IgE antibody serologic assays are one of the tools used for precise allergy testing (12-14). In this study, 87% of 15 AIP patients were positive for an allergen-specific IgE antibody, and the causative antigens were various. We were unable to find any data pertaining to positive rates of allergen-specific IgE antibody serologic assays in the Japanese general population; however, we speculate that the positive rate observed in this study is higher than that seen in the general population. Ito et al. reported three AIP patients in whom bronchial asthma preceded AIP; the asthma symptoms worsened at the onset of AIP and were accompanied by high serum IgE levels and positivity on allergen-specific IgE antibody serologic assays (15).

There were no significant differences between the assay-positive and -negative patients regarding clinical characteristics, possibly because the number of assay-negative patients was too small (n=2). However, Sah et al. reported that, in their study, there were no differences in the clinical profiles of AIP patients with and without peripheral eosinophilia (6), and Hirano et al. reported that there were no differences in the clinical profiles of AIP patients with and without elevation of the serum IgE levels (7). Peripheral eosinophilia and the serum IgE levels do not seem to reflect the disease activity.

This study revealed a higher level of allergen-specific IgE antibody serologic assay positivity than the incidence (36%) of clinical allergic disease found among the AIP patients enrolled. The reasons for this discrepancy are unknown; however, it has been reported that, in symptomatic self-selected populations, a positive test result significantly increases the probability that the patient is allergic, while the use of multiple allergen tests to screen unselected populations results in an unacceptable number of false-positive and false-negative results (12).

The small sample size of patients in whom assays were performed is the greatest limitation of this study. A second limitation is that other allergy tests, such as skin tests, were not performed. However, to our knowledge, this is the first report of the use of allergen-specific IgE antibody serologic assays in AIP patients. Further studies are therefore warranted to clarify the relationship between allergic phenomena and AIP.

In conclusion, the results of allergen-specific IgE antibody serologic assays were positive in 87% of the 15 AIP patients assayed in this study. Allergic mechanisms may be related to the occurrence of AIP.

The authors state that they have no Conflict of Interest (COI).

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Autoimmune pancreatitis complicated with inflammatory bowel disease and comparative study of type 1 and type 2 autoimmune pancreatitis

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Abstract

Background Two types of autoimmune pancreatitis (AIP) have been reported, lymphoplasmacytic sclerosing pancreatitis and idiopathic duct-centric chronic pancreatitis (IDCP), which are now recognized as type 1 and type 2 AIP, respectively. Since the clinical features of type 2 AIP have not been fully elucidated and this condition is frequently accompanied by inflammatory bowel disease (IBD), we performed a nationwide survey of patients with AIP complicated with IBD to precisely characterize this disease entity.

Methods We collected 138 cases of pancreatitis with complicating IBD from affiliated institutes specializing in AIP or IBD, and comparative study between the IDCP groups and type 1 AIP was performed.

Results Histological examination revealed 15 AIP cases to be IDCP of institutional diagnosis, among which 11 cases were upgraded to IDCP of central diagnosis by an expert pathologist. The IDCP group exhibited younger onset age, no gender bias, frequent abdominal pain, and normal IgG4 value, similar to those of type 2 AIP reported previously. We also witnessed a lower prevalence of jaundice in type 2 AIP than in type 1 AIP that corresponded to imaging findings of less frequent pancreatic head swelling and scarce bile duct stenosis.

Conclusions A characteristic feature of type 2 AIP compared with type 1 AIP is a low frequency of obstructive jaundice that is related to rare lower bile duct stricture due to lower prevalence of pancreatic head swelling. Contrary to type 1 AIP, lower bile duct stricture in this condition has no apparent relation to sclerosing cholangitis.

Keywords Autoimmune pancreatitis (AIP) · Lymphoplasmacytic sclerosing pancreatitis (LPSP) · Idiopathic duct-centric chronic pancreatitis (IDCP) · Granulocytic epithelial lesion (GEL) · Inflammatory bowel disease (IBD) · Ulcerative colitis (UC) · Crohn's disease

Members of the study group are listed in Appendix.

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Introduction

Autoimmune pancreatitis (AIP) is a specific type of pancreatitis whose pathogenesis has been implicated with autoimmune mechanisms [1]. In 1992, Toki et al. described the characteristic pancreatic duct findings of this condition, which they termed chronic pancreatitis with diffuse

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irregular narrowing of the main pancreatic duct [2]. Later, in 1995, Yoshida et al. proposed the concept of AIP based on apparent autoimmune phenomena [3]. In 2001 and 2002, Hamano et al. reported on the characteristic clinical findings of high serum IgG4 concentration and abundant IgG4-bearing plasma cell infiltration in the affected organ in AIP, which became useful serological and pathological hallmarks for AIP diagnosis [4, 5]. This type of AIP was reported mainly from Japan and Korea and became characterized by (1) elderly male preponderance, (2) similar imaging findings to pancreatic cancer, and (3) a close association with IgG4. AIP with pathological lymphoplasmacytic infiltration and fibrosis was referred to as lymphoplasmacytic sclerosing pancreatitis (LPSP) [6]. The inflammatory process of LPSP encroached on pancreatic and peripancreatic tissues, resulting in various degrees of parenchymal damage.

Elsewhere, another type of pancreatic lesion was being reported mainly from Europe and the USA as showing pathological neutrophil infiltration in the pancreatic duct epithelium that was referred to idiopathic duct-centric chronic pancreatitis (IDCP) [7], or AIP with granulocytic epithelial lesion (GEL) [8]. This type of lesion was proposed to be included in the AIP spectrum by several groups. However, the clinical features of IDCP/AIP with GEL differed considerably from those of LPSP, showing (1) young and middle age preponderance, (2) no gender bias, (3) complicating inflammatory bowel disease (IBD), and (4) no correlation with IgG4 [9, 10]. In the International Consensus Diagnostic Criteria (ICDC) for AIP established in 2011, AIP became classified as type 1 and type 2 based on the pathological subtypes of LPSP and IDCP/AIP with GEL, respectively. IDCP/AIP with GEL has since been widely accepted as a distinct type of AIP [11]. Similar imaging criteria are used for both type 1 and 2 AIP diagnosis in the ICDC [11]. Although imaging findings are indeed considered to resemble each other for the two conditions [12, 13], there have been few studies on the precise differences in clinical features between type 1 and type 2 AIP [12–16]. Especially in Japan, there is a scarcity of reports on type 2 AIP [14, 15, 17–20] and the clinical characteristics of this type of AIP have not been fully elucidated.

The Research Committee for Intractable Pancreatic Disease supported by the Ministry of Health, Labour, and Welfare of Japan (Chairman: Tooru Shimosegawa) conducted a national survey on type 2 AIP from July 2009 to March 2011 and preliminarily identified the clinical features of this condition, which included complicating IBD. However, both AIP and pancreatic diseases complicated with IBD have been reported to exhibit similar clinical features as type 2 AIP: Okano et al. described a case of pseudotumorous pancreatitis associated with ulcerative colitis (UC) that featured obstruction of the main pancreatic duct [21]. Toda et al. reported that among 79 patients with UC, five presented with magnetic resonance cholangiopancreatography (MRCP) finding of diffuse irregular narrowing as seen in AIP, whereas their serum IgG4 concentration was found to be within the normal range [22]. Nve et al. reported on the clinical outcome of a 25-year-old man with idiopathic fibrosing pancreatitis associated with UC who showed obstructive jaundice and diffuse enlargement of the pancreas [23], and Oishi et al. disclosed that irregular narrowing of the main pancreatic duct was found in 13 % of Crohn's disease cases with abnormal pancreatic imaging findings [17]. Ueki et al. reported that all seven cases of AIP complicated with IBD were classified as type 2 [20]. There have been few reports in which full pathological examination of type 2 AIP was done. As there are many patients with IBD in Japan, we hypothesized that a survey of AIP complicated with IBD would enable effective identification and investigation of type 2 AIP. Accordingly, we performed a collaborative study with The Research Committee for Intractable Pancreatic Disease (Chairman: Tooru Shimosegawa) and The Research Committee for Intractable Inflammatory Bowel Disease (Chairman: Mamoru Watanabe), both of which are supported by the Ministry of Health, Labour, and Welfare of Japan. While the former organization specializes in AIP, the latter focuses primarily on IBD. The present study sought to clarify the clinical features of type 2 AIP and compare them with those of type 1 AIP.

Materials and methods

Patients

Pancreatitis complicated with IBD

We collected cases of pancreatitis complicated with IBD, which included UC and Crohn's disease, from affiliated institutes. The pancreatic diseases assessed in this study were AIP, acute pancreatitis, and chronic pancreatitis that had been diagnosed and treated after 1995 when the concept of AIP had first been proposed. AIP was classified into two groups: IDCP of institutional diagnosis and that of central diagnosis. IDCP of institutional diagnosis corresponded to IDCP that was determined at individual institutes based on the ICDC for AIP 2011 with pathological examination of pancreatic tissue [18]. Among the cases with IDCP of institutional diagnosis, IDCP of central diagnosis was then established by careful examination of tissue slides by an expert pathologist (KN) who had proposed the concept of IDCP in 2003.

Type 1 AIP

For further analysis of IDCP complicated with IBD, 84 patients with type 1 AIP were selected as controls from a cohort recruited at Shinshu University and Kansai Medical School between 1992 and 2013, in which the diagnosis was based on the ICDC for AIP 2011 [11] and the Japanese Clinical Diagnostic Criteria for AIP 2011 [24], ten cases were available for pathological diagnosis and no cases were complicated with IBD.

Methods

Patient surveys

In a preliminary survey, we first asked member institutes about cases of acute pancreatitis, chronic pancreatitis, or AIP that were complicated with UC or Crohn's disease and had been confirmed by CT and MRI between 1995 and 2011. In a follow-up questionnaire, we then asked institutes that responded to the first survey to provide the detailed clinical features of each case. The completed reports were sent to the centers for clinical analysis at Shinshu University and Kansai Medical School.

Analysis of clinical features

We analyzed the collected patient data to elucidate the precise clinical features of pancreatitis complicated with IBD. To clarify IDCP in comparison with type 1 AIP, AIP with complicating IBD was further subdivided into IDCP of institutional diagnosis and that of central diagnosis, as described above.

Analysis of imaging findings collected from patients with IDCP of central diagnosis

We obtained CT, MRI, and ERCP images from institutes reporting cases with IDCP of central diagnosis and examined them for characteristic imaging findings.

Pathological analysis

When available, tissue slides were gathered for central pathological examination at Kurashiki Central Hospital. Tissue specimens with IDCP of institutional diagnosis were also obtained for review. The diagnosis of type 2 AIP was based on established ICDC histological criteria.

Statistical analysis

The Chi-squared test or Fisher's exact test were adopted for comparisons of categorical variables. The Mann–Whitney U test was used for comparisons of continuous variables. A p value of <0.05 was considered to be statistically significant. Statistical analyses were performed using StatFlex 6.0 software (Artech Co., Ltd., Osaka, Japan).

Ethics

Inquiries, patient data, and tissue slides for analysis were all handled anonymously. This study was approved by the respective ethics committees of each participating institute.

Results

Overall preliminary survey results

We initially sent inquiries to 132 institutes about the type and number of cases of pancreatitis with IBD they had encountered and received replies from 85 institutes (64 %). Based on the first survey, we sent a second questionnaire concerning detailed clinical features to 43 institutes and received the case data of 138 patients with pancreatitis complicated with IBD from 36 institutes (81 %).

The patient diagram flow chart for 138 patients complicated with IBD is shown in Fig. 1. The clinical characteristics of our 138 patients demonstrated a male/female ratio of 83/55 and mean disease onset age of 36.7 ± 17.2 years (Supplementary Table S1). IBD complications consisted of 90 cases of UC and 48 cases of Crohn's disease. Fifty-eight patients were clinically diagnosed as having acute pancreatitis, 28 as having chronic pancreatitis, and 52 as having AIP. The 52 cases of AIP consisted of seven confirmed cases based on the Japanese

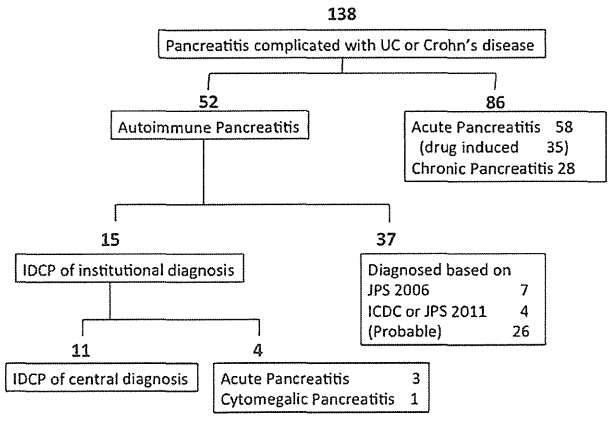

Fig. 1 The patient diagram flow chart for 138 patients complicated with IBD, IDCP: idiopathic duct-centric chronic pancreatitis, JPS 2006: Japanese Clinical Diagnostic Criteria of AIP 2006, ICDC: International Consensus Diagnostic Criteria, JPS 2011: Japanese Clinical Diagnostic Criteria for AIP 2011

Clinical Diagnostic Criteria of AIP 2006, and 19 confirmed and 26 probable cases based on the ICDC or the Japanese Clinical Diagnostic Criteria for AIP 2011. Histological examination was possible for 28 AIP cases and revealed 15 to be IDCP of institutional diagnosis, of which 11 were IDCP of central diagnosis. Among the 58 cases of acute pancreatitis, 35 were closely associated with immunosuppressive drugs used for IBD, such as mesalazine and azathioprine. Although patients with IDCP may have been included in the acute pancreatitis or chronic pancreatitis groups, a shortage of tissue samples made pathological examination and confirmed diagnosis of IDCP difficult. Accordingly, acute pancreatitis and chronic pancreatitis complicated with IBD were not analyzed in the present study.

Central pathological review

Tissue samples from 28 patients were obtained and reviewed by an expert pathologist. Two samples were from a pancreas resection and 26 were from a biopsy. In total, two (one each from a resected tissue and biopsy) and nine (all from a biopsy) cases fulfilled the level 1 and level 2 criteria for type 2 AIP, respectively. These 11 cases comprised the IDCP of central diagnosis group in this study. Cases with level 1 histological findings displayed neutrophilic infiltration in the duct epithelium (GEL) (Fig. 2a), while those meeting the level 2 criteria exhibited neutrophilic infiltration within lobules. Although numerous ductules were formed and infiltrated by neutrophils within lobules in the latter cases, we did not regard this finding to be GEL since these small ducts were considered to be acinar-ductular metaplasia rather than genuine intralobular ducts (Fig. 2b).

Four cases in the IDCP of institutional diagnosis group were excluded from the IDCP of central diagnosis group during central pathological review. Three of these cases displayed histological features of acute pancreatitis, such as acinar cell disappearance, edema, and/or fibroblastic proliferation, but neutrophilic infiltration was minimal. Although we considered that these findings may not have necessarily excluded a diagnosis of IDCP, the cases also did not meet the ICDC level 2 criteria for IDCP. The remaining excluded case showed marked destruction of the pancreas. As a cytomegalic inclusion body was identified, we concluded that this patient had cytomegalic pancreatitis (Fig. 1). None of the cases that underwent histological examination were diagnostic for type 1 AIP.

Analysis of AIP complicated with IBD

Overall analysis

Overall analysis of 52 patients with AIP revealed a male preponderance and median age of disease onset of 35 years (Supplementary Table S2). AIP was preferentially complicated with UC over Crohn's disease. Clinical features at presentation were jaundice in several cases consistent with slight elevation of transaminase and biliary enzymes. Diarrhea was present in 22 patients. Abdominal pain was noted in 29 patients, who also showed high serum amylase concentration. Although serum immunoglobulin concentrations were generally normal, seven patients had high serum IgG4, indicating that various conditions, such as type 1 AIP, may have been included in this group. Many patients exhibited pancreatic swelling and pancreatic duct narrowing, but few displayed lower bile duct stenosis, which likely accounted for the low prevalence of jaundice. Half of the patients had received corticosteroid therapy apart from the immunosuppressive drugs mesalazine and azathioprine.

Analysis of the clinical features of IDCP and type 1 AIP

Among the 52 patients with AIP, the IDCP group was established via pathological examination of pancreatic

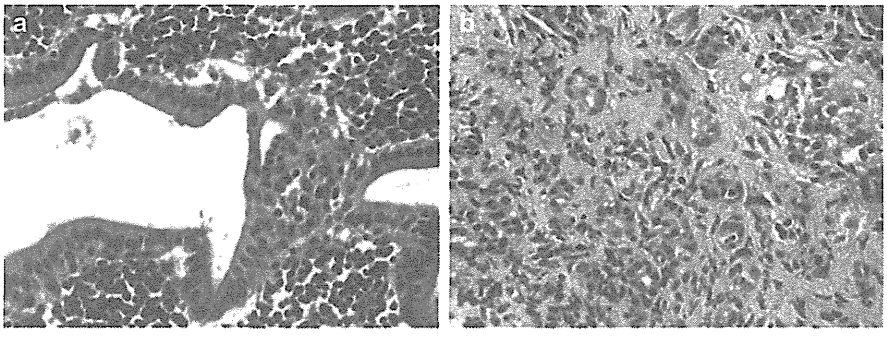

Fig. 2 Histological findings. **a** Level 1 histology of the ICDC for AIP 2011 with neutrophilic infiltration within the duct lumen and epithelium, which is termed GEL, in a resected tissue sample. **b** Level 2 histology with neutrophilic infiltration in the lobules. Small ductules corresponding to acinar-ductular metaplasia are infiltrated by neutrophils, which are not regarded as GEL in this study

Table 1 Comparison of clinical features and symptoms between idiopathic duct-centric chronic pancreatitis (IDCP) and type 1 autoimmune pancreatitis (AIP)

	IDCP				Type 1 AIP
	Institutional diagnosis		Central diagnosis		
	$n = 15$	p	$n = 11$	p	$n = 84$
Clinical feature					
Sex (male/female)	7/8	0.0289	5/6	0.0649	64/20
Age at onset (years)	31 (17–84)	0.0001	29 (17–84)	0.0001	65 (38–84)
UC[a]/Crohn's disease (+/+)	11/4		8/3		
Symptom (+/−)					
Abdominal pain	9/6	0.6043	7/4	0.5457	41/43
Abdominal tenderness	7/8	0.0055	5/6	0.0181	11/73
Backache	3/11	0.3911	2/8	0.611	10/74
Jaundice	0/15	0.0059	0/11	0.0156	31/53
Weight loss	3/11	1	3/7	0.6809	16/68
Fever	1/14	1	1/10	0.3939	3/81
Diarrhea	4/11	0.0055	4/7	0.0014	2/82
Constipation	0/15	1	0/11	1	0/84

[a] Ulcerative colitis

tissues and its clinical features were compared with those of type 1 AIP.

Gender and age

The IDCP groups of institutional diagnosis and central diagnosis consisted of 15 and 11 patients, respectively, and had an approximately equal male-to-female ratio (Table 1). This was markedly different from type 1 AIP, for which a male preponderance was seen. IDCP manifested at a significantly younger age of disease onset (approximately 30 years) than did type 1 AIP (65 years). Similarly to clinically diagnosed AIP, complicating UC was roughly three times more frequent than Crohn's disease in the IDCP groups.

Symptoms

No patients presented with jaundice in the IDCP groups, which was significantly different from type 1 AIP (Table 1). Although there were no remarkable differences in the prevalence of abdominal pain between the IDCP groups and type 1 AIP, abdominal tenderness was significantly more prevalent in IDCP. Diarrhea was predictably more frequent in the IDCP groups compared with type 1 AIP due to IBD complications.

Blood tests

In accordance with the absence of jaundice in the IDCP groups, serum concentrations of total bilirubin, biliary enzymes, and transaminase were significantly lower than those in type 1 AIP, except for that of total bilirubin in IDCP of central diagnosis (Table 2). The serum concentration of amylase in the IDCP groups was significantly higher than that in type 1 AIP.

Blood immunology tests

The serum concentrations of IgG and IgG4 in the IDCP groups were significantly lower than those in type 1 AIP, whereas the concentration of IgM in the IDCP groups was significantly higher (Table 2).

Imaging tests

The prevalences of pancreatic swelling and duct narrowing in the IDCP groups were significantly lower than those in type 1 AIP (Table 3). A lower prevalence of pancreatic head swelling in the IDCP groups was evident as compared with type 1 AIP. In accordance with the absence of jaundice, the prevalence of lower bile duct stenosis was significantly lower in the IDCP groups.

Treatment and recurrence

Prednisolone treatment was given to approximately half of IDCP group patients, which was significantly less frequent than in type 1 AIP (Table 4). Other immunosuppressant drugs were also used for patients in the IDCP groups, but their effects on pancreatic manifestations were unclear. There were no significant differences in the prevalence of recurrence between the IDCP and type 1 AIP groups.

Other organ involvement

The complication of other organ involvement, such as Mikulicz's disease, chronic thyroiditis, sclerosing cholangitis, and retroperitoneal fibrosis, which represent the predominant members of IgG4-related disease, tended to be less frequent in the IDCP groups, with significant differences noted for Mikulicz's disease and sclerosing cholangitis (Table 4).

Analysis of imaging findings collected from patients with IDCP of central diagnosis

The imaging findings of six patients with IDCP of central diagnosis were available for further analysis. Whereas pancreatic head swelling was found in three patients, lower

Table 2 Comparison of blood test results between idiopathic duct-centric chronic pancreatitis (IDCP) and type 1 autoimmune pancreatitis (AIP)

	IDCP				Type 1 AIP
	Institutional diagnosis		Central diagnosis		
	$n = 15$	p	$n = 11$	p	$n = 84$
AST	17 (8–297)	0.002	17 (8–297)	0.005	34 (4–730)
ALT	16 (7–504)	0.0037	14 (7–504)	0.0063	52 (9–833)
T-Bil	0.6 (0.2–0.9)	0.0088	0.7 (0.3–0.9)	0.052	0.8 (0.3–17.2)
ALP	226 (106–2,688)	0.0097	226 (106–2,688)	0.0166	496 (73–2,855)
γ-GTP	17 (8–934)	0.0019	17 (8–934)	0.0059	12 (8–1543)
BUN	6.0 (0.7–16.8)	0.0159	6.0 (3–16.8)	0.011	14 (6.6–58)
Creatinine	0.6 (0.4–1.1)	0.0177	0.6 (0.4–1.0)	0.0123	0.8 (0.4–7.9)
Amylase	241 (74–1541)	0.0004	241 (83–962)	0.0012	83 (17–1470)
IgG	1,271 (950–2433)	0.0002	1,255 (1,180–2,035)	0.0056	1,997 (918–5,247)
IgG4	34 (3–86)	0.0001>	24 (4–86)	0.0002	442 (18–2,696)
IgA	235 (138–637)	0.7078	235 (138–407)	0.955	245 (52–624)
IgM	112 (44–248)	0.0115	112 (44–248)	0.0313	81 (23–274)
ANA[a]	6/9	0.4835	4/7	0.4449	42/31
RF[b](+/−)	0/13	0.0612	0/9	0.1924	17/49

[a] Anti-nuclear antibody

[b] Rheumatoid factor

Table 3 Comparison of imaging findings between idiopathic duct-centric chronic pancreatitis (IDCP) and type 1 autoimmune pancreatitis (AIP)

	IDCP				Type 1 AIP
	Institutional diagnosis		Central diagnosis		
	(+/−)	p	(+/−)	p	(+/−)
Pancreatic swelling	10/5	0.0001	8/3	0.0012	84/0
	(Head/body/tail) 8/7/5		(Head/body/tail) 6/7/5		(Head/body/tail) 63/46/56
Pancreatic duct narrowing	12/3	0.0245	8/3	0.0107	81/2
	(Head/body/tail) 9/8/5		(Head/body/tail) 5/6/3		(Head/body/tail) 55/34/48
Pancreatic cyst	2/13	1	1/10	1	9/75
Pancreatic stone	0/15	0.3539	0/11	0.5901	8/76
Lower bile duct stenosis	2/13	0.0001>	1/10	0.0001>	64/20

bile duct stenosis was seen in only one patient who showed no jaundice but serum elevation of biliary enzymes and transaminase (Supplementary Table S3, figure S4 and S5), as reported previously [18]. Intra-ductal ultrasonography (IDUS) results from this patient revealed no bile duct wall thickening at either strictured or normal portions in cholangiography findings (Fig. 3), indicating no complication of sclerosing cholangitis.

Discussion

AIP complicated with IBD

The present study aimed to clarify the clinical features of type 2 AIP (IDCP/AIP with GEL) in Japan by analyzing pancreatic diseases complicated with IBD, among which type 2 AIP is preferentially found. We collected patient records for cases of acute pancreatitis, chronic pancreatitis, and AIP. Although type 2 AIP may also exhibit acute or chronic pancreatitis, the present analysis focused on the AIP group only because histological examination of acute and chronic pancreatitis proved difficult. Pancreatic diseases that are complicated with IBD consist mainly of acute pancreatitis and less frequently of chronic pancreatitis and AIP [17, 25]. Similarly to the present study, previous reports have shown that the main cause of acute pancreatitis was immunosuppressive drugs in addition to duodenal lesions from Crohn's disease and other idiopathic etiologies [17, 25]. The prevalence of AIP in pancreatic diseases complicated with IBD is reportedly low, as indicated in Oishi's report that irregular narrowing of the

Table 4 Comparison of treatment, relapse, and extra-pancreatic lesions between idiopathic duct-centric chronic pancreatitis (IDCP) and type 1 autoimmune pancreatitis (AIP)

	IDCP				Type 1 AIP
	Institutional diagnosis		Central diagnosis		
	(+/−)	*p*	(+/−)	*p*	(+/−)
Treatment					
Prednisolone	7/8	0.0029	5/6	0.0073	71/13
Azathioprine	2/11	0.0168	2/8	0.0103	0/84
Biliary drainage	1/14	0.0607	0/11	0.0302	28/56
Pancreatic resection	1/14	0.3925	1/10	0.3116	2/82
Relapse	2/12	0.7276	2/8	1	19/65
Extra-pancreatic lesion					
Mikulicz's disease	0/14	0.0022	0/10	0.0040	40/44
Chronic thyroiditis	0/14	0.2041	0/10	0.3474	13/71
Sclerosing cholangitis	1/14	0.0001>	1/10	0.0001>	63/21
Retroperitoneal fibrosis	0/14	0.2041	0/10	0.3474	18/66
Inflammatory pseudotumor	0/14	1	0/10	1	1/83
Prostatitis	0/14	0.1183	0/10	0.2097	15/69
Asthma	0/14	0.3488	0/10	0.3758	10/74
Polyarthritis	1/12	0.134	1/8	0.0968	0/84
Erythema nodosa	0/13	1	0/9	1	0/84

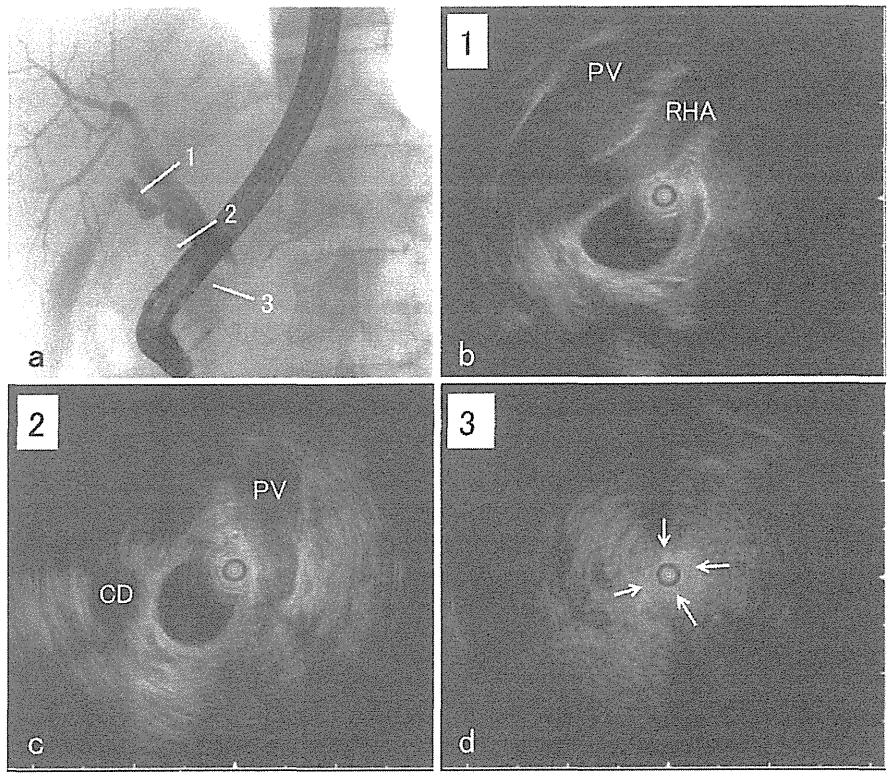

Fig. 3 IDUS findings of case 2 in supplementary Table S3 for IDCP of central diagnosis showing lower bile duct stenosis. **a** Cholangiography showing the position of IDUS, **b** IDUS finding for position 1, **c** IDUS finding for position 2, **d** IDUS finding for position 3. *PV* portal vein, *RHA* right hepatic artery, *CD* cystic duct. *Arrows* indicate strictured bile duct lumen and IDUS

pancreatic duct was found by ERCP in only two of 16 patients with abnormal pancreatic imaging among 255 cases of Crohn's disease [17]. Ueki et al. also reported that type 2 AIP was found in 5 (0.5 %) of 961 patients with UC and 2 (0.3 %) of 790 patients with Crohn's disease [20]. However, in collaboration with specialized organizations, we were able to collect a more substantial number of subjects with AIP complicated with IBD for analysis, though our analysis cannot make clear the prevalence of type 2 AIP in IBD cases because of collection bias mainly due to enrolled institutes that specialized in AIP or IBD.

Although AIP with complicating IBD demonstrated several clinical features of type 2 AIP, it was apparent that this group contained a variety of conditions that indicated probable AIP in half of the cases; indeed, a male preponderance and seven patients with high serum IgG4 concentration suggested that type 1 AIP was also included in this group. Thus, AIP complicated with IBD may be thought to include mainly type 2 AIP and to a lesser extent other conditions, including type 1 AIP. Park et al. reported that among six AIP patients with UC, four were diagnosed as having IDCP and two showed serum elevation of IgG and IgG4 [26]. Here, histological examination was possible for 28 cases of AIP complicated with IBD, which revealed 15 cases with IDCP of institutional diagnosis and ultimately 11 cases with IDCP of central diagnosis. A total of four cases (26.7 %) were excluded from the IDCP of central diagnosis group, reflecting the difficulty of histological IDCP diagnosis in general practice. Ikeura et al. reported on a case of type 1 AIP with histologically proven LPSP and prominent neutrophilic infiltration in the acinar lobules and epithelium of interlobular ducts, i.e., LPSP with GEL [19]. Therefore, we could not exclude the possibility that some cases of type 2 AIP were included with those having a biopsy diagnosis of simple acute pancreatitis due to sampling error or an atypical histological feature dependent on inflammatory stage because IDCP of institutional diagnosis and that of central diagnosis showed virtually identical clinical features.

It was mentioned that type 2 AIP was quite rare in the Japanese population [24], though correct incidence for it remains unclear because of diagnostic difficulty due to poor histological confirmation described above. In an international survey of 1,064 patients with AIP, type 2 AIP consisted of 86 patients (8 %) and the proportion of patients diagnosed with type 2 AIP was lower in Asian countries (3.7 %) compared with European (12.9 %) and North American (13.7 %) countries [27]. In Korean experience, however, type 2 AIP in all histologically confirmed AIP cases may not be as rare as originally thought, with an estimated prevalence rate of 28.8 % (15/52) [12]. These reports imply that the incidence of type 2 AIP in Japanese population seems to be more than expected previously, if extensive survey with detailed histological confirmation is done.

Characteristic clinical features of type 2 AIP and type 1 AIP

To clarify the characteristic features of type 2 AIP in Japan, this comparative study of clinical features between an IDCP group (composed of IDCP of institutional diagnosis and that of central diagnosis) and type 1 AIP was performed. The characteristics of type 2 AIP have been reported mainly from Europe and the USA to be different from those of type 1 AIP, including a younger onset age of 30–40 years, no gender bias, frequent symptoms of abdominal pain, and no relation to IgG4 [9, 10, 12, 28, 29]. The present study uncovered comparable results in that the IDCP group showed an onset age of 30–35 years, no gender bias, frequent abdominal pain with high serum amylase concentration, and no serum elevation of IgG4. Treatment with prednisolone was performed in half of the IDCP patients and achieved a favorable result. Immunosuppressive drugs for IBD had also been given to some patients, but their effect on pancreatic lesions was unclear. IDCP has been reported to have an excellent response to corticosteroids and no or few relapses during follow-up [12, 20, 27, 28]. Accordingly, we noted no significant difference in the frequency of relapse between the IDCP and type 1 AIP groups.

Low prevalence of jaundice in the IDCP group

The present study revealed that a characteristic feature of type 2 AIP was a low prevalence of jaundice compared with type 1 AIP that was closely associated with a decreased frequency of pancreatic head swelling and lower bile duct stenosis. Previous studies have similarly reported infrequent jaundice in type 2 AIP [12, 20, 27, 28]. They also reported imaging findings of type 2 AIP identical to those of type 1 AIP [12], but we wonder if this is truly the case since a difference in the frequency of obstructive jaundice should theoretically correspond to associated imaging findings, such as the decreased pancreatic head swelling and lower bile duct stenosis that were evident in this study.

Two mechanisms have been proposed to underlie the lower bile duct stenosis frequently encountered in type 1 AIP: compression stricture caused by pancreatic head swelling [30] and luminal stricture caused by bile duct thickening due to IgG4-related sclerosing cholangitis [31]. The IDCP group exhibited less frequent pancreatic head swelling that resulted in infrequent compression stricture of the bile duct and a correspondingly low frequency of jaundice. In addition, analysis of the CT, MRI, and ERCP images from six patients with IDCP of central diagnosis

revealed pancreatic head swelling in three patients (50 %), whereas pancreatic head swelling was found in 75 % of our patients with type 1 AIP. Analysis of IDUS images from one IDCP patient with lower bile duct stenosis showed no bile duct wall thickening at the portions of either strictured or non-strictured cholangiography findings, which indicated an absence of sclerosing cholangitis [31]. Accordingly, the major reason for a low frequency of jaundice in type 2 AIP is regarded to be a lower frequency of pancreatic head swelling and a corresponding decrease in the incidence of compression stricture of the lower bile duct, and bile duct wall thickening due to sclerosing cholangitis likely does not contribute to its formation.

In conclusion, we evaluated the clinical characteristics of type 2 AIP by analyzing pancreatic diseases complicated with IBD and uncovered several results that were similar to previous reports from Europe and the USA. We also revealed that a characteristic feature of type 2 AIP was a significantly lower frequency of obstructive jaundice compared with type 1 AIP that was related to rare lower bile duct stricture and a relatively lower prevalence of pancreatic head swelling. Furthermore, the lower bile duct stricture found in type 2 AIP has no apparent relation to intraluminal stricture due to bile duct wall thickening or sclerosing cholangitis.

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Conflict of interest The authors declare that they have no conflict of interest.

Appendix

Study Group for Pancreatitis Complicated with Inflammatory Bowel Disease organized by The Research Committee for Intractable Pancreatic Disease (Chairman: Tooru Shimosegawa) and The Research Committee for Intractable Inflammatory Bowel Disease (Chairman: Mamoru Watanabe), both of which are supported by the Ministry of Health, Labour, and Welfare of Japan.

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Preferential M2 macrophages contribute to fibrosis in IgG4-related dacryoadenitis and sialoadenitis, so-called Mikulicz's disease

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IL-10;
CCL18

Abstract IgG4-related dacryoadenitis and sialoadenitis (IgG4-DS) is characterized by bilateral swelling of glandular tissues with extensive fibrosis, and is immunologically considered a Th2-predominant disease. Recent studies reported that alternatively activated (M2) macrophages enhanced Th2 immune responses and fibrosis by production of pro-fibrotic factors (IL-10, IL-13 and CCL18). Therefore, we examined the association between M2 macrophages and fibrosis in submandibular glands from 7 patients with IgG4-DS, 10 patients with chronic sialoadenitis, 10 patients with Sjögren's syndrome, and 10 healthy subjects. The number of M2 macrophages in SMGs from patients with IgG4-DS was also significantly higher than in the other groups. Double immunofluorescence staining showed that IL-10 and CCL18 expression co-localized with M2 macrophage-marker (CD163). Furthermore, the SMG fibrosis score was positively correlated with the frequency of M2 macrophages in only IgG4-DS. These results indicate that IL-10 and CCL18 secreted by preferential M2 macrophages possibly play a key role in the development of severe fibrosis in IgG4-DS.

Abbreviations: MD, Mikulicz's disease; SMG, Submandibular gland; LG, lacrimal gland; SS, Sjögren's syndrome; IgG4-RD, IgG4-related disease; IgG4-DS, IgG4-related dacryoadenitis and sialoadenitis; Th2, helper T type 2; eGC, ectopic germinal center; OSCC, oral squamous cell carcinoma; CS, chronic sialoadenitis; MT, Masson's trichrome; Treg, regulatory T cell.

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1. Introduction

Mikulicz's disease (MD), first reported by Mikulicz in 1888 [1], is characterized by firm swelling of submandibular glands (SMGs) and lacrimal glands (LGs), and has been considered to be a subtype of Sjögren's syndrome (SS) because of these histopathological similarities [2]. However, Yamamoto et al. reported that patients diagnosed with MD also had high serum levels of IgG4 and marked infiltration of IgG4-positive plasma cells in salivary glands [3]. Moreover, several reports also demonstrated that these findings were accompanied by autoimmune pancreatitis (AIP) [4], sclerosing cholangitis (SC) [5], tubulointerstitial nephritis (TIN) [6], interstitial pneumonia [7], Hashimoto's thyroiditis [8] and Küttner tumor [9]. These diseases are now collectively called "IgG4-related disease (IgG4-RD)" and we have described this concept and provided up-to-date information regarding this emerging disease entity in a recent review [10]. Furthermore, recent studies have also referred to MD as IgG4-related dacryoadenitis and sialoadenitis (IgG4-DS) [11].

Regarding the immunological aspect of IgG4-RD, it is well known that IgG4 is induced by T helper type 2 cell (Th2) cytokines such as interleukin (IL)-4 and IL-13. We previously reported that analysis of peripheral $CD4^+$ Th cells from patients with IgG4-DS revealed a deviation in the Th1/Th2 balance favoring Th2 [12] and that Th2 cells play a key role in the production of IgG4 and formation of ectopic germinal centers (eGCs) [13,14]. In addition, Watanabe et al. reported that abnormal immune responses might enhance the Th2 response via Toll-like receptors expressed by macrophages, contributing to the immunopathology of IgG4-RD [15]. Recent studies reported that rituximab targeting CD20 plasma cells appeared to be an effective treatment strategy for IgG4-RD, but plasmablasts lack surface expression of CD20 and thus demonstrate a resistance to direct depletion by rituximab and IgG4-positive plasmablast. Macrophage might play an effective role in IgG4 production produced by IgG4-positive plasmablast. Recently, innate immune system such as macrophages has received a lot of attention to the initiation of IgG4-RD [16]. At least two distinct subtypes of macrophages have been identified; the classically activated (M1) macrophage stimulated by Th1 responses and the alternatively activated (M2) macrophage stimulated by Th2 responses. M2 macrophages contribute to angiogenesis, suppression of adaptive immunity, and wound healing and fibrosis [17,18]. Histologically, the critical features of IgG4-RD are severe fibrosis with dense lymphoplasmacytic infiltration of the salivary glands and other lesion [19]. However, to our knowledge, no published reports have investigated the mechanism promoting severe fibrosis in IgG4-RD. In this study, we examined the distribution of macrophage subsets and the expression of pro-fibrotic factors in salivary glands to clarify the contribution of macrophages to the pathogenesis of IgG4-DS.

2. Material and methods

2.1. Patients

SMG samples were collected from 7 patients with IgG4-DS (five men and two women; mean age ± standard deviation

Table 1 Clinical characteristics of 7 patients with IgG4-related dacryoadenitis and sialoadenitis (IgG4-DS).

No.	Age	Sex	Disease duration	Complications	Swollen glands					Complaint		Histological findings		Serological test								
					LG	PG	SMG	SLG	PLG	LSG	Dry eye	Dry mouth	IgG4/IgG (%)	IgG4+ cells (/HPF)	RF (U/ml)	ANA	IgG (mg/dl)	IgG4 (mg/dl)	IgA (mg/dl)	IgE (IU/ml)	IgM (mg/dl)	Anti-SS-A (U/ml)	Anti-SS-B (U/ml)
1	58	F	6 M	–	–	–	+	+	–	–	–	–	***73.0***	***28***	4	***80***	1188	***151***	193	***178***	56	–	–
2	68	F	9 M	AIP, TIN	–	–	+	+	–	+	–	–	***52.3***	***42***	5	***160***	***6758***	***1500***	78	13	81	–	–
3	39	M	2 Y	–	+	–	+	–	–	+	–	–	***64.2***	***77***	5	–	1534	***188***	170	***1619***	99	–	–
4	69	M	3 M	HT	+	–	+	+	–	+	–	–	***61.2***	***32***	ND	–	***1662***	***458***	97	60	79	–	–
5	74	M	4 M	AIP, Prostatitis	+	–	+	+	–	+	–	–	***50.0***	***85***	ND	40	***4217***	***524***	177	29	60	–	–
6	55	M	3 Y	AIP, IP	+	–	+	+	–	–	–	–	***70.0***	***18***	4	–	***2092***	***510***	148	ND	70	–	–
7	69	M	4 M	AIP	–	–	+	+	–	+	–	–	***63.2***	***79***	ND	–	***1675***	***484***	229	***283***	44	–	–

Abbreviations: LG, lachrymal gland; PG, parotid gland; SMG, submandibular gland; SLG, sublingual gland; PLG, palatine gland; LSG, labial salivary gland; TIN, tubulointerstitial nephritis; HT, higher tension; AIP, autoimmune pancreatitis; IP, interstitial pneumonitis; –, negative; ND, not done; bold italic means higher than normal values.

(SD), 61.7 ± 12.1 years), 10 patients with chronic sialoadenitis (CS) caused by sialolith (five men and five women; 51.5 ± 17.2 years), 10 patients with SS (five men and five women; 61.5 ± 14.9 years), and 10 patients with oral squamous cell carcinoma (OSCC) as a control group (five men and five women; 58.4 ± 16.3 years) who were referred to the Department of Oral and Maxillofacial Surgery, Kyushu University Hospital between 2010 and 2013. Patients underwent the following procedures: (1) open SMG biopsies for IgG4-DS and SS patients as described by Moriyama et al. [20]; (2) submandibulectomy for CS patients; and (3) neck dissection for OSCC patients. SMGs from OSCC patients were histologically normal and had no clinical evidence of metastasis. IgG4-DS was diagnosed according to both the "Comprehensive diagnostic criteria for IgG4-related disease" [10] and "Diagnostic criteria for IgG4-related Mikulicz's disease" [21]. The clinical and serological characteristics of the 7 patients with IgG4-DS are summarized in Table 1. SS was diagnosed according to both the Research Committee on SS of the Ministry of Health and Welfare of the Japanese Government (1999) [22] and the American–European Consensus Group criteria for SS [23]. Each patient showed objective evidence of salivary gland involvement based on the presence of subjective xerostomia and a decreased salivary flow rate, abnormal findings on parotid sialography and focal lymphocytic infiltrates in the labial salivary glands. There was no documented history of treatment with steroids, infection with HIV, HTLV-1, hepatitis B virus, or hepatitis C virus infection, sarcoidosis or any other immunodepressants in any of the patients. None of the patients had evidence of malignant lymphoma at the time of the study. The comparison of clinical and serological characteristics between the patients included in this study with SS and IgG4-DS is summarized in Table 2.

This study design was approved by the Ethics Committee of Kyushu University, Japan, and written informed consent was obtained from all of the patients and healthy controls (IRB serial number: 25-287).

Table 2 Comparison of clinical and serological findings between primary Sjögren's syndrome and IgG4-DS.

	SS (n = 10)	IgG4-DS (n = 7)
Mean age (years)	61.5 ± 14.9	61.7 ± 12.1
Men:women	5:5	5:2
Frequency of elevated serum IgG	60.0% (6/10)	71.4% (5/7)
<Mean ± SD (mg/dL)>	<2135.3 ± 757.0>	<2732.3 ± 2037.4>
Frequency of elevated serum IgG4	ND	100.0% (7/7)
<Mean ± SD (mg/dL)>	<ND>	<545.0 ± 448.6>
Frequency of elevated ANA	100.0% (10/10)	42.9% (3/7)
Anti-SS-A/Ro	100.0% (10/10)	0.0% (0/7)
Anti-SS-B/La	40.0% (4/10)	0.0% (0/7)
Frequency of other organs diagnosed with IgG4-RD	None	Pancreas: 57.1% (4/7) Kidney: 14.3% (1/7) Lung: 14.3% (1/7) Prostate: 14.3% (1/7)

Abbreviations: SS, Sjögren's syndrome; IgG4-RD, IgG4-related disease. Data are presented as the mean ± SD.

2.2. Immunohistochemical analysis

For immunohistochemical analysis, 4-μm formalin-fixed, paraffin-embedded sections were prepared and stained with a conventional avidin–biotin complex technique as previously described [13]. Anti-CD68 (catalog # ab955; Abcam, Cambridge, MA, USA) and CD163 (catalog # NCL-CD163; Leica Biosystems, Nussloch GmbH, Germany) mouse monoclonal antibodies were used to analyze the protein expression of CD68 and CD163, respectively. Anti-CCL18 (catalog # ab104867; Abcam), IL-10 (catalog # ab34843; Abcam) and IL-13 (catalog # HPA042421; Atlas Antibodies AB, Stockholm, Sweden) rabbit polyclonal antibodies were used to analyze the protein expression of CCL18, IL-10 and IL-13, respectively. Tissue sections were sequentially incubated with primary antibodies for 2.5 h then with biotinylated anti-mouse IgG and anti-rabbit IgG secondary antibodies (Vector Laboratories, Burlingame, CA, US), avidin–biotin–horseradish peroxidase complex (Vector Laboratories), and 3,3′-diaminobenzidine (Vector Laboratories). Mayer's hematoxylin was used for counterstaining. Photomicrographs were obtained using a light microscope equipped with a digital camera (BZ-9000 series; Keyence, Tokyo, Japan).

2.3. RNA extraction and complementary DNA (cDNA) synthesis

Total RNA was prepared from whole LSGs and SMGs by the acidified guanidinium–phenol–chloroform method. One microgram of total RNA was used for the synthesis of cDNA. Briefly, RNA was incubated for 1 h at 42 °C with 20 U of RNase inhibitor (Promega, Madison, WI, USA), 0.5 μg of oligo-1218 (Pharmacia, Uppsala, Sweden), 0.5 mM of each deoxyribonucleotide triphosphate (dNTP) (Pharmacia), 10 mM of dithiothreitol (DTT), and 100 U of RNA reverse transcriptase (Life Technologies, Gaithersburg, MD, USA).

2.4. Quantitative estimation of mRNA by real-time PCR

The mRNA levels of the cytokines and chemokines were analyzed quantitatively by real-time PCR using Light Cycler Fast Start DNA Master mix SYBR Green III (Roche Diagnostics, Mannheim, Germany) in a Light Cycler real-time PCR instrument (version 3.5; Roche Diagnostics). The cytokines and cell surface markers analyzed were CD68, CD163, CCL18, IL-10, and IL-13. The primer sequences used were as follows: β-actin (260 bp), forward 5′-GCA AAG ACC TGT ACG CCA AC-3′, reverse 5′-CTA GAA GCA TTT GCG GTG GA-3′; CD68 (199 bp), forward 5′-TCA GAA TGC ATC CCT TCG AG-3′, reverse 5′-GAT GAG AGG CAG CAA GAT GG-3′; CD163 (168 bp), forward 5′-TGA TTT CGG ACT TCT CTC TGG-3′, reverse 5′-ACT GGG CAG AGT GAA AGA TG-3′; CCL18

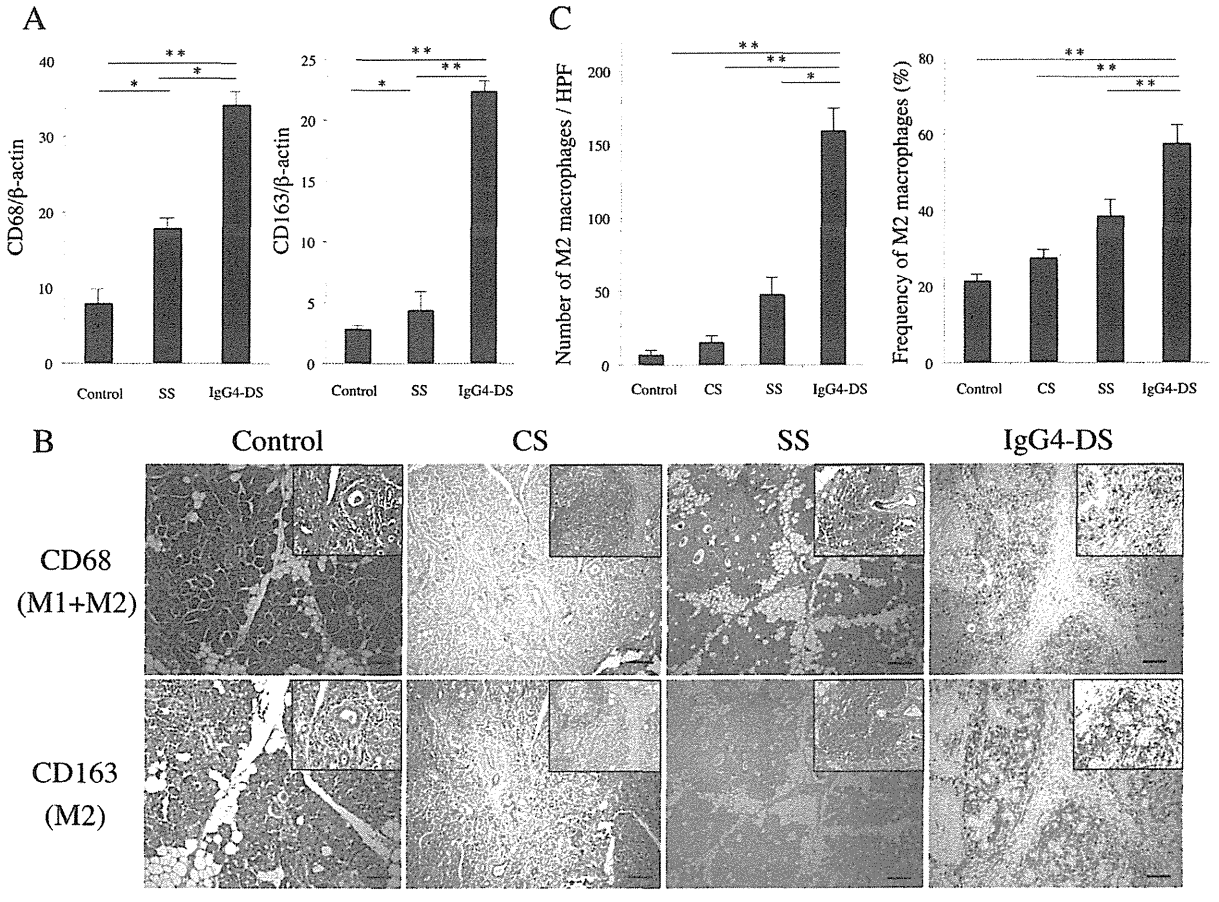

Figure 1 M1 and M2 macrophage localization in submandibular glands (SMGs). (A) mRNA expression levels of macrophage markers (CD68, M1 and M2 macrophages; CD163, M2 macrophages) were examined in SMGs from controls (n = 10), patients with Sjögren's syndrome (SS) (n = 10) and IgG4-related dacryoadenitis and sialoadenitis (IgG4-DS) (n = 7). Macrophage markers were quantitatively estimated as described in the Materials and methods section. Significant differences between groups were determined by the Mann–Whitney *U* test (*$p < 0.05$, **$p < 0.01$). (B) Distribution of M1 and M2 macrophages in SMGs from representative controls and patients with chronic sialoadenitis [33], SS and IgG4-DS. Counterstaining was performed with Mayer's hematoxylin (blue). Higher magnifications are displayed at the upper right. Scale bars, 100 μm. (C) Number and frequency of M2 macrophages per high-power field (HPF) were counted in 4-mm^2 sections from five different areas as described in the Materials and methods section. Statistically significant differences between groups were determined by Mann–Whitney *U* tests (*$p < 0.05$, **$p < 0.01$).

(187 bp), forward 5′-AGC TCT GCT GCC TCG TCT AT-3′, reverse 5′-CAG GCA TTC AGC TTC AGG TC-3′; IL-10 (144 bp), forward 5′-TGA GAA CCA AGA CCC AGA CA-3′, reverse 5′-AAG GCA TTC TTC ACC TGC TC-3′; and IL-13 (240 bp), forward 5′-GGT CAA CAT CAC CCA GAA CC-3′, reverse 5′-TTT ACA AAC TGG GCC ACC TC-3′. The relative mRNA level was calculated after normalizing to the housekeeping gene β-actin.

2.5. Double immunofluorescence analysis

For double immunofluorescence analysis, 4-μm formalin-fixed, paraffin-embedded sections were prepared and stained. Sections were incubated with the primary antibody, CCL18 (Abcam), IL-10 (Abcam), or IL-13 (Atlas Antibodies AB) at room temperature for 2 h after blocking with 1% BSA for 1 h, then incubated with secondary antibody (1:100 dilution of Alexa 488 USA) for 30 min. The sections were washed well, blocked with 1% BSA blocking buffer for 40 min, and incubated with the primary antibodies, CD163 (clone EDHu-1; AbD Serotec, Raleigh, NC, USA) at room temperature for 2 h. After incubation, the sections were incubated with secondary antibodies (1:100 dilution of Alexa 568) for 30 min at room temperature. Slides were mounted (VectaMount, Vector Laboratories) and kept in the dark. DAPI was used to stain nuclei. Images were taken using a Keyence microscope (BZ-9000 series), setting the background fluorescence level with the negative controls.

2.6. Evaluation of the severity of fibrosis

To evaluate fibrosis histologically, Masson's trichrome (MT) staining (Polysciences, Warrington, PA, USA) was performed.

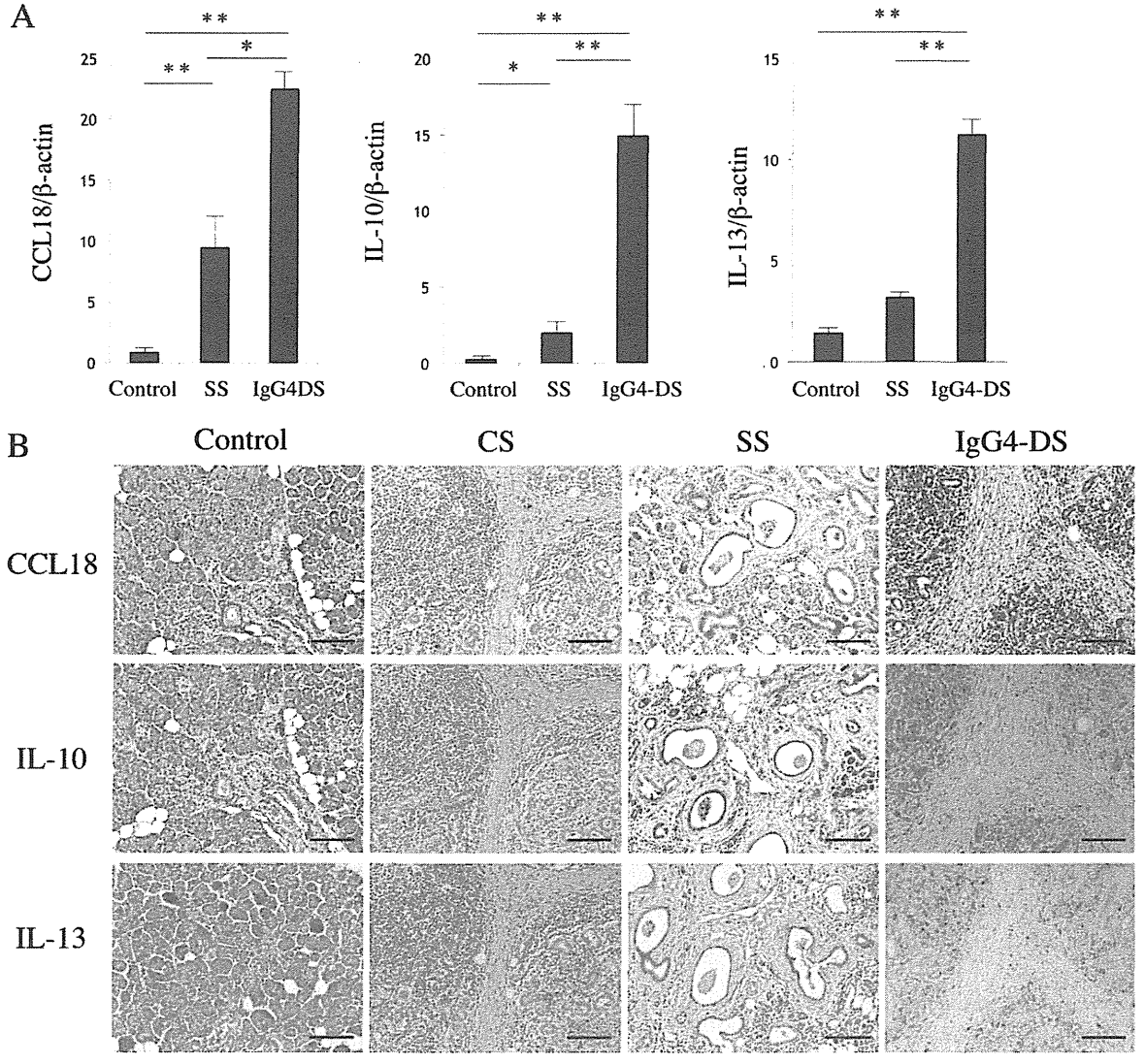

Figure 2 Localization of pro-fibrotic factors (CCL18, IL-10, and IL-13) in SMGs. (A) mRNA expression levels of pro-fibrotic factors in SMGs from controls (n = 10), patients with SS (n = 10) and IgG4-DS (n = 7). Expression levels of pro-fibrotic factors were estimated quantitatively as described in the Materials and methods section. Statistically significant differences between groups were determined by Mann–Whitney *U* tests (*$p < 0.05$, **$p < 0.01$). (B) Distribution of fibrosis factors in SMGs from representative controls and patients with CS, SS, and IgG4-DS. Counterstaining was performed with Mayer's hematoxylin (blue). Scale bars, 200 μm.

In short, 4-μm formalin-fixed, paraffin-embedded sections were prepared and stained. Connective and fibrosis tissues were selectively stained blue, whereas nuclei stained by Weigert's iron hematoxylin were dark brown to black and the cytoplasm was stained red. The fibrosis scores in SMGs were defined as the ratio of the fibrotic area (blue) to the whole stained area in a 4-mm^2 field of view, from five different areas.

2.7. Statistical analysis

The statistical significance of the differences between the groups was determined by the Mann–Whitney *U* test or Spearman's rank correlation as appropriate. All statistical analyses in this study were performed using JMP software, version 8 (SAS Institute, Cary, NC, USA). A p-value <0.05 was considered statistically significant.

3. Results

3.1. Expression of macrophage markers in SMGs

The mRNA levels of CD68 and CD163 in SMGs from SS and IgG4-DS patients were significantly higher than those in controls. Furthermore, the mRNA levels of CD68 and CD163 in IgG4-DS patients were significantly higher than those in SS

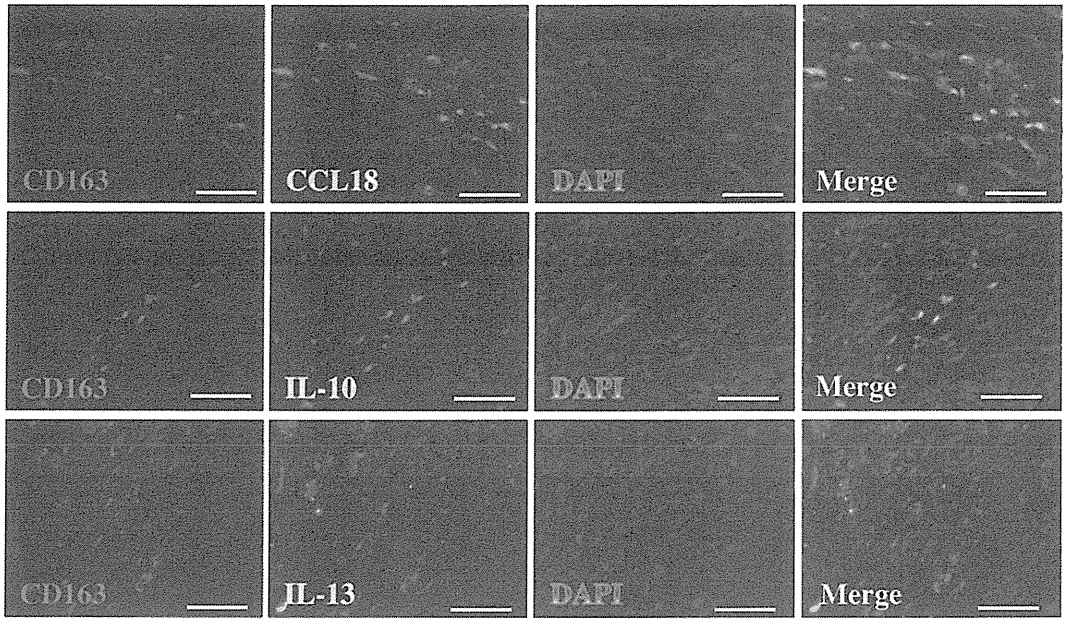

Figure 3 M2 macrophages produce pro-fibrotic factors in SMG in patients with IgG4-DS. Double immunofluorescence staining performed with CD163 (red), pro-fibrotic factors (CCL18, IL-10, and IL-13) [23], and DAPI for staining nuclei (blue) as described in the Materials and methods section. The images for CD163 and pro-fibrotic factors were merged (yellow). Scale bars, 50 μm.

patients (Fig. 1A). As specimens from CS patients were formalin-fixed and paraffin-embedded tissues, the mRNA levels in CS patients were not measured. The specimens were also examined after the immunohistochemical staining to evaluate the distribution of macrophages in SMGs. Expression of CD68 was weakly detected in/around areas of fibrosis or connective tissue in SMGs from controls, while it was strongly detected in/around areas of fibrosis in SS, CS and IgG4-DS patients. Expression of CD163 was not detected in controls, but was weakly detected in/around fibrotic lesions in SS and CS patients. Interestingly, expression of CD163 was strongly detected in/around areas of fibrosis in IgG4-DS patients (Fig. 1B). Moreover, the number of CD163-positive cells and the CD163/CD68 ratio in SMGs from IgG4-DS patients were significantly greater than those in the other groups (Fig. 1C).

3.2. Expression of pro-fibrotic factors in SMGs

As IL-10, IL-13, and CCL18 produced by macrophages promote fibrosis, we next compared the expression and distribution of these pro-fibrotic factors between controls and patients. The mRNA levels of CCL18, IL-10, and IL-13 in SMGs from SS and IgG4-DS patients were significantly greater than those in controls. Furthermore, the mRNA levels of CCL18, IL-10 and IL-13 in IgG4-DS patients were significantly higher than those in SS patients (Fig. 2A). Expression of CCL18, IL-10, and IL-13 could not be detected by immunohistochemistry in SMGs from controls or CS patients, but could be detected in/around ductal epithelial cells in SS patients. In contrast, expression of CCL18, IL-10, and IL-13 was strongly detected in/around areas of fibrosis in IgG4-DS patients (Fig. 2B).

3.3. Co-localization of M2 macrophage markers and pro-fibrotic factors in SMGs from IgG4-DS patients

To clarify whether M2 macrophages expressed the pro-fibrotic factors, double immunofluorescence staining with CD163 and CCL18, IL-10 or IL-13 was performed. As shown in Fig. 3, CD163-positive cells (red) were co-localized with CCL18 and IL-10 positive cells, while they only partly co-localized with IL-13-positive cells. Therefore, M2 macrophages might promote fibrosis in SMGs from IgG4-DS patients through increased production of IL-10 and CCL18.

3.4. Evaluation of fibrosis score in SMGs

Specimens were stained by MT staining to evaluate the degree of fibrosis in SMGs. IgG4-DS patients showed severe cordlike fibrosis with extensive eGC formation, while the other groups showed only mild or moderate periductal fibrosis (Fig. 4A). The fibrosis score of IgG4-DS was significantly higher than those in the other groups (Fig. 4B). Furthermore, the fibrosis score was positively correlated with the CD163/CD68 ratio in SMGs from IgG4-DS patients but not in those from the other groups (Fig. 4C).

3.5. Expression of macrophage markers in other organs from IgG4-DS patients

To confirm the changes in macrophage distribution in IgG4-RD patients, specimens from other involved organs including the lacrimal gland, pancreas, prostate gland, and pleura from IgG4-DS patients included in this study were examined by immunohistochemical staining. Expression of