to 1.83 after steroids. These findings suggest that the scoring system reflects disease activity of AIP. However, it is unclear whether the system can predict early AIP relapse. Cutoff values suggesting relapse are also unknown [23].

CQ-III-8. How are AIP relapses treated?

- Re-administration or dose-up of steroid is effective for treating AIP relapses.
- Remission can be obtained with the same prednisolone dose as the initial dose in most relapsed AIP cases, but it may be necessary to taper more gradually. (Level of recommendation: I)

Description Remission can be obtained with re-administration or dose-up of steroid in most relapsed AIP cases. According to Kamisawa et al. [10], 4 AIP patients who relapsed at pancreatic or extrapancreatic lesions during maintenance therapy obtained remission with dose-up (30 mg/day) of steroid. Nishino et al. reported that bile duct stenosis and swelling of the salivary glands relapsed during steroid tapering in 1 and 3 patients respectively, but they improved with dose-up steroid. They also tapered the steroid more gradually (1 mg/2 weeks) as compared with the speed of initial therapy in relapsed cases [22]. At the Mayo Clinic, second relapse occurred in 4 of 11 patients with first relapse, despite slow steroid tapering after the second induction therapy. They also reported that immunomodulatory drugs such as azathioprine (initial dose of 50 mg/day, increasing to 2–2.5 mg/kg) and mycophenolate mofetil (initial dose of 500 mg twice daily, increasing to 750 mg twice daily) were effective in 7 relapsed AIP patients, and none of these patients relapsed (median follow-up period on immunomodulatory drugs alone, 6 months; range, 2–19 months) [20]. Although immunomodulatory drugs appear to prevent relapse and to maintain remission, indications for these drugs should be judged carefully based on their adverse effects.

CQ-III-9. Do pancreatic exocrine and endocrine functions improve after steroid therapy in AIP patients?

- Pancreatic exocrine and endocrine functions improve after steroid therapy in some AIP patients. Many AIP patients with type 2 diabetes mellitus before AIP onset showed worsening of diabetes mellitus control after steroid therapy. (Level of recommendation: A)

Description Many AIP patients have associated pancreatic exocrine and endocrine dysfunction [2, 7, 11, 24–26]. It has been reported that improvement of pancreatic exocrine and endocrine function was detected after steroid therapy in 38% [22] to 50% [25] and 25% [22] to 45% [25] of AIP patients, respectively. It has also been suggested as a mechanism of improvement in pancreatic exocrine and endocrine functions after steroid therapy that steroid suppresses lymphoplasmacytic cell infiltration and fibrosis, permitting the attenuation of blood flow [26] and further regenerating islet cells by suppression of cytokine production [27]; however, the precise mechanisms remain unclear.

Diabetes mellitus control worsens in 75% of AIP patients with type 2 diabetes mellitus before AIP onset after steroid therapy [25]. DM also develops after steroid therapy in some AIP patients [24, 25]. We should therefore take occurrence of DM into consideration in patients who continuously undergo steroid therapy.

CQ-III-10. Is the prognosis of AIP good?

- The prognosis of AIP appears to be good over the short-term with steroid therapy.
- It is unclear whether the long-term outcome is good, because there are many unknown factors, such as relapse, pancreatic exocrine or endocrine dysfunction, and associated malignancy. (Level of recommendation: B)

Description The relapse rate of AIP is reported to be 10% [10] to 53% [20] in patients treated with steroids, and 28% [28] to 35% [20] in those without steroid therapy.

AIP responds well to steroid therapy, and remission can be induced in most AIP patients. However, with respect to the long-term outcome, there are many unknown factors, such as relapse, pancreatic exocrine or endocrine dysfunction, and associated malignancy.

Nishino et al. [22] reported that pancreatic atrophy developed in 33% of 12 patients, and 1 patient developed early gastric cancer after 29 months of steroid therapy, while another patient developed advanced rectal cancer after 13 months of steroid therapy. According to Hirano et al., unfavorable events occurred in 32% of AIP patients treated with steroid therapy during an average 41-month follow-up period, and they occurred in 70% of those without steroid therapy during an average follow-up of 61 months. Furthermore, 1 patient treated with steroid therapy died of acute myelocytic leukemia, 1 patient not treated with steroid therapy died of lung cancer, and 1 patient not treated with steroid therapy died of pancreatic cancer [19]. Kubota et al. [3] also reported 4 patients who were diagnosed as having a malignancy during follow-up (pancreatic cancer, $n = 2$; breast cancer, $n = 2$; gastric cancer, $n = 1$). Kamisawa et al. [10] reported that marked atrophy of the pancreas was observed in 30% of AIP patients during follow-up. Park et al. [16] reported that 13 (33%) of 40 patients treated with steroids relapsed during a median follow-up period of 40 months, with 7 relapsing on the maintenance dose of prednisolone (2.5–7.5 mg/day), and the remaining 6 patients relapsing while off steroids. According to Ghazale et al. [20],

16 (53%) of 30 patients treated with steroids relapsed during a median follow-up period of 30 months. They also reported that 7 of 53 AIP patients died and that pancreatic cancer and metastatic pancreatic cancer developed.

In 37 AIP patients who underwent pancreatoduodenectomy, no patients relapsed during a median follow-up period of 33 months, and 68% subjectively rated their quality of life as better [29]. On the other hand, among 29 surgically resected AIP patients, 8 (28%) relapsed at a median time to recurrence of 11 months during a median follow-up period of 38 months [28]. Schneldorfer et al. [30] reported that in 8 surgically resected AIP patients, improved quality of life (QOL) was seen in almost half of patients, but 2 (25%) patients relapsed.

CQ-III-11. Is there any relationship between AIP and pancreatic cancer?

- There are a few papers reporting an AIP case developing pancreatic cancer, but it is unclear whether there is a relationship between AIP and pancreatic cancer. (Level of recommendation: B)

Description It has been reported that chronic pancreatitis is one of the risk factors for pancreatic cancer [31]. It has been reported that some AIP patients developed pancreatic atrophy or pancreatic stones [32, 33]. AIP occurred predominantly in the elderly males. It is necessary to observe whether there is an association with pancreatic cancer and other malignancies in AIP patients treated with steroid for a long period, since steroid therapy is immunosuppressive. Periodic checks of serum tumor markers are necessary during follow-up.

There have been 6 recent papers reporting AIP cases developing pancreatic cancer [34–39]. The locations of these cancers were the pancreatic head ($n = 1$), body ($n = 3$), and tail ($n = 2$). All patients were males, and average age was 72 years (62–80 years). Three pancreatic cancers were diagnosed simultaneously with AIP, and the other 3 cancers were diagnosed 3–5 years after the onset of AIP. Kamisawa et al. [40] reported frequent and significant K-ras mutations in the pancreas of AIP patients. However, it is unclear whether there is a relationship between AIP and pancreatic cancer.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Long-term outcome of autoimmune pancreatitis

Kazushige Uchida · Shujiro Yazumi · Akiyoshi Nishio · Takeo Kusuda · Masaki Koyabu · Masanori Fukata · Hideaki Miyoshi · Yutaku Sakaguchi · Toshiro Fukui · Mitsunobu Matsushita · Makoto Takaoka · Kazuichi Okazaki

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Abstract
Purpose Autoimmune pancreatitis (AIP) is a unique form of pancreatitis and can be complicated with various extrapancreatic lesions. Little is known about the long-term clinical course of AIP. Here we aimed to document the clinical course of AIP.
Methods For this study, we recruited 21 patients, averaging 66.5 years in age (range, 19–84 years) and observed them at a mean interval of 40.8 months (range, 18–130 months). Three of the patients were also diagnosed with retroperitoneal fibrosis, 3 had sialoadenitis, 2 had chronic thyroiditis, 1 had interstitial nephritis, and 1 had interstitial pneumonia. Three of the patients underwent surgical therapy, 12 patients received methylprednisolone (PSL) treatment, and the 6 remaining patients received no treatment.
Results Enlargement of the pancreas was attenuated in all the PSL-treated patients. Seven of the 21 patients showed pancreatic atrophy, of whom 2 were non-PSL-treated patients. Three patients developed chronic pancreatitis. One patient was diagnosed with pancreatic cancer after 50 months of PSL therapy.
Conclusions As with chronic pancreatitis patients, AIP patients should be observed closely for abnormality in pancreatic function.

Keywords Autoimmune pancreatitis · Chronic pancreatitis · Pancreatic cancer

K. Uchida · A. Nishio · T. Kusuda · M. Koyabu · M. Fukata · H. Miyoshi · Y. Sakaguchi · T. Fukui · M. Matsushita · M. Takaoka · K. Okazaki (✉)
Department of Gastroenterology and Hepatology, Kansai Medical University, 10-15 Fumizono-cho, Moriguchi, Osaka 570-8506, Japan
e-mail: okazaki@takii.kmu.ac.jp

S. Yazumi
Department of Gastroenterology and Hepatology, Graduate School of Medicine, Kyoto University, Sakyo-ku, Kyoto, Japan

Introduction

Chronic pancreatitis (CP), of which about 30–40% of cases are idiopathic [1], is a disorder characterized by chronic inflammation and fibrosis of the pancreas that leads to irreversible pancreatic dysfunction and finally to pancreatic insufficiency. Sarles et al. [2] observed the first case of pancreatitis with hypergammaglobulinemia. In 1995, Yoshida et al. [3] first proposed the concept of "autoimmune pancreatitis (AIP)," in which patients show diffusely enlarged pancreas, narrowing pancreatogram, increased serum IgG, the presence of autoantibodies, fibrotic changes with lymphocytic infiltration, and the efficacy of steroid treatment. Since that time, many AIP cases have been reported by Japanese gastroenterologists, and AIP has been accepted as a new clinical entity [4–6]. Many reports have shown that steroid treatment was very effective against AIP with increased immunoglobulin (Ig) G, IgG4, or autoantibodies. Recent studies have clarified that extrapancreatic lesions, such as sclerosing cholangitis, sclerosing sialoadenitis, interstitial nephritis, and retroperitoneal fibrosis, are often observed in AIP, suggesting that AIP may not be a discrete entity [7–9]. Imaging studies of patients with AIP show characteristically diffuse enlargement of the pancreas and irregular narrowing of the main pancreatic duct [10, 11]. Typical immunological abnormalities include increased levels of serum gammaglobulin, IgG, and IgG4,

and the presence of autoantibodies. Histopathological findings show lymphoplasmacytic sclerosing pancreatitis, which is a fibrotic change entailing dense infiltration of lymphocytes and IgG4-positive plasmacytes [12]. However, little is known about the prognosis of AIP patients. In the present study, we report the outcome and clinical features of our experiences with patients with AIP under long-term observation.

Methods

We diagnosed 52 patients with AIP at Kansai Medical University and affiliated hospitals. All of these patients were diagnosed according to the clinical diagnostic criteria for AIP proposed by the Research Committee of Intractable Diseases of the Pancreas supported by the Japanese Ministry of Health, Labor, and Welfare, and the Japan Pancreas Society [13]. All 52 patients fulfilled diagnostic criteria 1 and 2. In brief, they exhibited diffuse enlargement of the pancreas and diffuse narrowing of the main pancreatic duct with an irregular wall. They also showed high levels of serum gammaglobulin (normal range, > g/dl), IgG (normal range, >1800 mg/dl), or IgG4 (normal range, >135 mg/dl). However, histological findings of the pancreas were not confirmed in any of them.

Of the 52 patients, 21 (14 male, 7 female, averaging 66.5 years in age; range, 19–84 years) were followed up for 18 months or more (mean period, 40.8 months; range, 18–130 months). Extrapancreatic lesions were observed in 10 patients: retroperitoneal fibrosis was diagnosed in 3 patients, sialoadenitis in 3, thyroiditis in 2, interstitial nephritis in 1, and interstitial pneumonia in 1. Four patients had associated diabetes mellitus (DM), and 1 had nonalcoholic steatohepatitis (NASH; Table 1).

Three patients underwent surgical treatment: these patients underwent left-lobe hepatectomy, pancreatoduodenectomy, and bilio-jejunostomy when AIP or associated conditions were misdiagnosed as cholangioductal cancer, pancreatic cancer, and mass-forming pancreatitis, respectively. Twelve patients were treated with oral steroid (methylprednisolone; PSL), and 6 patients were observed without treatment. The initial dose of oral PSL was 30 mg/day, decreasing by 5 mg/day every 2 weeks. The maintenance doses of PSL were 10 mg/day in three patients, 7.5 mg/day in two, and 5 mg/day in five; maintenance

Table 1 Clinical profiles of 21 patients

Patient number	Onset age (years)	Sex	Treatment	Follow-up period (months)	Associated diseases
1	64	M	PSL	20	Thyroiditis, sialoadenitis, DM
2	77	M	PSL	50	Pancreatic cancer
3	71	M	PSL	31	
4	66	M	PSL	79	DM
5	66	F	Bilio-jejunostomy; steroid pulse	130	Retroperitoneal fibrosis Hashimoto's disease
6	62	F	None	32	Sialoadenitis
7	63	M	PSL	34	
8	71	M	PSL	27	Retroperitoneal fibrosis
9	71	F	None	21	NASH
10	77	F	PD	48	
11	54	M	None	39	Inflammatory pseudotumor (liver)
12	70	M	None	21	
13	19	F	None	57	
14	73	M	Hepatectomy	27	Retroperitoneal fibrosis
15	65	M	PSL	23	
16	74	M	PSL	83	DM
17	65	M	PSL	23	DM
18	84	F	PSL	19	
19	81	M	PSL	47	
20	73	M	None	35	
21	51	F	PSL	18	Interstitial pneumonia Mikulicz's disease

PSL treatment with methylprednisolone, *PD* pancreatoduodenectomy, *DM* diabetes mellitus, *NASH* nonalcoholic steatohepatitis

therapy with oral PSL was discontinued in two of the patients. We observed six patients without administering oral steroid therapy, because one patient had NASH and five patients showed only pancreatic body and/or tail enlargement without extrapancreatic lesions. No patients had any complications during the follow-up period.

To evaluate clinical courses, levels of pancreatic enzymes, the glycosylated hemoglobin value (HbA1c), and morphological changes as indicated by computed tomography (CT) were studied. Pancreatic size was evaluated by CT according to the method of Heuck et al. [14]. The width of the pancreas along its longest axis was measured on CT images and compared with the transverse diameter of the vertebral body. The pancreatic size on the first CT image was defined as 100%. Pancreatic atrophy was defined according to the criteria of of Heuck et al. [14]. In brief, the vertebral body diameter was regarded as 100% and from this the corresponding pancreatic diameter was calculated. Pancreatic atrophy was defined as a ratio of the vertebral body to the pancreatic body of 20% or less.

Results

Morphological changes of the pancreas

In all 12 patients treated with oral PSL, the enlargement of the pancreas improved. After 18 months, the average pancreatic size had decreased to 51.3% of that before treatment (Fig. 1a). Pancreatic atrophy developed in 5 of the 12 patients treated with PSL and in 2 of the 6 patients without medication. The pancreatic size was not changed in 2 of the patients without PSL treatment (Fig. 1b). The incidence of pancreatic atrophy in the steroid-treated group was higher than that in the nontreated group. Pancreatic calcification was observed in 1 patient after 12 months of PSL therapy. Chronic pancreatitis with dilation of the main pancreatic duct (MPD) developed in 2 patients after PSL therapy; in 1 after 12 months and in 1 after 19 months. Pancreatic cyst occurred in 1 patient after 24 months of PSL therapy.

Pancreatic enzymes

Serum trypsin levels (100–550 ng/ml) were monitored periodically in 10 of the 21 observed patients. In 1 of these 10 patients, serum trypsin levels decreased after PSL therapy, while in 1 patient, serum trypsin levels increased after PSL therapy. In 3 of the 10 patients, serum trypsin levels decreased to below the normal limit, while in 1 patient, serum trypsin levels decreased after PSL therapy (Fig. 2a). Elastase I levels (<400 ng/dl) were monitored in 12 of the 21 patients. In 4 of these 12 patients, elastase I

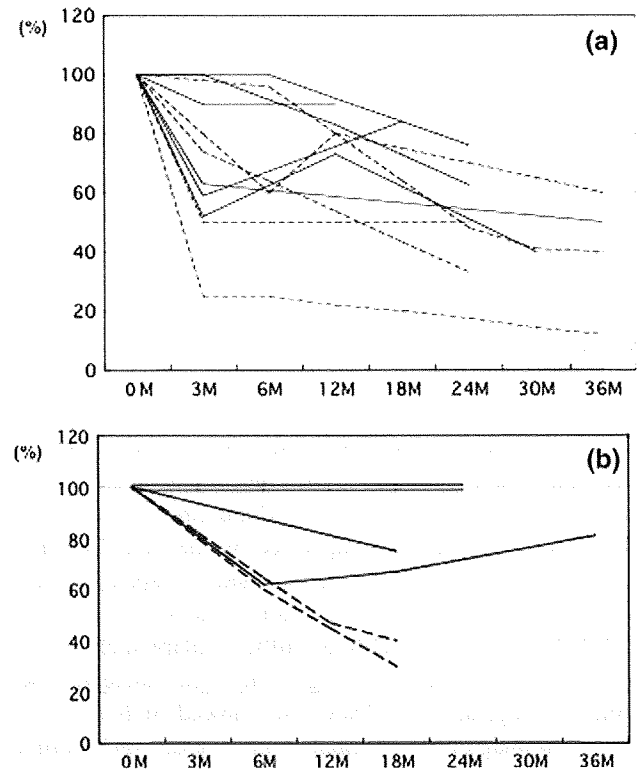

Fig. 1 Follow up of pancreatic enlargement in patients with steroid therapy (**a**) and without steroid therapy (**b**). Pancreatic enlargement was evaluated by computed tomography (CT). The width of the pancreas along its maximum longitudinal axis was measured on CT images and compared with the transverse diameter of the vertebral body. The pancreatic size on the first CT image was defined as 100%. In all 12 PSL-treated patients (**a**), the enlargement of the pancreas was attenuated; after 24 months, the average pancreatic size had decreased to 51.3% of the pancreatic size before treatment. Pancreatic atrophy developed in 5 of these patients (*dashed lines*). In 2 of the patients without PSL treatment, there were no changes in pancreatic size. Two of the patients without PSL treatment showed pancreatic atrophy (*dashed lines*). *M* months

levels fell to within normal limits: in 3 patients after PSL therapy and in 1 patient naturally without PSL therapy (Fig. 2b).

Pancreatic exocrine function

In our short-term follow-up series, after 6 months with steroid treatment, pancreatic exocrine function showed improvement in 11 patients, as determined by the urine exocrine *N*-benzoyl-L-tyrosyl-*p*-aminobenzoic acid (BT-PABA) test (73.1–90.1%) (Fig. 3). In the long-term follow-up series, pancreatic exocrine function was monitored by the BT-PABA test in 10 of the 21 patients (Fig. 4). Four of them showed improvement of pancreatic exocrine function by steroid therapy, while 6 (3 with steroid and 3 without steroid therapy) showed progressive dysfunction.

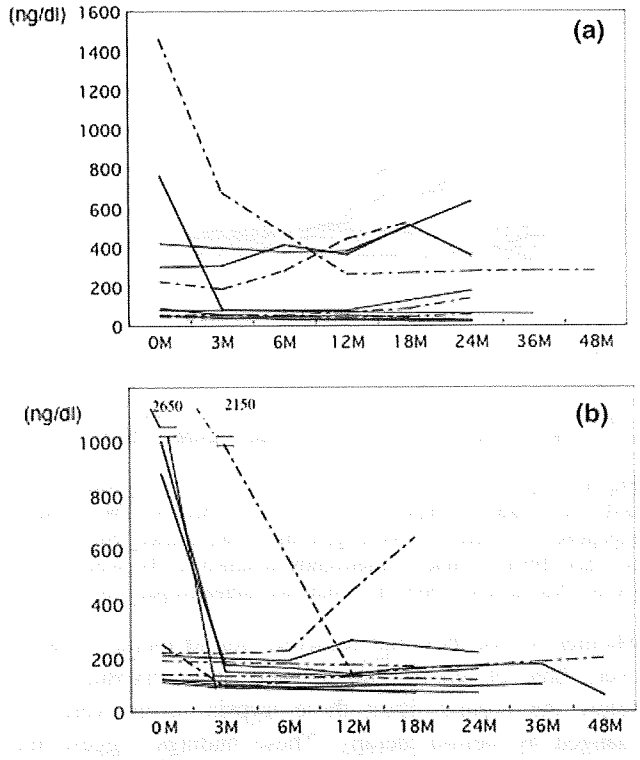

Fig. 2 Long-term follow up of serum pancreatic enzyme levels. Serum trypsin (**a**) was elevated in 2 of 10 patients tested at regular intervals after PSL therapy. The concentration decreased in patients both after PSL therapy and in the natural course without PSL therapy. In 3 patients, the levels decreased below the normal limit. One patient showed elevation of serum trypsin after PSL therapy. Elastase I (**b**) was elevated in 4 of 12 patients tested. In 3 patients levels returned to normal after PSL therapy. In 1 patient levels were normalized naturally. In 1 patient without PSL therapy, elastase I was elevated. *Dashed lines* show the results for untreated patients

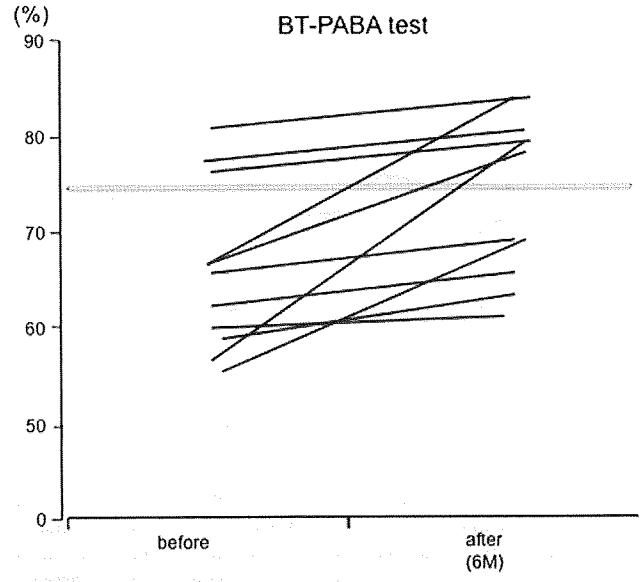

Fig. 3 Short-term follow up of pancreatic exocrine function. After 6 months of steroid treatment, pancreatic exocrine function showed improvement in 11 patients, as determined by the urine exocrine *N*-benzoyl-L-tyrosyl-*p*-aminobenzoic acid (*BT-PABA*) test

Pancreatic endocrine function

The short-term effect of PSL treatment on DM associated with AIP was evaluated in all 52 patients after 3 months of steroid therapy. Steroid therapy improved HbA1c levels (4.3–5.8%) in 6 of 9 patients with AIP but worsened them in the remaining 3 patients (Fig. 5). HbA1c levels were monitored in 13 of the 21 observed patients in the long-term follow-up series. Six of these 13 patients were found to have associated DM. Insulin therapy was effective for 1 of these patients. After PSL therapy, 3 of the patients experienced a temporary decline in glycemic control, but their HbA1c levels subsequently returned to pretreatment levels (Fig. 6).

Recurrence of AIP

Two patients relapsed despite receiving maintenance steroid therapy. One patient showed swelling of the bilateral hilar lymph nodes during maintenance therapy with 5 mg/day PSL, and showed improvement after the PSL dose was increased to 10 mg/day. Another patient relapsed with stricture of the bilioenteric anastomosis after biliojejunostomy, and was treated with two courses of steroid pulse therapy (500 mg/day of PSL for 3 days per week). After the steroid pulse therapy, the intrahepatic bile duct dilatation improved dramatically; nevertheless, the patient was continuing on a 10 mg/day maintenance dose of oral PSL at the time of this writing [15].

Long-term prognosis

In our long-term follow-up series, three patients developed chronic pancreatitis. Two patients died of pancreatic cancer (follow-up period, 4 years and 2 months) [16], and hepatic failure unrelated to AIP (2 years and 3 months; Fig. 7).

Discussion

Because PSL therapy is effective for resolving the clinical symptoms and morphological changes in AIP, including extrapancreatic lesions, many AIP patients undergo steroid therapy. However, little is known about the prognosis of AIP. In the present study, pancreatic enlargement was attenuated by steroid therapy in all treated patients. However, marked atrophy of the pancreas developed in 7 of the cohort of 21 (33.3%) patients, and in 5 of the 12 patients treated with steroid therapy (41.7%). These findings

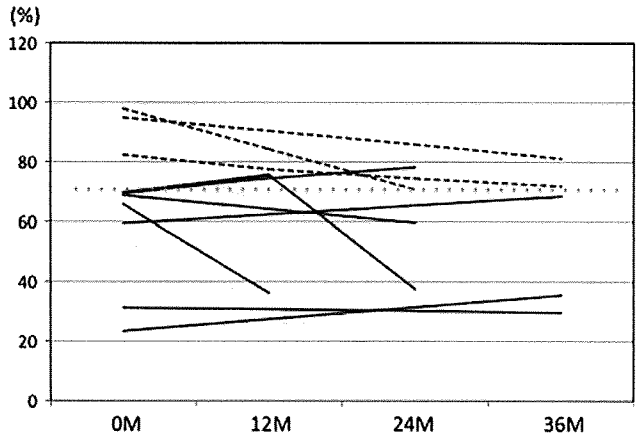

Fig. 4 Long-term follow up of pancreatic exocrine function in patients with autoimmune pancreatitis (AIP).Pancreatic exocrine function was monitored by the BT-PABA test in 10 of 21 patients. Four of them showed improvement of pancreatic exocrine function by steroid therapy, while 6 (3 with steroid and 3 without steroid therapy) showed progressive dysfunction. *Dashed lines* show the results for untreated patients

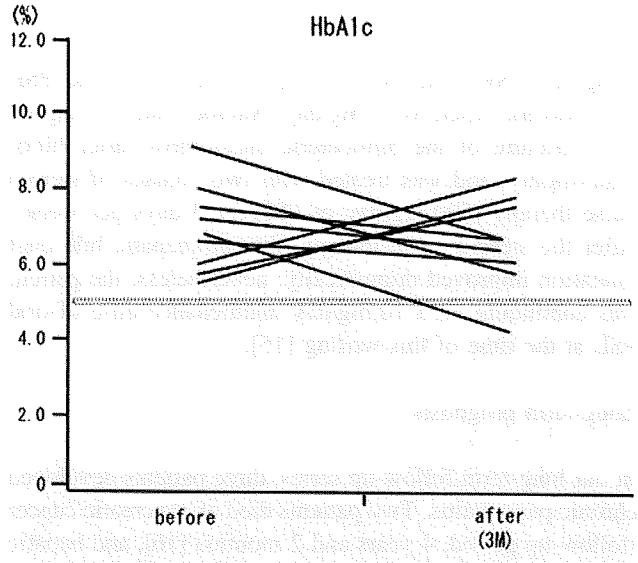

Fig. 5 Short-term follow up of HbA1c levels. The short-term effect of PSL treatment on diabetes mellitus (DM) associated with AIP in all 52 patients was evaluated after 3 months' steroid therapy. Steroid therapy improved HbA1c levels in 6 of 9 patients with AIP but worsened them in the remaining 3 patients

suggest that steroid treatment may induce pancreatic atrophy in patients diagnosed with AIP.

In general, the endocrine and exocrine dysfunction in AIP patients has been reported to be reversible with PSL therapy. In our short-term study, we observed the recovery of pancreatic function, as indicated by the BT-PABA test. In our long-term series, pancreatic exocrine function was improved in four of ten patients by steroid therapy, while it progressively decreased in the other patients irrespective of

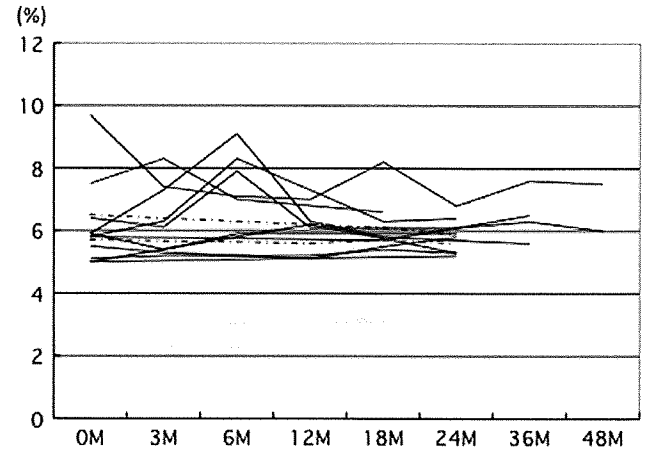

Fig. 6 Long-term follow up of HbA1c levels. In one patient with DM, the condition improved with insulin therapy. Two patients experienced a transient loss of glycemic control after PSL therapy, but their HbA1c values subsequently returned to the pretreatment levels. *Dashed lines* show the results for untreated patients

whether or not they had received steroid therapy. Moreover, three of ten patients showed serum trypsin levels below the normal limit; these trypsin levels were not changed by steroid therapy. These findings suggest that steroid therapy, administered early, may prevent the decrease of pancreatic exocrine function.

It is also known that chronic pancreatitis and pancreatic stones may develop in patients with AIP [17]. In our patient group, pancreatic stone was observed in 1 patient after 12 months of PSL therapy. In 3 of the 21 (14.3%) AIP patients, chronic pancreatitis developed, with irregular dilation of the main pancreatic duct; 1 patient had had PSL therapy and 2 had not. Takayama et al. reported that pancreatic stone formation was detected in 19% of 42 AIP patients during follow up [18] and more frequently (55%) in relapsing AIP patients. These findings, when taken together, suggest that relapse may be a risk factor for chronic pancreatitis and pancreatic stone formation, due to obstruction of the pancreatic duct, as well as some immunological mechanism. In other words, AIP may have the potential to progress to chronic pancreatitis.

It has been reported that pancreatic endocrine function is frequently decreased in AIP patients, and PSL therapy is occasionally effective for attenuating this dysfunction [17, 19, 20]. In our patients, HbA1c levels improved in two patients after 3 months' administration of PSL, but worsened in one patient. However, in our long-term follow-up series, DM was not necessarily improved by PSL therapy. One patient showed improved glycemic control with insulin therapy. In the other AIP patients, HbA1c values were not changed by PSL therapy, or the PSL therapy did not bring about any improvement of DM in these patients. These findings suggest that the efficacy of steroid treatment may be heterogeneous, and may be dependent on the stage and

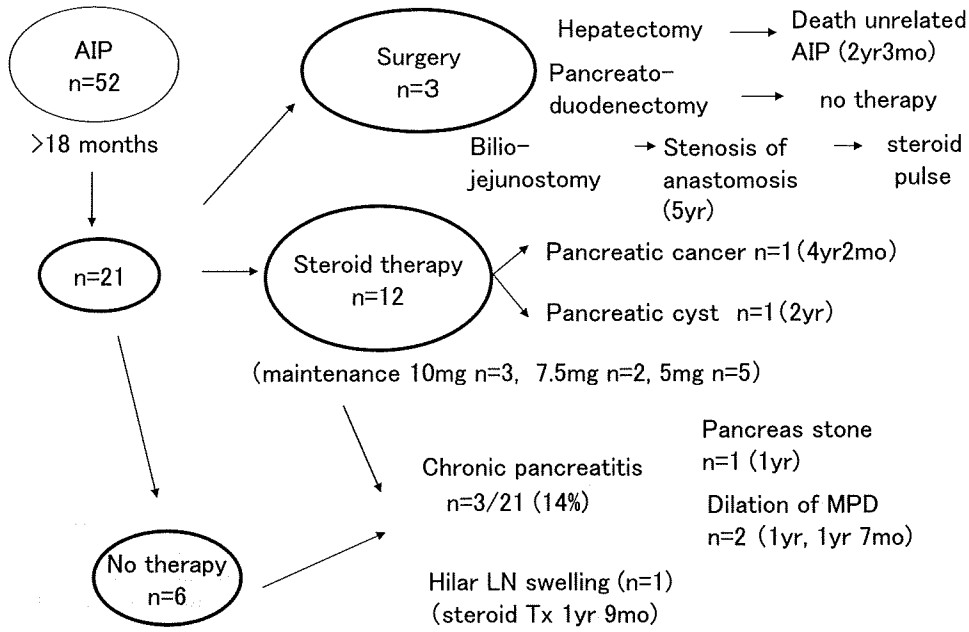

Fig. 7 Long-term prognosis of AIP. Of the 52 patients with AIP, 21 were followed up for 18 months or more (mean period, 40.8 months; range, 18–130 months). Three patients underwent surgical treatment: these patients underwent left-lobe hepatectomy, pancreatoduodenectomy, and bilio-jejunostomy when AIP or associated conditions were misdiagnosed as cholangioductal cancer, pancreatic cancer, and mass-forming pancreatitis, respectively. Twelve patients received steroid therapy (oral PSL), and 6 patients were observed without treatment. The initial dose of oral PSL was 30 mg/day, decreasing by 5 mg/day every 2 weeks. The maintenance doses of PSL were 10 mg/day in 3 patients, 7.5 mg/day in 2, and 5 mg/day in 5; maintenance therapy with oral PSL was discontinued in 2 of the patients. In our follow-up series, 3 patients developed chronic pancreatitis. Two patients died of pancreatic cancer (follow-up period 4 years and 2 months), and hepatic failure unrelated to AIP (2 years and 3 months). *Periods shown in parentheses* are periods after completion of treatment. *MPD* Main pancreatic duct, *LN* lymph node, *Tx* treatment, *yr* year(s), *mo* months

activity of AIP, probably being more effective in the early active phase.

Pancreatic and extrapancreatic lesions often relapse in patients treated with steroids. In our series, pancreatic or extrapancreatic lesions relapsed in 2 of the 21 patients. In 1 patient, swelling of the hilar lymph nodes occurred during PSL maintenance therapy (5 mg/day), and the lymphadenopathy disappeared with an increase in the PSL dose (10 mg/day). In the other patient, stricture of the bilioenteric anastomosis occurred 60 months after bilio-jejunostomy, and was attenuated with steroid therapy. The recurrence rate of AIP was reported to be about 17% [15]. Hirano et al. [17] reported that about 2 years after the diagnosis of AIP, 16 of 23 patients without steroid therapy (70%) developed unfavorable events, including obstructive jaundice in 2, while 6 of 19 patients (32%) with steroid therapy developed unfavorable events, including interstitial pneumonia in 3, and recurrence of obstructive jaundice in 3. Thus, it seems AIP patients who relapse during maintenance therapy should be re-treated with high-dose steroid therapy.

The long-term clinical course of AIP still remains unclear. Until recently, it has been thought that AIP has a good prognosis that responds well to steroid therapy, and it was unclear whether or not prolonged AIP was a risk factor for the development of malignancy. Nishino et al. reported two cases where the patients developed malignancy (gastric cancer and rectal cancer) during steroid therapy [18]. Kamisawa et al. reported two cases of malignancy (pulmonary cancer and esophageal cancer) [19]. We experienced pancreatic cancer in one of our patients after 50 months of steroid therapy. Inoue et al. also reported a case of pancreatic cancer in patients with AIP [21]. As a matter of fact, there is no evidence that AIP is a risk factor for pancreatic cancer. Generally, AIP is found commonly among the elderly, indicating that age may be a dominant factor. Although these observations are not conclusive, they may suggest that patients with AIP should be observed closely with regard to the development of pancreatic and other cancers.

In conclusion, AIP treated with PSL generally has a good long-term outcome. However, some patients with AIP may develop pancreatic stones and the conventional type of chronic pancreatitis, as well as pancreatic malignancy. Further study is needed to elucidate the relationship between AIP and pancreatic cancer.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Analysis of regulatory T cells and IgG4-positive plasma cells among patients of IgG4-related sclerosing cholangitis and autoimmune liver diseases

Masanori Koyabu · Kazushige Uchida · Hideaki Miyoshi · Yutaku Sakaguchi · Toshiro Fukui · Hiroki Ikeda · Makoto Takaoka · Junko Hirohara · Akiyoshi Nishio · Yoshiko Uemura · Shinji Uemoto · Kazuichi Okazaki

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Abstract
Objectives Patients with autoimmune pancreatitis (AIP) characteristically show elevated serum levels of immunoglobulin G4 (IgG4) and abundant infiltration of IgG4-positive plasmacytes in the involved organs. The most common involved organ showing extrapancreatic lesions is the bile duct, which exhibits sclerosing cholangitis (SC). However, the role of IgG4 in the development of IgG4-related SC (IgG4-SC) remains unclear. To clarify the role of IgG4 in IgG4-SC, we have performed an immunohistochemical analysis of the bile duct.
Methods Laboratory and immunohistochemical findings of liver biopsy specimens obtained from patients with IgG4-SC, primary sclerosing cholangitis (PSC), autoimmune hepatitis (AIH), and primary biliary cirrhosis (PBC) were compared. The biopsy specimens were first stained with anti-IgG1, anti-IgG4, and anti-Foxp3 (forkhead box P3) antibodies, and the ratio of IgG4-, IgG1-, and Foxp3-positive cells, respectively, to infiltrated mononuclear cells (IgG4/Mono, IgG1/Mono, Foxp3/Mono) was assessed.
Results The ratio of IgG4/IgG1-positive plasma cells was significantly higher in specimens obtained from patients with IgG4-SC than in those from patients with PSC, AIH, and PBC. The Foxp3/Mono ratio in patients with PBC was significantly higher than that in patients with IgG4-SC and PSC. In patients with IgG4-SC, the number of Foxp3-positive cells was significantly correlated with the number of IgG4-positive cells; in the other patient groups, there was no correlation.
Conclusions The IgG4/IgG1 ratio in the liver may be a useful marker for differential diagnosis of IgG4-SC and PSC. In IgG4-SC, abundant infiltration of regulatory T cells (Tregs) may affect the switching of B cells to IgG4-producing plasmacytes, and there is a possibility that the function of Tregs is different in IgG4-SC and other liver diseases (PSC, AIH, and PBC).

Keywords Autoimmune pancreatitis · IgG4 · IgG4-related diseases · Regulatory T cell · Sclerosing cholangitis

M. Koyabu · K. Uchida · H. Miyoshi · Y. Sakaguchi · T. Fukui · H. Ikeda · M. Takaoka · J. Hirohara · A. Nishio · K. Okazaki (✉)
Division of Gastroenterology and Hepatology, The Third Department of Internal Medicine, Kansai Medical University, 10-15 Fumizono, Moriguchi, Osaka 570-8507, Japan
e-mail: okazaki@hirakata.kmu.ac.jp

Y. Uemura
Department of Clinical Sciences and Laboratory Medicine, Kansai Medical University, Moriguchi, Osaka, Japan

S. Uemoto
Division of Hepato-Pancreato-Biliary Surgery and Transplantation, Department of Surgery, Graduate School of Medicine, Kyoto University, 54 Shogoin Kawara-cho, Sakyo-ku, Kyoto, Kyoto 606-8507, Japan

Introduction

Sarles et al. [1] reported the first case of pancreatitis with hypergammaglobulinemia in 1961. This was followed by the occasional report of the coexistence of pancreatitis with other autoimmune diseases. In 1995, Yoshida et al. [2] proposed the concept of autoimmune pancreatitis (AIP): a clinical condition in which patients show a diffusely enlarged pancreas, narrowing pancreatogram, increased serum immunoglobulin (Ig)G, presence of autoantibodies, fibrotic changes with lymphocytic infiltration, and steroidal

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efficacy. Many AIP cases have since been reported by Japanese investigators, and AIP has been accepted as a new clinical entity [3, 4]. Patients with AIP often show discomfort in the epigastrium, obstructive jaundice due to bile-duct stricture, and diabetes mellitus. It is more common among middle-aged and elderly men. Patients with AIP often also have extrapancreatic lesions, such as biliary lesions, sialoadenitis, retroperitoneal fibrosis, enlarged celiac and hilar lymph nodes, chronic thyroiditis, and interstitial nephritis [5–8], which suggests that AIP may be a systemic disorder. In 2001, Hamano et al. [9] reported that patients with AIP have high serum IgG4 concentrations. In 2006, Kamisawa et al. [10] proposed the existence of IgG4-related sclerosing disease, which has recently led to immunoglobulin G4-related sclerosing cholangitis (IgG4-SC) being recognized as a disease entity characterized by sclerosing inflammation with abundant IgG4-positive plasma cells; most cases reported to date have been associated with AIP [11]. The cholangiographic findings on radiological images of patients with IgG4-SC and primary sclerosing cholangitis (PSC) are similar [8, 12]. However, while IgG4-SC responds dramatically to steroid therapy, PSC, even in patients receiving medication, remains a progressive disease that involves the intra- and extra-hepatic bile ducts and leads to biliary cirrhosis. Therefore, it is important that the clinician is able to distinguish between IgG4-SC and PSC.

Attention has recently focused on the relationship between various autoimmune diseases and regulatory T cells (Tregs), which are present in human peripheral blood [13–15], intestinal lamina propria [16], and the thymus [15]. $CD4^{+}CD25^{high}$ Tregs, which are characterized by the expression of a specific transcription factor, forkhead box P3 (Foxp3), play a key role in the autoimmune diseases. We have previously reported that increased numbers of $CD4^{+}CD25^{high}$ Tregs may influence IgG4 production and that naive Tregs may be involved in the pathogenesis of AIP [17]. However, the relationship between Tregs in the peripheral blood and the liver remains unclear. The aim of the study reported here was to clarify the role of IgG4 in IgG4-SC by comparing the immunohistochemical features of the liver in patients with IgG4-SC, PSC, autoimmune hepatitis (AIH), and primary biliary cirrhosis (PBC).

Patients and methods

Subjects

The patient cohort consisted of 16 IgG4-SC patients of the Kansai Medical University and its affiliated hospitals (16 patients untreated with corticosteroids; 15 men and one woman; mean age 63 years; range 31–81 years). Of these 16 patients, 14 had SC with AIP based on the Japanese clinical diagnostic criteria for AIP proposed by the Research Committee of Intractable Diseases of the Pancreas and accepted by both the Japanese Ministry of Health, Labor, and Welfare and the Japan Pancreas Society in 2006 [5] and on Asian criteria [18]. Two patients were diagnosed according to the Mayo criteria for IgG4-associated cholangitis [19].

We classified the IgG4-SC patients into two groups, intra-/extra-hepatic (intra-IgG4-SC) and extra-hepatic (extra-IgG4-SC), based on the nature of the biliary strictures as characterized by endoscopic retrograde cholangiopancreatography (ERCP) (Fig. 1). In those patients with intra-IgG4-SC ($n = 8$), strictures were present in the intra-hepatic or in both the intra-hepatic and extra-hepatic bile ducts. In those patients with extra-IgG4-SC ($n = 8$), the strictures were present only in the extra-hepatic bile duct. In our study, we analyzed only the eight intra-IgG4-SC patients. Prior to biliary drainage, we performed an intraductal ultrasonography (IDUS) in all patients to evaluate thickening of the bile duct wall. The IDUS demonstrated

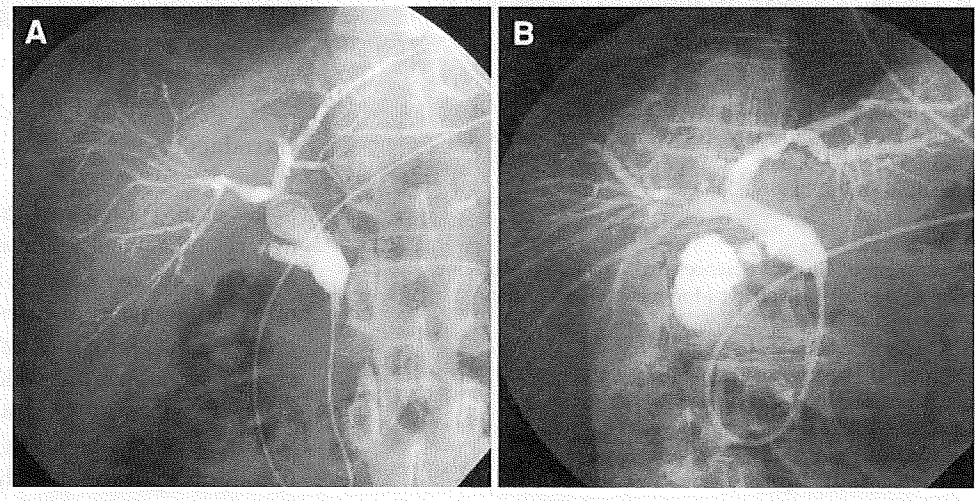

Fig. 1 Endoscopic nasobiliary drain (ENBD) cholangiogram of immunoglobulin G4-related sclerosing cholangitis (IgG4-SC). The IgG4-SC patients were classified into two groups (intra- and extra-IgG4-SC, respectively) based on the nature of the biliary strictures. **a** Stricture of the intra-hepatic and extra-hepatic bile duct (intra). **b** Stricture of the extra-hepatic bile duct (extra)

that it was the thickening of the bile-duct wall itself that caused the biliary strictures in all patients—and not extrinsic compression from inflammatory pancreatic tissue. We consequently diagnosed all patients with IgG4-SC.

The control group consisted of patients with PSC (n = 26; all patients treated with ursodeoxycholic acid; 15 men and 11 women; mean age 36 years; range 6–77 years) (Ludwig's stage 2, n = 4; stage 3, n = 8; stage 4, n = 14), AIH (n = 9; five patients untreated and four patients treated with corticosteroids; nine women; mean age 53 years; range 33–70 years), and PBC (n = 9; all patients untreated with ursodeoxycholic acid; nine women; mean age 53 years; range 35–67 years) (Table 1). All patients with AIH were diagnosed as definitely having AIH based on the scoring system established by the International Autoimmune Hepatitis Group and classified as having type 1 AIH [20, 21]. The diagnosis of PBC was based on internationally accepted criteria, and the antimitochondrial antibody status of each patient was verified [22]. Patients with overlap syndrome were excluded from this study. The diagnosis of PSC was based on typical cholangiographic and liver biopsy criteria [23]. Liver biopsy was performed in all IgG4-SC, AIH, and PBC patients and in three of the 26 PSC patients. In the remaining 23 PSC patients, a liver specimen was obtained during liver transplantation surgery. This study was approved by the Kansai Medical University's Ethics Committee.

Histopathology and immunohistochemistry

Formalin-fixed and paraffin-embedded specimens were prepared and used for histopathological and immunohistochemical studies. Sections measuring 4 μm were cut from each paraffin block and stained with hematoxylin and eosin, periodic acid–Schiff after diastase digestion, Azan–Mallory, reticulin, or orcein. The remaining material was used for immunohistochemical analysis. The immunostaining of IgG4 was performed using a monoclonal antibody for human IgG4 (ZYMED Laboratories, San Francisco, CA) and that of IgG1 and Foxp3 using the avidin–biotin complex (ABC) method with reagents obtained from Vector Laboratories (Burlingame, CA). The antibodies used to identify the inflammatory cells in the liver were the IgG1 antibody (Binding Site, Birmingham, UK) and Foxp3 (eBioscience, San Diego, CA). The deparaffinized sections were pretreated in ethylenediaminetetraacetic acid buffer (pH 8.0) in a pressure cooker at 100°C for 5 min. Following incubation with the first antibody at 4°C overnight, biotinylated rabbit anti-sheep serum IgG (Vector Laboratories) was used as the secondary antibody (sections for IgG1), and immunoreactive deposits were visualized with 3,3′-diaminobenzidine tetrahydrochloride. To correct for differences in the sizes of the portal tracts, we counted the numbers of immunohistochemically identifiable IgG1-, IgG4-, and Foxp3-positive cells and mononuclear cells contained within the portal tracts selected in each specimen under five different high power fields (hpf); two pathologists subsequently calculated the ratio between IgG1-, IgG4-, Foxp3-positive cells and infiltrated mononuclear cells in each case.

Table 1 Clinical profile and characteristics of the patient and control groups

Clinical profile	n	Sex (male/female)	Age (years)[a]
IgG4-SC	16	15/1	63 ± 3 (31–81)
Intra	8	7/1	59 ± 6 (31–75)
Extra	8	8/0	67 ± 3 (54–81)
PSC	26	15/11	36 ± 4 (6–77)*, **
AIH	9	0/9	53 ± 4 (33–70)
PBC	9	0/9	53 ± 4 (35–67)

IgG4-SC Immunoglobulin G4-related sclerosing cholangitis, *Intra* intra-hepatic or both intra-hepatic and extra-hepatic biliary strictures, *Extra* extra-hepatic biliary strictures, *PSC* primary sclerosing cholangitis, *AIH* autoimmune hepatitis, *PBC* primary biliary cirrhosis

* $p < 0.01$ vs. AIH and PBC; ** $p < 0.001$ vs. IgG4-SC, intra- and extra-hepatic

[a] Values are given as the mean ± standard error of the mean (SEM), with the range in parenthesis

Statistical analysis

For all studies, data are expressed as mean ± standard error of the mean (SEM). Differences were analyzed using the nonparametric Mann–Whitney rank test and Fisher's exact test, where p values <0.05 were considered to be significant.

Results

Patients profile

Patient age was significantly lower in the PSC group than in the other groups. There was one peak in the age distribution of the IgG4-SC patients between 60 and 70 years and two peaks in the age distribution of the PSC patients (one between 20 and 30 years, and the other between 40 and 50 years). Of the eight intra-IgG4-SC patients, seven were male; all AIH and PBC patients were female (Table 1).

Laboratory findings

The serum asparate aminotransferase and alanine aminotransferase values were elevated in all groups, with no difference in laboratory values among the groups. The

serum alkaline phosphatase level was significantly higher in the PSC group than in the AIH group, and the serum γ-glutamyl transpeptidase level was significantly higher in the PBC group than in the AIH group. The serum total bilirubin was significantly higher in the PSC group. The eosinophil level was significantly higher in the PBC and intra-IgG4-SC groups than in the PSC and AIH groups. There were no significant differences in the serum levels of IgA. The serum IgG4 values were elevated in patients with intra-IgG4-SC. Antinuclear antibodies were positive in 38% of the intra-IgG4-SC patients, 48% of the PSC patients, 78% of the AIH patients, and 22% of the PBC patients. Antimitochondrial antibodies were positive in 0% of the intra-IgG4-SC and PSC patients, 22% of the AIH patients, and 78% of the PBC patients (Table 2).

Immunohistochemical findings of IgG1 and IgG4

As shown in Figs. 2 and 3, the ratio of IgG4-positive plasma cells to infiltrated mononuclear cells (IgG4/Mono) was significantly higher in patients with intra-IgG4-SC (0.121 ± 0.069) than in those with PSC (0.02 ± 0.003; $p = 0.002$), AIH (0.013 ± 0.004; $p = 0.0052$), and PBC (0.013 ± 0.002; $p = 0.0052$; Fig. 3a). The ratio of IgG1-positive plasma cells to infiltrated mononuclear cells (IgG1/Mono) was significantly lower in patients with intra-IgG4-SC (0.041 ± 0.009) than in those with AIH (0.084 ± 0.014; $p = 0.0161$; Fig. 3b). The ratio of IgG4/Mono to IgG1/Mono (IgG4/G1) was significantly higher in patients with intra-IgG4-SC (3.084 ± 1.824) than in those with PSC (0.424 ± 0.068; $p = 0.0018$), AIH (0.169 ± 0.042; $p = 0.004$), and PBC (0.196 ± 0.02; $p = 0.0044$; Fig. 3c). The intra-IgG4-SC patients were found to have an IgG4/G1 ratio >1.

Immunohistochemical findings of Foxp3

As shown in Figs. 4 and 5, patients with PBC has a significantly higher ratio of Foxp3-positive cells to infiltrated mononuclear cells (Foxp3/Mono) (0.042 ± 0.008) than those with intra-IgG4-SC (0.013 ± 0.006; $p = 0.0007$) and PSC (0.006 ± 0.001; $p < 0.0001$). Patients with AIH (0.027 ± 0.009) had a significantly higher Foxp3/Mono ratio than those with PSC ($p = 0.0016$). The Foxp3/Mono ratio was significantly higher in patients with intra-IgG4-SC than in those with PSC ($p = 0.0314$; Fig. 5, dotted line).

Correlation between the Foxp3/Mono and IgG4/Mono ratios in patients with IgG4-SC

The Foxp3/Mono and IgG4/Mono ratios were found to be positively correlated in the group of patients with intra-IgG4-SC ($R = 0.75$), but there was no correlation found in the other patient groups (PSC, $R = 0.05$; AIH, $R = 0.07$; PBC, $R = 0.11$; Fig. 6).

Table 2 Laboratory findings of AIP-SC, PSC, AIH, and PBC patients

Laboratory parameters[a]	Intra-IgG4-SC ($n = 8$)	PSC ($n = 26$)	AIH ($n = 9$)	PBC ($n = 9$)	Statistical significance[b]
AST (13–35 U/l)	57 ± 15	135 ± 14	152 ± 69	144 ± 96	NS
ALT (5–35 U/l)	52 ± 17	93 ± 16	185 ± 77	132 ± 91	NS
ALP (107–340 U/l)	837 ± 313	1284 ± 228*	360 ± 57	626 ± 66	$p < 0.05$
γ-GTP (11–64 U/l)	242 ± 59	211 ± 34	136 ± 31	330 ± 99*	$p < 0.05$
T-Bil (0.2–1.2 mg/dl)	2.7 ± 1.4	15.8 ± 2.5***	2.5 ± 1.1	1.0 ± 0.3	$p < 0.001$
ALB (3.8–5.0 g/dl)	3.3 ± 0.2	3.2 ± 0.1	3.6 ± 0.2†	3.8 ± 0.1**, †	$p < 0.05$, 0.01
Eosinophils (%)	6.2 ± 0.9*	2.7 ± 0.7	1.0 ± 0.3	9.3 ± 2.5***	$p < 0.05$, 0.001
IgM (33–190 mg/dl)	97 ± 22 (6)	154 ± 24 (17)	316 ± 126	558 ± 132**, ***	$p < 0.01$, 0.001
IgA (110–410 mg/dl)	307 ± 85 (6)	509 ± 112 (16)	281 ± 44 (8)	280 ± 44 (8)	NS
IgG (870–1700 mg/dl)	2585 ± 345 (7)*	1780 ± 156 (20)	2323 ± 457	1685 ± 168	$p < 0.05$
IgG4 (4.8–105 mg/dl)	556 ± 224	–	–	–	–
ANA-positive	38% (3/8)	48% (10/21)	78% (7/9)*	22% (2/9)	$p < 0.05$
AMA-positive	0% (0/6)	0% (0/14)	22% (2/9)	78% (7/9)***	$p < 0.001$

Values are given as the mean ± SEM

[a] *AST* Aspartate aminotransferase, *ALT* alanine aminotransferase, *ALP* alkaline phosphatase, *γ-GTP* γ-glutamyl transpeptidase, *T-Bil* total bilirubin, *ALB* albumin, *ANA* antinuclear antibody, *AMA* antimitochondrial antibody

[b] Significance: *NS* not significant; ALP, *$p < 0.05$ vs. AIH; γ-GTP, *$p < 0.05$ vs. AIH; T-Bil, ***$p < 0.001$ vs. intra-IgG4-SC, AIH, and PBC; ALB, **$p < 0.01$ vs. PSC, †$p < 0.05$ vs. intra-IgG4-SC, †$p < 0.05$ vs. PSC; eosinophils, ***$p < 0.001$ vs. PSC and AIH, *$p < 0.05$ vs. AIH and PSC; IgM, **$p < 0.01$ vs. intra-IgG4-SC, ***$p < 0.001$ vs. PSC; IgG, *$p < 0.05$ vs. PSC and PBC; ANA, *$p < 0.05$ vs. PBC; AMA, ***$p < 0.001$ vs. intra-IgG4-SC, PSC, and AIH

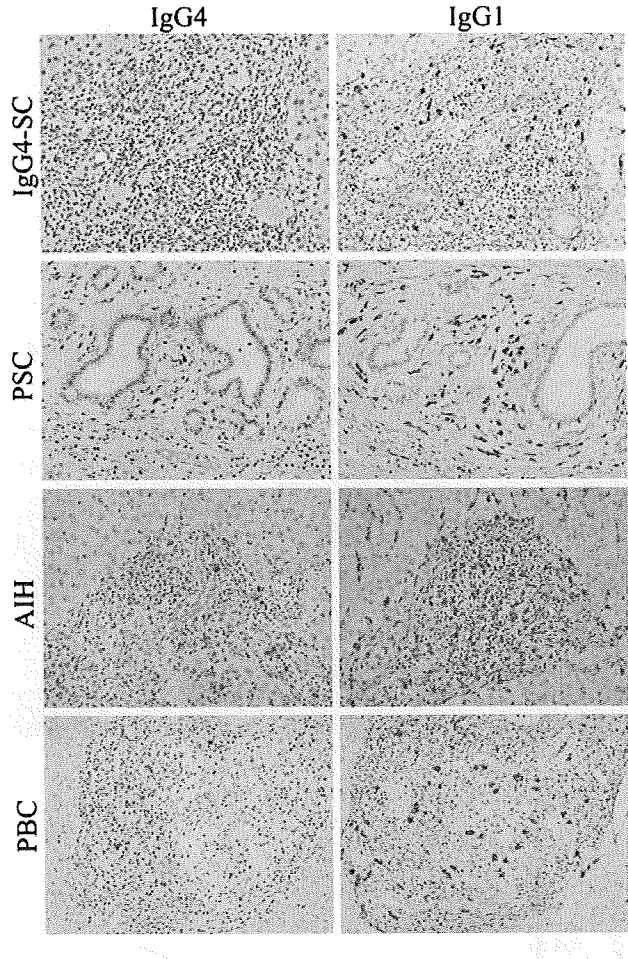

Fig. 2 Immunostaining of formalin-fixed, paraffin-embedded liver sections obtained from patients with IgG4-SC, primary sclerosing cholangitis (*PSC*), autoimmune hepatitis (*AIH*), and primary biliary cirrhosis (*PBC*). Representative liver sections of IgG4-SC, PSC, AIH, and PBC patients show immunostaining of IgG4 and IgG1. The density of IgG4-positive cells is higher than that of IgG1-positive cells in the IgG4-SC liver sections. In the liver sections of PSC, AIH and PBC patients, the density of IgG1-positive cells is higher than that of IgG4-positive cells (×200)

Comparison between immunohistochemical findings in the liver specimens of AIH patients with and without steroid therapy

In terms of the Foxp3/Mono, IgG4/Mono, IgG1/Mono, and IgG4/G1 ratios, AIH patients not receiving steroid therapy showed an increasing tendency relative to those receiving steroid therapy, but the differences were not significant.

Discussion

In general, AIP is currently accepted to be a unique distinctive disease in which histopathological findings show an abundant infiltration of IgG4-positive plasma cells and fibrosis, a condition denoted as lymphoplasmacytic

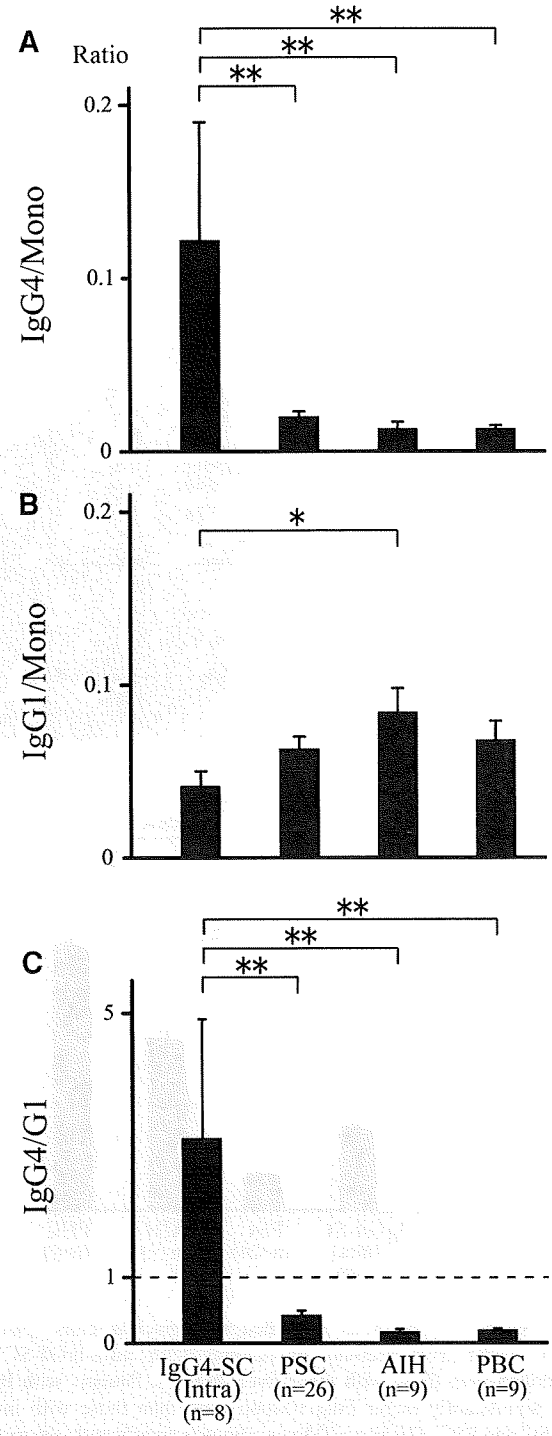

Fig. 3 Ratios of IgG4-positive plasma cells/infiltrated mononuclear cells (*IgG4/Mono*), IgG1-positive plasma cells/infiltrated mononuclear cells (*IgG1/Mono*), and IgG4/Mono to IgG1/Mono (*IgG4/G1*) in the liver of patients with intra-IgG4-SC in comparison with those with other liver diseases. **a** The IgG4/Mono ratio was significantly higher in patients with intra-IgG4-SC than in those with other liver diseases. **b** The IgG1/Mono ratio was significantly lower in patients with intra-IgG4-SC than in those with AIH. **c** The IgG4/G1 was significantly higher in patients with intra-IgG4-SC than in those with other liver diseases. Data are expressed as the mean ± standard error of the mean (SEM). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, *NS* not significant

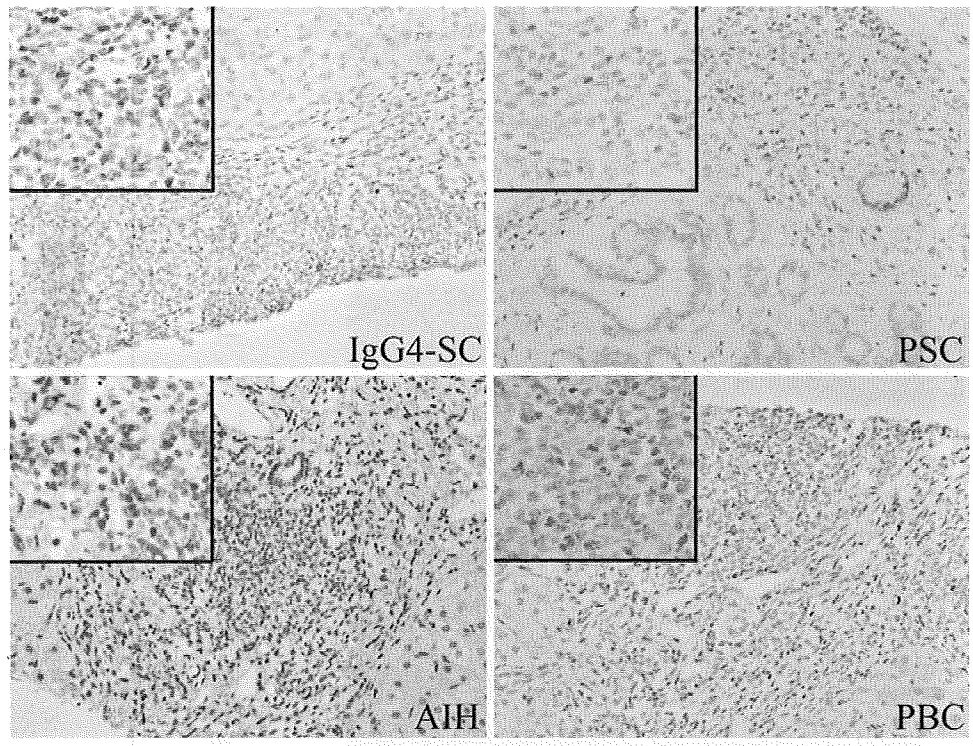

Fig. 4 Immunostaining of forkhead box P3 (Foxp3) in liver sections from IgG4-SC, PSC, AIH, and PBC patients. Foxp3-positive cells were found to be scattered among the lymphoid infiltrates in the portal tracts of the IgG4-SC, AIH, and PBC sections but among those of the PSC sections (×200)

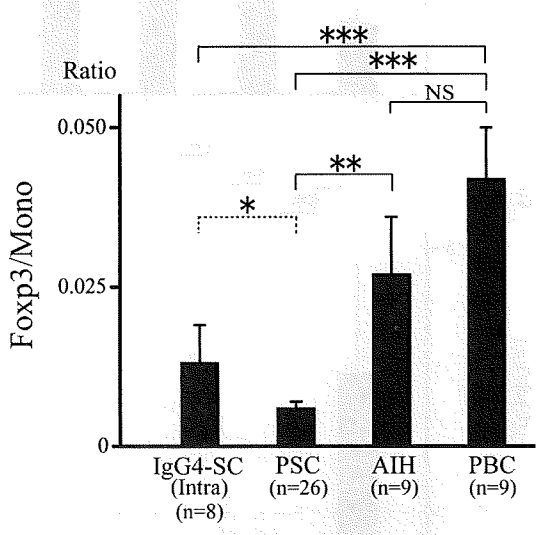

Fig. 5 Ratios of Foxp3 to infiltrated mononuclear cells (*Foxp3/Mono*) in the liver sections of patients with intra-IgG4-SC in comparison with those with other liver diseases. Patients with PBC had a significantly larger Foxp3/Mono ratio than those with intra-IgG4-SC and PSC. The Foxp3/Mono ratio was significantly decreased in PSC patients compared with those with intra-IgG4-SC, AIH, and PBC. *Dotted line* Comparison between intra-IgG4-SC and PSC patients. Data are expressed as the mean ± SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, *NS* not significant

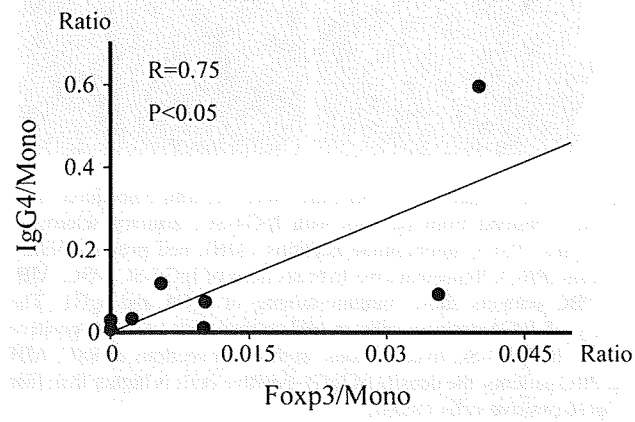

Fig. 6 Correlation between the Foxp3/Mono and IgG4/Mono ratio in patients with intra-IgG4-SC. The Foxp3/Mono and IgG4/Mono ratios are positively correlated in the intra-IgG4-SC patients ($R = 0.75$), but there is no correlation in the other patient groups

sclerosing pancreatitis (LPSP), and the clinical manifestations dramatically respond to steroid treatment. In addition to pancreatic lesions, patients with AIP have occasional extrapancreatic lesions, such as SC, sclerosing sialoadenitis, and retroperitoneal fibrosis (all of which are similar to LPSP). The novel features of this systemic disease have been proposed to be IgG4-related sclerosing disease [10], systemic IgG4-related plasmacytic disease [24], and IgG4-positive multi-organ lymphoproliferative syndrome [25]. Among these, IgG4-SC is the most common and serious extrapancreatic lesion resulting in obstructive jaundice. Prior to the concept of AIP being established, IgG4-SC was often misdiagnosed as PSC complicating chronic pancreatitis. Therefore, differential diagnosis between IgG4-SC and PSC is important. Although IgG4-SC is usually associated with pancreatic lesions, some patients with IgG4-SC have no apparent pancreatic changes or other organ

involvement [26, 27]. The cholangiographic findings in patients with IgG4-SC and PSC have been compared and summarized in a number of studies [12, 28, 29]; the following are only found in PSC patients: a band-like stricture, a beaded appearance, a pruned-tree appearance, and a diverticulum-like formation. Long stenosis, segmental stricture, and a long stricture with prestenotic dilatation are significantly more common in IgG4-SC patients. A shaggy appearance and stricture of the hilar region are occasionally present in both groups. Histological studies have revealed that IgG4-SC patients have fibrosis with lymphoplasmacytic and eosinophil infiltration with mild fibrosis and a significantly increased number of IgG4-positive plasma cells than PSC patients [30, 31]. In one recent study, the immunohistochemical analysis of the liver biopsy specimens revealed that IgG4-positive plasma cell infiltration was significantly more severe in IgG4-SC than in PSC patients [28, 32]. Our immunohistochemical findings on IgG4 are consistent with the results of these earlier studies. However, we did not observed abundant IgG4-positive plasma cell infiltration in all our cases of IgG4-SC. In our study, we classified IgG4-SC patients into two groups based on the intra-/extra-hepatic (intra-IgG4-SC) or extra-hepatic (extra-IgG4-SC) nature of the biliary strictures. The infiltration of IgG4-positive plasma cells was more severe in patients with intra-IgG4-SC than in those with extra-IgG4-SC (data not shown). In terms of IgG4 serum levels, there were no significant differences between intra- (556 ± 224; $n = 8$) and extra-IgG4-SC (341 ± 61; $n = 8$). Moreover, IgG4-positive plasma cell infiltration in both classes of IgG4-SC was high compared to that of IgG1-positive plasma cell infiltration, whereas in other liver diseases (PSC, AIH, PBC), IgG1-positive plasma cell infiltration was higher than that of IgG4. Taken together, our study shows that the ratio of IgG4/Mono to IgG1/Mono (IgG4/G1) in intra-IgG4-SC patients was significantly higher than that in patients with other liver diseases (PSC, AIH, and PBC). In recent studies, IgG4-associated AIH was differentiated from other recognized types of AIH [33, 34]. Chung et al. [34] reported that patients with IgG4-associated AIH showed increased IgG serum levels and a marked response to prednisolone therapy. In our study, one of the nine AIH patients showed infiltration of abundant IgG4-positive plasma cells in the liver (>10 IgG4-positive plasma cells/hpf) and increased serum levels of IgG (2886 mg/dl).

In our study, we also examined the local infiltration of $Foxp3^+$ Tregs in the liver of intra-IgG4-SC, AIH, PSC, and PBC patients because $Foxp3^+$ Tregs have been reported to be involved in the development of various autoimmune diseases. Several recent studies have demonstrated the presence of $CD4^+CD25^{high}$ Tregs in patients with autoimmune liver diseases, such as AIH and PBC [35–39], but these results are still open to discussion in terms of PBC [35–37]. Lan et al. [35] recently reported a decrease in the level of Tregs in PBC patients and suggested that Tregs may play a role in the loss of immune tolerance in PBC; however, other investigators [36, 37] have reported a relative increase of Tregs in PBC patients. Sasaki et al. [36] reported that the level of Foxp3, interleukin (IL)-10, and transforming growth factor beta (TGF-β) mRNA expression was higher in the livers of PBC patiens than in normal healthy livers and that the amount of infiltrating $Foxp3^+$ Tregs in portal tracts paralleled the degree of portal inflammation. In contrast, the level of $CD4^+CD25^+$ Tregs suppressing the effector Th1 and Th2 responses were decreased in peripheral blood samples from AIH patients [39]. Our data show a significantly increased infiltration of $Foxp3^+$ Tregs in the liver of intra-IgG4-SC, AIH, and PBC patients compared with PSC patients and a significantly increased number of $Foxp3^+$ Tregs in PBC patients than in those with intra-IgG4-SC and PSC. In addition, we found that the number of infiltrated Foxp3-positive cells was positively correlated with the number of IgG4-positive cells in intra-IgG4-SC patients, but not in those with other liver diseases (PSC, AIH, and PBC). Data obtained in previous studies showed that the level of $Foxp3^+$ Tregs decreased in the liver of PBC patients as the histological stage of the disease advanced [35–37]. Of our 26 cases of PSC, 23 liver specimens were obtained during liver transplantation surgery, indicating that the histological stages in our cases were advanced. Further studies are necessary in order to be able to draw a reliable conclusion on the relationship between $Foxp3^+$ Tregs and PBC because there is a possibility that staging of PSC may affect the severity of infiltration of Tregs similar to that observed in PBC.

We previously reported that circulating Th1 type $CD4^+$ T cells, but not Th2 type $CD4^+$ T cells [40], and $CD4^+CD25^{high}$ Tregs were increased in the peripheral blood of AIP patients [17]. Recent studies of immune tolerance and allergy show that high-dose antigen exposures can cause both immune deviations of the Th2 response in favor of a Th0/Th1 and the generation of IL-10- and TGF-β-producing Tregs. During high-dose antigen exposures, the activation and/or maintenance of the usual Th2 T cell response is inhibited. Additionally, IL-10 induces preferential switching of the B cell response in favor of producing IgG4 antibodies, and possibly IgA antibodies, under the influence of TGF-β [41]. $CD4^+CD25^+$ Tregs also produce IL-10 to educate antigen presenting cells [42]. Therefore, increased Treg levels may correlate with the production of IL-10 in the involved organs, which in turn may influence the switching of B cells to IgG4-producing plasmacytes and the production of serum IgG4. We have previously reported that serum levels of IL-10 and TGF-β in AIP patients were not different from those in healthy and

other disease controls (alcoholic and idiopathic chronic pancreatitis patients) [17]. In other experiments, there was no difference in the serum levels of TGF-β among patients with PSC [43], PBC [44], and healthy controls but serum TGF-β levels in AIH patients were higher than those in healthy controls [45]. Zen et al. [11] reported that Tregs producing IL-10 and TGF-β infiltrated the liver of patients with IgG4-SC and that Foxp3-positive cells were lower in PSC patients than in IgG4-SC patients. However, different from IgG4-SC, our data suggest that such a mechanism is unlikely in AIH or PBC. It still remains unclear why there is difference in the relationship between IgG4-positive cells and Tregs in patients with IgG4-SC, PSC, AIH, and PBC. There are at least two possibilities explaining these differences: (1) it may be due to an originally different subpopulation of Tregs [46–49]; (2) it may be due to different activity of Tregs, such as acting Tregs and resting Tregs [50]. In contrast to murine $Foxp3^+$ Tregs, human $Foxp3^+$ cells may not be functionally homogenous [46, 47]. In general, high amounts of IL-10-producing Tregs, which also produce TGF-β, are well known as type 1 regulatory (Tr1) cells. There is some evidence (based on CD25 expression on $CD4^+$Tr1 cells) in adult humans that constitutive $CD4^+CD25^+$ Tregs and inducible IL-10- and TGF-β-secreting Tr1 cells represent overlapping populations [41]. Furthermore, some $Foxp3^+$ cells are phenotypically naive (e.g., $CD45RA^+$), being present in cord blood as well as in the peripheral blood of adults, and suppressive in vitro [48], whereas other $Foxp3^+$ cells phenotypically resemble memory T cells (e.g., $CD45RA^-$) and possibly originate from peripheral memory $Foxp3^-CD4^+$ T cells [49], in which case they may use different suppressive mechanisms by secreting different immunosuppressive cytokines, such as IL-10 and TGF-β [51]. Taken together, our data may support a hypothesis that decreased naive Tregs may be involved in the pathogenesis of IgG4-SC, resulting in the activation of Th1 type immune responses, while a high-dose antigen (carbonic anhydarase II or lactoferrin, etc.) may induce $CD4^+CD25^{high}$ Tregs from the peripheral blood [52]. This mechanism correlates with the production of IL-10 switching B cells to IgG4-producing plasmacytes in the chronic active phase, resulting in the suppression of both Th2 and Th2 type immune cells [53].

In conclusion, the IgG4/G1 ratio may be another useful marker for the differential diagnosis between intra-IgG4-SC and PSC. We have also demonstrated that the infiltration of $Foxp3^+$ Tregs in the liver was significantly increased in the livers of patients with intra-IgG4-SC, AIH, and PBC relative to those of patients with PSC and that there is a possibility that the function of infiltrated $Foxp3^+$ Tregs is different in intra-IgG4-SC and other liver diseases (PSC, AIH, and PBC). Further studies are needed to clarify the real function of $Foxp3^+$ Tregs infiltrating into the liver of patients with intra-IgG4-SC and other autoimmune liver diseases as well as whether IL-10 or TGF-β is upregulated in the local microenvironment or not.

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