Table 1 Clinical characteristics of eight KT-S (−) patients

				Swollen glands				Serological test			Immunohistological test					
No.	*Age*	*Sex*	*Disease duration*	*LG*	*PG*	*SMG*	*SLG*	*LSG*	*IgG*	*IgG4 (mg dl^{-1})*	*Anti-SS-A*	*Anti-SS-B*	*Number of IgG4 + cells (/HPF)*	*Frequency of IgG4 + cells (%)*	*Other IgG4-RD*	*Treatment*	*Lesions of Recurrence*
1	67	M	1 M	–	–	U	–	–	1244	220	–	–	133	633	–	None	SMGs, LGs
2	59	F	3 Y	B	–	B	–	–	2236	452	...	...	85	82.5	AIP, RPF	PSL	–
3	64	F	6 M	–	–	B	–	–	2016	19.5	–	–	102	72.8	–	PSL	–
4	67	F	4 M	B	–	B	–	–	1223	232	–	–	112	76.3	–	None	–
5	56	F	3 M	–	–	U	–	–	961	24.9	...	...	137	65.2	–	None	–
6	59	M	5 M	–	–	U	–	–	ND	ND	–	–	121	61.7	–	None	–
7	57	F	4 M	...	...	U	–	–	1188	151	–	–	52	71.2	–	None	–
8	62	M	4 M	–	–	U	–	–	1682	ND	...	...	4	4.9	–	None	–

KT-S (−), Küttner tumour (KT) without sialolith; lacrimal gland; PG, parotid gland; SMG, submandibular gland; SLG, sublingual gland; LSG, labial salivary gland; B, bilateral; U, unilateral; IgG4-RD, IgG4-related disease; AIP, autoimmune pancreatitis; RPF, retroperitoneal fibrosis; PSL, prednisolone; –, negative; ND, not done; bold and italicised numbers indicate abnormal values.

Table 2 Comparison of clinical and laboratory findings between KT-S (+) and KT-S (−) patients

	KT-S (+) n = *46*	*KT-S (−)* n = *8*	*P value*
Mean age (years)	49.8 ± 20.4	61.3 ± 4.6	0.30649
Sex (Male:female)	20:26	2:6	0.44913
Lesion part of SMG (unilateral: bilateral)	46:0	5:3	0.00226*
Duration disease (months)	50.7 ± 70.0	7.8 ± 11.5	0.10007
Serum IgG4 (mg ml^{-1})	ND	183.2 ± 162.1	
IgG4-positive cells/IgG-positive cells (%)	3.0 ± 2.0	62.5 ± 24.3	0.00071†
IgG4 + cells (/HPF)	1.5 ± 1.3	93.2 ± 95.4	0.00022†
Complicated of other IgG4-RD	0% (46/46)	12.5% (1/8)	0.14815
Recurrence (%)	0% (0/46)	12.5% (1/8)	0.14815

KT-S (+), KT with sialolith; KT-S (−), KT without sialolith; HPF, high-power field; IgG4-RD, IgG4-related disease.
*Student's *t*-test.
†Fisher's test.

of the six KT-S (−) patients had high serum IgG4 levels (the IgG4 level was not determined in two KT-S (−) patients and all KT-S (+) patients). The average serum IgG4 level among the six KT-S (−) patients was 183.2 ± 162.1 mg ml^{-1}. No patients in the KT-S (+) group had a history of other IgG4-RD, while one of the eight KT-S (−) patients had other IgG4-RD (AIP and RPF). During follow-up, no KT-S (+) patients demonstrated recurrence, while one of the eight KT-S (−) patients demonstrated relapse of the bilateral swelling of the SMGs and expansion of the LGs. Two of the eight KT-S (−) patients were treated with prednisolone, and then, the swelling of the SMGs was immediately disappeared.

Histological findings in the SMGs

Representative sialographic findings in the SMGs of both KT-S (+) and KT-S (−) patients are shown in Figure 2. KT-S (+) showed strong non-IgG4 lymphocytic infiltration and severe widespread fibrosis. Seven of the eight KT-S (−) patients showed selective infiltration of IgG4-positive cells [IgG4-positive cells/IgG-positive cells >0.4 based on 'Diagnostic criteria for IgG4-related Mikulicz's disease' (Umehara *et al*, 2012b)] and severe cordlike fibrosis with formation of ectopic germinal centres (eGCs). In contrast, one of the eight KT-S (−) patients showed moderate widespread fibrosis and diffuse lymphocytic infiltration without eGCs and a very small number of IgG-positive and IgG4-positive cells. The frequency and number of IgG4-positive cells in the SMGs of KT-S (−) patients were significantly higher than those of KT-S (+) patients (Figure 3).

Discussion

Küttner tumour presents with sclerosis of the bilateral or unilateral SMGs as first described by Küttner *et al* (Küttner, 1896). In 1976, Seifert and Donath, (1977) demonstrated that KT could be histopathologically diagnosed by strong lymphocytic infiltration and fibrosis in the

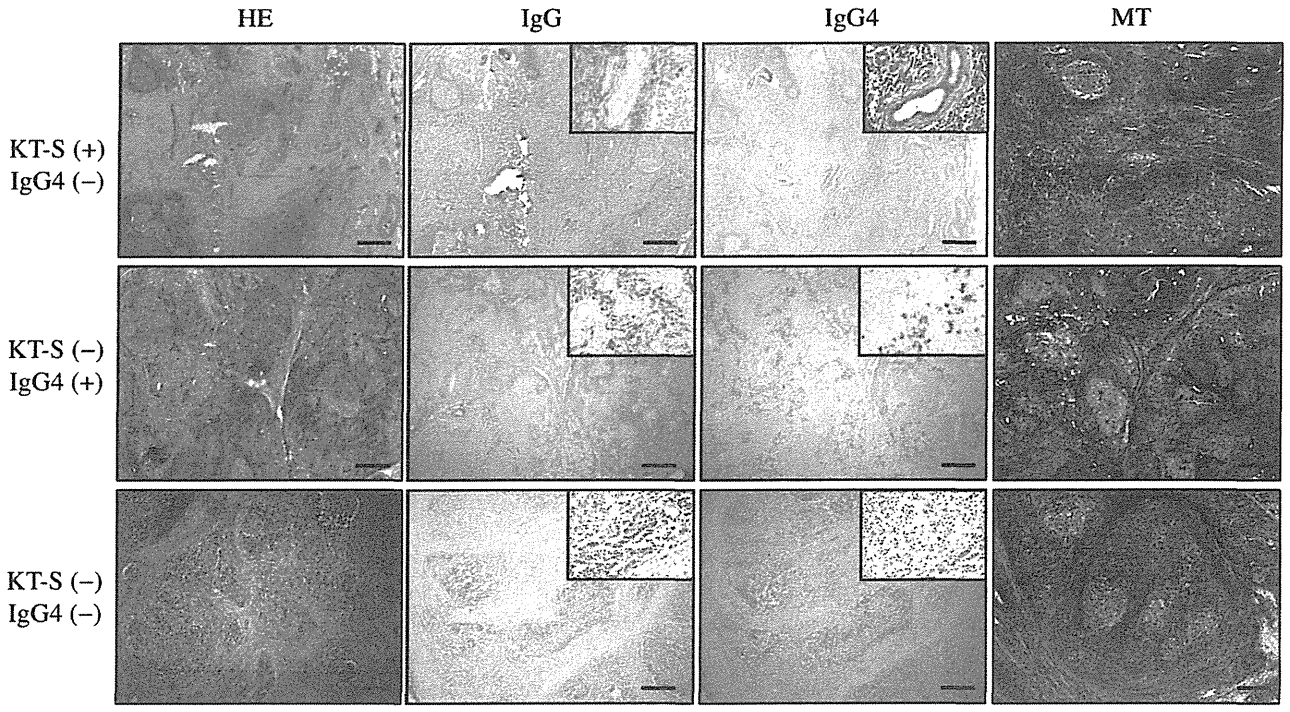

Figure 2 Histological findings in submandibular glands of patients with CS. IgG4 (+), IgG4-positive plasma cells/IgG-positive plasma cells >0.4; IgG4 (−), IgG4-positive plasma cells/IgG-positive plasma cells ≤0.4; MT, Masson's trichrome staining; Scale bars, 400 μm

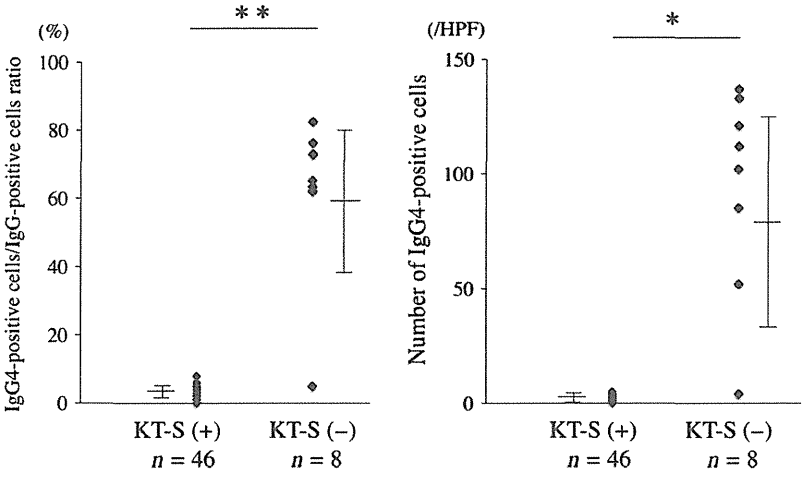

Figure 3 IgG4 production of patients with Küttner tumour (KT)-S (+) and KT-S (−). HPF, high-power field. The bar shows the mean value ± standard deviation (s.d.). $^{*}P < 0.05$, $^{**}P < 0.01$ (Mann–Whitney *U*-test)

SMGs either with or without sialolith. However, Kitagawa *et al*, (2005) reported that 12 patients diagnosed with chronic sclerosing sialoadenitis or KT showed high levels of IgG4 and strong infiltration of IgG4-positive plasma cells without sialolith. In addition, the complications in these patients frequently included other IgG4-RD. In the present study, we thus examined the involvement of IgG4 in CS regardless of the presence or absence of sialolith. We found that the histological findings in seven of the eight KT-S (−) patients in this study were consistent with the previous histological findings for KT, whereas the remaining KT-S (−) patient and all KT-S (+) patients showed strong lymphocytic infiltration without infiltration of IgG4-positive plasma cells. These results suggest that KT-S (−) is closely associated with IgG4-DS and KT-S (+) with non-IgG4-DS, so-called sialolithiasis. However, KT must be carefully diagnosed by SMG biopsy as well as the presence of sialolith, unilateral or bilateral swelling, and increased serum IgG4 levels because some KT-S (−) patients showed unilateral swelling of the SMG, normal serum IgG4 levels and slight infiltration of IgG4-positive cells. With regard to fibrosis, some of IgG4-RD including AIP and IgG4-tubulointerstitial nephritis often showed 'storiform fibrosis' in the lesions (Yoshita *et al*, 2012). However, these features were rarely seen in SMGs from IgG4-DS patients. As shown in Figure 2, there was no difference in the degree of fibrosis between KT-S(+) and KT-S(−) patients. These results suggest that evaluation of

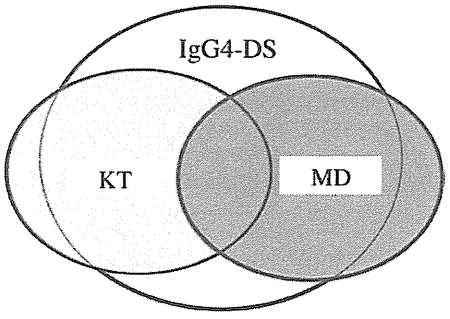

Figure 4 Clinical relevance of Küttner tumour, MD and IgG4-DS. MD, Mikulicz's disease; IgG4-DS, IgG4-related dacryoadenitis and sialoadenitis.

fibrosis pattern might be useful for diagnosis. Moreover, our previous studies demonstrated that IgG4-DS could be quickly and simply diagnosed using diagnostic criteria in conjunction with salivary gland imaging findings such as those of sonography (Shimizu *et al*, 2009; Moriyama *et al*, 2013).

In conclusion, this study suggests the clinical relevance of KT, MD and IgG4-DS (summarised in Figure 4), and as a result, KT could be considered as part of MD. However, we had selected just 54 cases diagnosed as KT or CS by SMG biopsies in this study period because many cases presenting with swelling of SMGs were diagnosed by clinical findings without biopsies. Therefore, evaluating greater numbers of patients with CS will help to elucidate the clinical and histological differences among these diseases, which might eventually lead to clarification of the pathogenesis of KT.

Acknowledgements

This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (26293430) and Takeda Science Foundation.

Author contributions

S Furukawa and M Moriyama defined the intellectual content. S Furukawa, M Moriyama and A Tanaka carried out the literature search. S Furukawa, T Maehara, JN Hayashida and Y Goto carried out the experimental studies. S Kawano, H Shiratsuchi, Y Ohyama, M Ohta and Y Imabayashi acquired the data. S Furukawa and T Kiyoshima analysed the data. S Furukawa, M Moriyama and A Tanaka carried out the statistical analysis. M Moriyama prepared and edited the manuscript.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Journal of Autoimmunity 51 (2014) 81–88

Contents lists available at ScienceDirect

Journal of Autoimmunity

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

Review

T helper subsets in Sjögren's syndrome and IgG4-related dacryoadenitis and sialoadenitis: A critical review☆

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ARTICLE INFO

Article history:
Received 10 July 2013
Accepted 15 July 2013

Keywords:
T helper subset
IgG4-related disease
IgG4-related dacryoadenitis and sialoadenitis
Sjögren's syndrome
Cytokine

ABSTRACT

IgG4-related disease (IgG4-RD) is a systemic disease characterized by the elevation of serum IgG4 and infiltration of IgG4-positive plasma cells in multiple target organs, including the pancreas, kidney, biliary tract and salivary glands. In contrast, Mikulicz's disease (MD) has been considered a subtype of Sjögren's syndrome (SS) based on histopathological similarities. However, it is now recognized that MD is an IgG4-RD distinguishable from SS and called as IgG4-related dacryoadenitis and sialoadenitis (IgG4-DS). Regarding immunological aspects, it is generally accepted that CD4+ T helper (Th) cells play a crucial role in the pathogenesis of SS. Since it is well known that IgG4 is induced by Th2 cytokines such as interleukin (IL)-4 and IL-13, IgG4-DS is speculated to be a unique inflammatory disorder characterized by Th2 immune reactions. However, the involvement of Th cells in the pathogenesis of IgG4-DS remains to be clarified. Exploring the role of Th cell subsets in IgG4-DS is a highly promising field of investigation. In this review, we focus on the selective localization and respective functions of Th cell subsets and discuss the differences between SS and IgG4-DS to clarify the pathogenic mechanisms of these diseases.

Sjögren's syndrome (SS) is an autoimmune disease characterized by lymphocytic infiltration into the salivary and lacrimal glands with concomitant autoantibody production and destruction of the glandular tissue. Patients typically experience symptoms of dry mouth (xerostomia) and dry eyes (keratoconjunctivitis sicca). Because of its characteristic lymphocytic infiltration and destruction of the salivary and lacrimal glands, SS is considered to be an ideal disease for studying patterns of cytokine production at the site of organ-specific autoimmune damage [1]. SS occurs alone as primary SS, or as secondary SS when underlying other connective tissue diseases [2]. Immunohistochemical studies demonstrated that the salivary glands are predominantly infiltrated by CD4+ T helper (Th) cells at an early stage of SS, and these cells are therefore thought to play a crucial role in the induction and/or maintenance of the disease [3]. In advanced stage, B cells predominate and these infiltration extends to occupy the acinar epithelium and further progress to hypergammaglobulinemia and B cell lymphoma [4]. Recent studies have suggested a central role of the epithelium in orchestrating the immune reaction by expressing HLA antigens, adhesion and costimulatory molecules, cytokines, and chemokines. Therefore, SS has been proposed as an etiological term "autoimmune epithelitis" [4–7], and it is of interest to examine the involvement of interaction between CD4+ Th cells and the epithelium in the initiation and progression of the disease process. Th cell populations comprise functionally distinct subsets characterized by specific patterns of cytokines and transcription factors. At least six Th subsets exist: Th0, Th1, Th2, Th17, regulatory T (Treg), and follicular helper T (Tfh) cells [8], which are suggested to be involved in the pathogenesis of SS [9–12].

On the other hands, Mikulicz's disease (MD) has been considered to be a subtype of SS based on histopathological similarities between the two diseases [13]. However, MD has a number of differences compared with typical SS including: 1) difference of gender distribution (MD occurs in both men and women, while SS occurs mainly in women); 2) persistent enlargement of lacrimal and salivary glands; 3) normal or mild salivary secretion dysfunction; 4) good responsiveness to corticosteroid treatment; 5) hypergammaglobulinemia and low frequency of anti SS-A and SS-B antibodies by serological analyses; and 6) multiple GC formation in

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http://dx.doi.org/10.1016/j.jaut.2013.07.007

glandular tissue (Table 1). Previously, we reported that SS was characterized by periductal lymphocytic infiltration with atrophy or severe destruction of the acini, while MD showed non-periductal lymphocytic infiltration with hyperplastic GCs and mild destruction of the acini (Fig. 1) [14]. Fifteen of 66 patients with SS (23%) and 12 of 20 patients with MD (60%) showed ectopic GC formation in labial salivary glands (LSGs). Patients with MD showed a significantly higher frequency, higher number and larger size of GCs compared with SS patients [15]. In addition, Yamamoto et al. [16–18] reported that patients with MD had elevated levels of serum IgG4 and infiltrating IgG4-positive plasma cells in the gland tissues. Similar findings have been observed in autoimmune pancreatitis (AIP) [19], sclerosing cholangitis [20], tubulointerstitial nephritis [21], Ridel's thyroiditis [22] and Küttner's tumor [23]. These diseases are now referred to as IgG4-related disease (IgG4-RD) [24,25]. We recently described the concept of IgG4-RD and provided up-to-date information regarding this emerging disease entity [26]. Recent studies have referred to MD as IgG4-related dacryoadenitis and sialoadenitis (IgG4-DS) [15,27] (Table 2).

IgG4 molecules are symmetrical homobivalent antibodies that can exchange half-molecules (heavy and light chain) specific for two different antigens ("Fab-arm exchange"), which results in losing the ability to cross-link antigens and to form immune complexes [28]. In addition, IgG4 also can bind the Fc fragment of other IgG molecule, particularly other IgG4 molecules ("Fc–Fc interactions"). These IgG4 Fc–Fc interactions proceed to Fab-arm exchange reaction and may contribute to the anti-inflammatory activity, which includes a poor ability to induce complement and cell activation caused by low affinity for C1q (Fig. 2) [29]. Another characteristic is that IgG4 is a Th2-dependent immunoglobulin and has low affinity for its target antigen. interleukin (IL)-4 directs naive human B cell immunoglobulin isotype switching to IgG4 and IgE production [30]. We previously reported that peripheral CD4+ Th cells from patients with IgG4-DS revealed a deviation in the Th1/Th2 balance to Th2 and elevated expression of Th2-type cytokines [15,31,32]. Therefore, IgG4-DS is suggested to have a Th2-predominant phenotype. This review article will emphasize recent studies seeking to understand the role of Th cell subsets in primary SS and IgG4-DS.

1. Cytokine profiles of CD4+ Th cells

1.1. Th1/Th2 paradigm

Th1 cells support cell-mediated immunity and produce IL-2, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α, which induce inflammatory responses responsible for killing intracellular parasites and perpetuating autoimmune responses. However, excessive inflammatory responses can lead to uncontrolled tissue damage. Th2 cells produce IL-4, IL-5, and IL-13, which provide help for humoral immunity and promote IgE secretion and eosinophilic responses. Th2 responses can counteract Th1-mediated microbicidal action. Thus, the Th1/Th2 balance plays an important role in immunoregulation. In contrast, Th0 cells are characterized by the production of both Th1 and Th2 cytokines and are considered precursors of Th1 and Th2 cells. Several studies have revealed that autoimmune diseases are caused by disruption to the Th1/Th2 balance [33,34]. The relationship of Th1/Th2 imbalance to the pathogenesis of SS has been widely investigated. Polarized Th1 responses were associated with the immunopathology of SS [9]. High numbers of IFN-γ-positive CD4+ T cells were detected in the salivary glands of SS patients and intracellular cytokine analysis demonstrated the polarization of Th cells to a Th1 phenotype [35]. Furthermore, we reported that IL-2 and IFN-γ were consistently detected in all SS patients, while IL-4 and IL-5 were only detected in patients with high levels of B cell accumulation in the salivary glands [10,36]. Recently, Theander et al. [37] reported that the detection of GC-like structures (B cell accumulation) in LSG biopsy specimens from primary SS patients could be used as a highly predictive and easy-to-obtain marker for B cell lymphoma development. Taken together, these studies suggest that Th1 cytokines are essential for the induction and/or maintenance of SS, whereas Th2 cytokines may be involved in disease progression, especially local B cell activation. Our clinical data was demonstrated that Th1 and Th2 cytokine concentrations were significantly higher in saliva from SS patients than from controls, and the levels of Th2 cytokines were closely associated with increased lymphocytic accumulation in LSGs. Thus, the measurement of cytokines in saliva may be useful for diagnosis and to reveal disease status [12].

IgG4-DS patients frequently have a history of bronchial asthma and allergic rhinitis with severe eosinophilia and elevated serum IgE levels [38]. It is well known that allergic immune responses are induced by allergen-specific Th2 cytokines, such as IL-4 and IL-13, which promote the secretion of IgG4 and IgE by B cells [39]. Recent studies indicated that Th2 immune reactions contributed to IgG4-DS [15,32,40] and IgG4-related tubulointerstitial nephritis [31,41]. The expression profile of cytokines suggested that IgG4-DS was characterized by a deviation of the Th1/Th2 balance to a Th2 phenotype and elevated expression of Th2 cytokines. Contrary to our results, Ohta et al. [42] reported a strong predominance of Th1 and cytotoxic type 1 cells in the salivary glands from IgG4-DS patients. They concluded that disruption of the Th1/Th2 balance might be due to differences in the specimens examined or the severity of the disease.

Chemokines are important for leukocyte activation and chemotaxis. Interactions between chemokines and chemokine receptors promote the selective local infiltration of specific cells into inflamed areas. Furthermore, chemokines are intimately involved in maintenance of the Th1/Th2 balance and immune responses in cardiac allograft rejection [43], atopic keratoconjunctivitis [44], and cutaneous lupus erythematosus [45]. Chemokines also play a key role in lymphoid neogenesis in target organs [46]. Immunohistochemical staining in our studies indicated that Th2-type chemokines including macrophage-derived chemokine (MDC)/CCL22 and thymus and activation regulated chemokine (TARC)/CCL17, natural ligands for CCR4 on Th2 cells, were detectable in and around the ductal epithelial cells and GCs, while CCR4 was expressed on infiltrating lymphocytes in LSGs in both SS and IgG4-DS patients. Thus, interactions of CCR4 with MDC and TARC may play a critical role in the accumulation of Th2 cells and subsequently, the progression of SS and IgG4-DS [12,32]. In contrast, interferon gamma induced protein 10 (IP-10)/CXCL10, natural ligand for CXCR3 on Th1 cells, was detected in and around the ductal epithelial cells, while CXCR3 was only expressed on infiltrating lymphocytes in LSGs from SS patients [47].

Table 1
Clinical and laboratory findings of Sjögren's syndrome (SS) and IgG4-related dacryoadenitis and sialoadenitis (IgG4-DS). § IgG4 positive plasma cells/IgG positive plasma cells >50%.

	SS	IgG4-DS
Peak age of onset	40's and 50's	60's
Sex	Male ≪ Female	Male ≦ Female
Salivary secretion dysfunction	Moderate or severe	None or mild
Glandular swelling	Recurrent	Persistent
Sialography	Apple-tree sign	Parenchymal defect
$IgG4^+$ plasma cell infiltration§	Positive	Negative
Serum IgG	Often high	High
Serum IgG4	Normal	High
Serum complement	Normal	Often low
Anti SS-A/SS-B antibody (+)	High rate	Rare
Antinuclear antibody (+)	Often	Rare

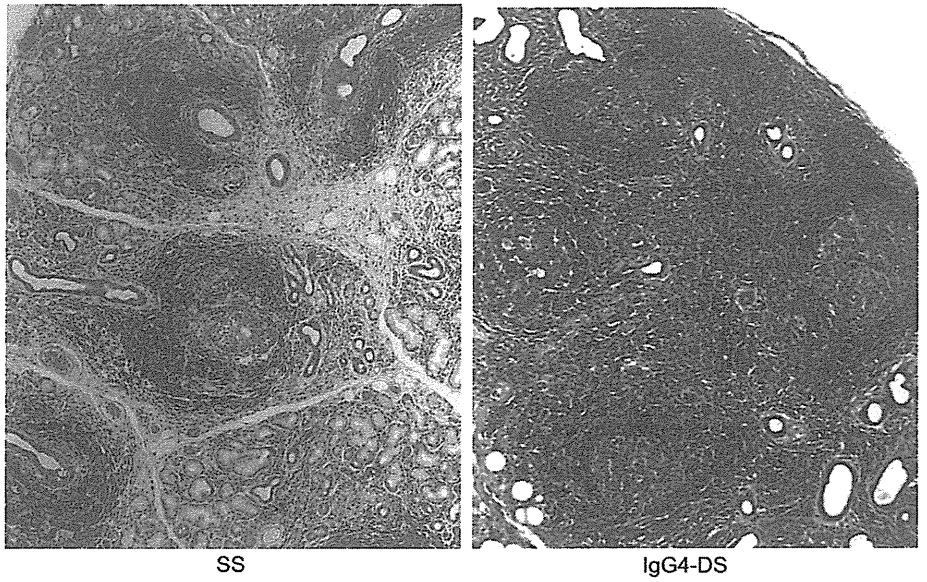

Fig. 1. Histopathological findings in salivary glands from patients with Sjögren's syndrome (SS) and IgG4-related dacryoadenitis and sialoadenitis (IgG4-DS). SS is characterized by periductal lymphocytic infiltration with atrophy or severe destruction of the acini, while IgG4-DS shows non-periductal lymphocytic infiltration with hyperplastic GCs and mild destruction of the acini. Abbreviations: GC, germinal center.

1.2. Th17 cells

The Th1/Th2 paradigm was recently expanded by the identification of Th17 cells, a subset of CD4+ Th cells characterized by their ability to produce IL-17. Several studies have reported that IL-17 was detected in epithelial and infiltrating mononuclear cells in LSGs from patients with SS. In addition, Th17 cells are "tissue seeking" and intimately involved in the initiation of SS [48]. Youinou et al. [49] reported that Th17 cells orchestrate autoreactive GCs. However, Our previous data in selectively extracted lesions from LSGs by laser capture microdissection showed that the expressions of Th17-related molecules in infiltrating lymphocytes outside ectopic GCs were higher than inside ectopic GCs [36]. Interestingly, a subset of Th17/Th1 cells identified in the gut of Crohn's disease patients may co-express IFN-γ and IL-17 [50]. Both Th1 and Th17 cells were involved in the pathogenesis of SS [51], and the early induction of a CD4+ Th1/Th17 pathway caused the systemic release of IL-17 in mice [52]. Our previous data suggest that both Th1 and Th17 cells present around the ductal epithelial cells might be of critical importance in the initiation of SS. Furthermore, the destruction of epithelial by Th1 and Th17 cells are thought to play an important pathogenetic role by the occurrence of infiltrating lesions in various epithelial tissues as well as the increased epithelial expression of various immunoactive molecules. Thus, SS has been described as "autoimmune epithelitis" [6]. In contrast, Th17-related molecules were rarely expressed in patients with IgG4-DS [32,36]. As mentioned above, IgG4-DS showed non-periductal lymphocytic infiltration and mild destruction of the epithelial cells. These findings were speculated that IgG4-DS might be a "non- autoimmune epithelitis".

Table 2
Role of Th subsets in IgG4-related disease (IgG4-RD). Abbreviations: Th, T helper; MD, Mikulicz's disease; AID, activation-induced cytidine deaminase; LSG, labial salivary gland; Tc1, T cytotoxic type 1; Tfh, follicular helper T; NLR, nucleotide-binding oligomerization domain-like receptor; TLR, Toll-like receptor; AIP, autoimmune pancreatitis; BAFF, B-cell activating factor belonging to the tumor necrosis factor family; APRIL, a proliferation-inducing ligand; Treg, regulatory T; TGF-β, transforming growth factor β.

Principal findings	Reference
Overexpression of IL-21 by Th2 cells play a key role in germinal center formation and IgG4 production in IgG4-DS.	[15]
Peripheral CD4+ T cells from the patient with MD reveal the deviation of the Th1/Th2 balance to Th2.	[31]
Th2 and regulatory immune reactions play a key role of IgG4 production in MD.	[32]
The production of IgG4 antibodies appears to be driven in part by Th2 cytokines that mediate allergic responses and IgE production.	[38]
Th2 cells are involved in the pathogenesis of IgG4-related lacrimal gland enlargement.	[39]
Overexpressions of IL-10, TGF-β, and AID in LSGs play important roles in the pathogenesis of IgG4-RD, such as IgG4-specific class-switch recombination and fibrosis.	[81]
IgG4-related tubulointerstitial nephritis shows amplification of IL-10 and TGF-β.	[41]
Th1 and Tc1 cell populations and IL-17 expression are involved in the mechanism of pathogenesis of IgG4-related sclerosing sialadenitis.	[42]
IgG4-related interstitial nephritis shows Tfh cells in enhancing a skewed B-cell terminal maturation and of CD20 + B cells in disease progression.	[66]
Activation of NLR and TLR in monocytes from AIP patients induces IgG4 production by B cells.	[76]
BAFF and APRIL are useful markers for predicting disease activity in IgG4-RD.	[78]
The progression and induction of AIP was supported by increased memory Treg and Th2 immune responses.	[80]

1.3. Regulatory T cells

Treg cells, identified by the expression of Foxp3, are essential for the maintenance of immunological self-tolerance and immune homeostasis to prevent the development of various inflammatory diseases. It achieves this either by direct contact with effector immune cells and/or by secreting anti-inflammatory cytokines such as IL-10 and transforming growth factor (TGF)-β. Treg cells exert their effects through the modulation of both T and B cell responses. Two subsets of Treg cells, CD4+ CD25+ Foxp3+ Treg cells [53] and IL-10–producing Tr1 cells [54] are crucial for regulating effector T cell functions. CD4+ CD25+ Foxp3+ Treg cells can prevent

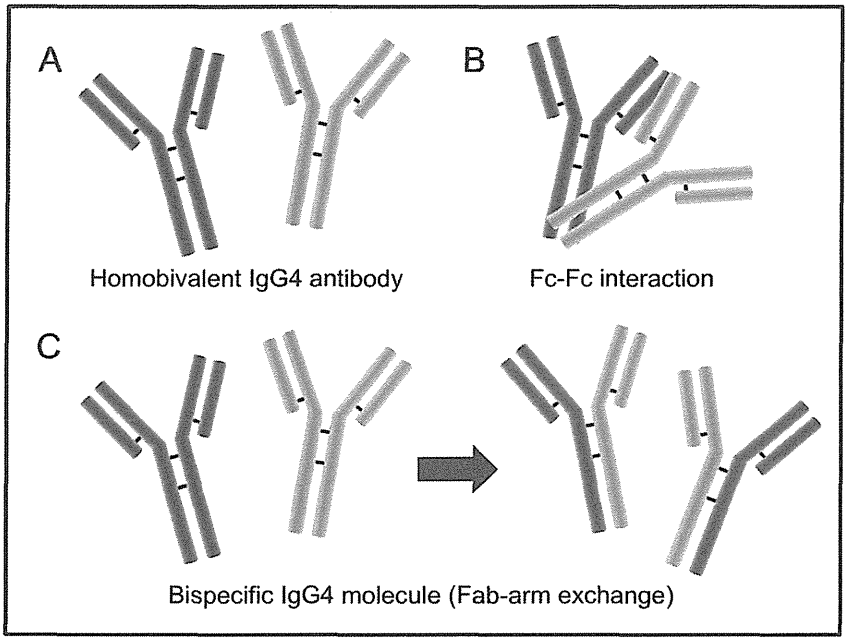

Fig. 2. Unique structure of IgG4 antibody. A, IgG4 antibody consists of two heavy chains and two light chains. B, Fc fragment of IgG4 can interact with the Fc fragment of another IgG4 molecule. C, Exchange of half-molecules (Fab-arm exchange) results in IgG4 combining two different specificities in a single molecule (bispecific antibody).

autoimmune hepatitis and primary biliary cirrhosis [55]. Mice with defects in Treg cell generation often develop T cell-mediated systemic autoimmune responses that affect multiple organs. Kolkowski et al. [56] demonstrated that salivary glands in SS constitutively expressed IL-10 and TGF-β. Other studies reported a significant reduction of Tregs in LSGs and peripheral blood from SS patients that might be involved in the pathogenesis of salivary gland destruction [57,58]. In contrast, Gottenberg et al. [59] reported increased Treg cell numbers in the peripheral blood of SS patients. Therefore, it is unclear whether Tregs are involved in the pathogenesis of SS. According to recent data, Foxp3+ T-regulatory cell frequency in the salivary glands of SS patients correlates with inflammation grade and certain risk factors for lymphoma development [60]. While in early and moderate infiltrations a compensatory control of Tregs in response to Th17 expansion seems to occur, in advanced SS lesions Tregs may fail to control the immune mediated tissue injury [7,61]. Increased levels of Treg cells in salivary glands from SS patients might suggest negative feedback is more active than in healthy subjects. Therefore, Treg cells might be not involved in the initiation of disease.

Zen et al. [62] reported that significant numbers of CD4+ CD25+ Foxp3+ Tregs infiltrated the affected tissues in cases of autoimmune pancreato-cholangitis (AIPC), which is one of IgG4-RD. Furthermore, another study demonstrated that IL-10 decreased IL-4-induced IgE switching but increased IL-4-induced IgG4 production [63]. We found that IL-4, IL-10, and Foxp3 were positively correlated with the IgG4/IgG ratio in the salivary glands from patients with IgG4-DS [32]. These results suggest that Th2 and regulatory immune reactions might play key roles in IgG4 production.

2. Role of IL-21 in SS and IgG4-DS

2.1. Follicular helper T cells

Tfh cells were recently identified as a unique Th phenotype, expressing high levels of CXCR5, a chemokine receptor [64]. Several studies reported that Tfh cells control the functional activity of effector Th cells and promote ectopic GC formation by IL-21, which contributed to impaired B cell differentiation [65,66]. Once GCs are formed, Tfh cells are required for their maintenance and the regulation of B cell differentiation into plasma cells and memory B cells. Several studies in SS patients demonstrated that IL-21 was increased in serum and high levels of IL-21 receptor were present on the surface of most B cells [67]. Furthermore, IL-4 and IL-21 receptors knockout mice have greatly reduced IgG responses, indicating that IL-21 co-operates with IL-4 to regulate humoral immune responses [68]. We previously observed that Tfh-related molecules, CXCR5 and B-cell lymphoma 6 protein (Bcl-6), were highly expressed on infiltrating lymphocytes in ectopic GCs of LSG lesions from both SS and IgG4-DS patients [15,36]. These results provide strong support for Tfh cells in the progression of disease as a lymphoproliferative disorder, particularly in the growth and activation of ectopic GC formation (Fig. 3).

IL-21 was mainly produced by Th2 and Th17 cells in addition to Tfh cells [68,69]. Interestingly, high IL-21 expression was only detected outside ectopic GCs in patients with IgG4-DS in our immunohistological analyses. The expression patterns of Th2-related molecules (IL-4, CCR4 and c-Maf) in LSGs were similar to that of IL-21 in patients with IgG4-DS. In contrast, Th17-related molecules were rarely expressed in patients with IgG4-DS. Furthermore, IL-21 positively correlated with the number of GCs formed in LSGs from patients with IgG4-DS [15]. Taken together, these findings suggest that excessive IL-21 production by Th2 cells in salivary glands from IgG4-DS patients might induce Bcl-6 expression in B cells resulting in multiple GC formation. Furthermore, IL-21 directly inhibited IL-4-induced IgE production [70], and IgG4 class switching was induced by co-stimulation with IL-4 and IL-21 in humans and mice [71]. In addition, IL-21 induced IL-10 production by mitogen-stimulated peripheral blood mononuclear cells in humans [72]. Therefore, we speculate that IL-21 correlates with IL-4 and IL-10 for IgG4 class switching. In the current study, we found that IL-21 positively correlated with the IgG4/IgG ratio in immunohistochemically positive cells

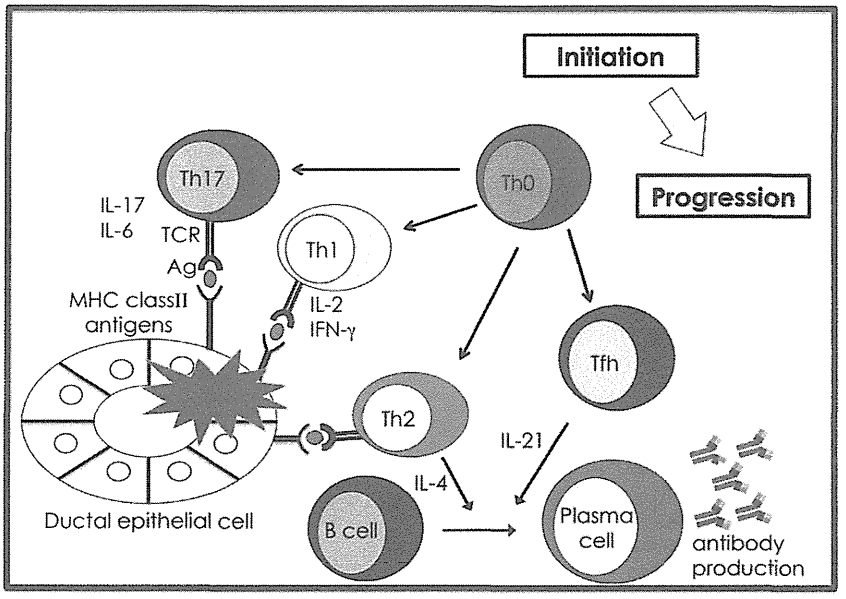

Fig. 3. Schematic model of Th cell network in SS. Th1 and Th17 cells are involved in early stages of disease, while Th2 and Tfh cells are associated with GC formation in the late stage. Abbreviations: Th, T helper; Tfh, follicular helper T.

[15] suggesting that IL-21 might also be involved in the class switching of IgG4 in IgG4-DS [73].

2.2. Innate immunity in IgG4-DS

Macrophages act as cells in the immune response to foreign invaders of the body, by presenting pathogenic antigens to antigen-specific Th cells. Historically, they have been classified into two distinct macrophage phenotypes, "classically activated" proinflammatory (M1) and "alternatively activated" anti-inflammatory (M2) macrophages [74]. M2 macrophages are activated by IL-4, produce high levels of IL-10 and are important for debris scavenging, wound healing and fibrosis. These polarized macrophage populations can also contribute to systemic diseases [75]. Watanabe et al. [76] demonstrated that abnormal innate immune responses induced via Toll-like receptor signaling in macrophages might enhance Th2 immune responses and the immunopathogenesis of IgG4-RD. Our current studies observed that IgG4-DS patients showed predominant infiltration by M2 macrophages that secreted IL-10 and IL-13 in salivary glands.

Dendritic cells (DCs) are professional antigen presenting cells that bridge innate and adaptive immunity. Expression of

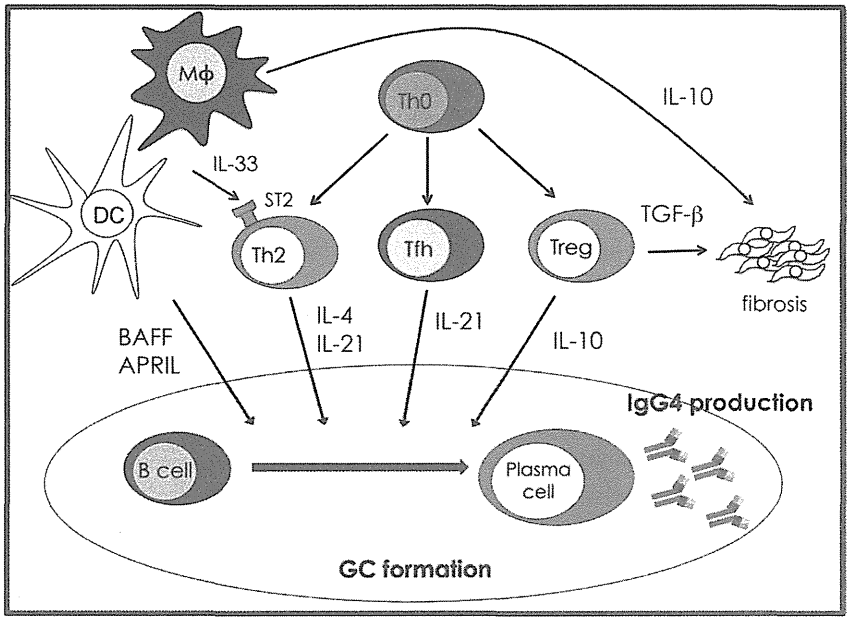

Fig. 4. Schematic model of Th cell and innate immune network in IgG4-DS. Th2, Treg, and Tfh cells play key roles in GC formation and IgG4 production. Dendritic cells and macrophages promote Th2 immune reaction by IL-33 as well as BAFF and APRIL. Abbreviations: Treg, regulatory T; BAFF, B cell activating factor belonging to the tumor necrosis factor family; APRIL, a proliferation-inducing ligand.

DC-derived TNF-family ligands such as a proliferation-inducing ligand (APRIL) and B cell activating factor belonging to the tumor necrosis factor family (BAFF) is induced by innate immune signals to promote the differentiation and activation of plasma cells [77]. In IgG4-RD patients, serum BAFF and APRIL levels were significantly higher than in healthy individuals [78]. BAFF and APRIL may contribute to progressive plasmacyte infiltration and ectopic GC formation in the target organs of patients with IgG4-RD. In addition, BAFF and APRIL enhance IgG4 and IgE class switching in the presence of IL-4 [79]. Th2 cytokine production was increased in the tissues of patients with autoimmune pancreatitis [80]. Therefore, BAFF and APRIL may contribute to the pathogenesis of IgG4-RD in concert with Th2 cells. Although IgG4-RD was considered to be a Th2-dependent disease [40,41,81], the mechanism of Th2 polarization has yet to be elucidated. IL-33 is a recently identified cytokine that directly stimulates ST2, IL-33 receptor, expressed by Th2 cells to produce IL-4, IL-5, and IL-13 [82]. Moreover, the genetic polymorphism of IL-33 in humans is associated with allergic diseases [83]. Our current studies suggest that IL-33 production by DCs and M2 macrophages might play a key role in Th2 cytokine production and the pathogenesis of IgG4-DS (Fig. 4).

3. Conclusions

Research accumulated in recent years makes it increasingly clear that the immunological backgrounds are entirely different between SS and IgG4-DS. However, additional research is required to elucidate further the pathogenesis of IgG4-DS, especially the development of a mouse model of IgG4-DS. Although Glucocorticoids are the standard treatment for IgG4-RD, Yamamoto et al. [84] reported that the relapse rate of IgG4-DS during steroid therapy is 26.8%. A more thorough understanding of the complex mechanisms of IgG4-DS, especially the role of Th subset-related cytokines, could lead to the development of novel pharmacological strategies aimed at disrupting the cytokine network and inhibiting the initiation and/or progression of IgG4-DS. Finally, it should be noted that while this thesis focuses primarily on T cells, that there have recently been other extensive reviews and hypotheses published on Sjogren's syndrome, reflecting its increased interest not only to basic immunologists, but also to rheumatologists [4,85–116].

Competing interests

The authors declare no competing interests.

Author contributions

All authors provided substantial contributions to discussions of content, and to reviewing and editing the manuscript before submission. M Moriyama researched the data and wrote the article.

Acknowledgments

This work was supported by the "Research on Measures for Intractable Diseases" Project, a matching fund subsidy from the Ministry of Health Labour and Welfare, Japan.

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Japan College of Rheumatology
MODERN RHEUMATOLOGY

http://informahealthcare.com/mor
ISSN 1439-7595 (print), 1439-7609 (online)

Mod Rheumatol, 2015; 25(1): 105–109

DOI: 10.3109/14397595.2014.903596

informa
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ORIGINAL ARTICLE

Clinical features of patients with IgG4-related disease complicated with perivascular lesions

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Abstract

Objective. To define the clinical features of IgG4-related disease (IgG4-RD) complicated with perivascular lesions.

Methods. The clinical features of seven patients with IgG4-RD and perivascular lesions diagnosed at the University of Tsukuba Hospital between October 2008 and October 2013, were analyzed, including clinical background, results of imaging studies, satisfaction of the 2011 comprehensive diagnostic criteria (CDC) for IgG4-RD, laboratory data, distribution of perivascular lesions, involvement of other organs, and response to steroid therapy.

Results. We studied six men and one woman with a mean age of 66.9 ± 6.7 years (± SD). Six of seven patients were diagnosed as definite IgG4-RD, while the seventh was considered possible IgG4-RD, based on the CDC for IgG4-RD. Serum IgG4 levels at diagnosis were higher than 135 mg/dl in all seven patients (mean, 933 ± 527). Serum C-reactive protein (CRP) levels were elevated in two only (mean, 1.42 ± 3.56 mg/dl). The perivascular lesions were located in the pulmonary artery ($n = 1$), thoracic aorta ($n = 2$), abdominal aorta ($n = 6$), coronary ($n = 1$), celiac ($n = 1$), superior mesenteric ($n = 1$), renal ($n = 2$), inferior mesenteric ($n = 5$), and iliac ($n = 3$) arteries. In addition to perivascular lesions, six patients showed involvement of other organs. All seven patients were treated with prednisolone (0.6 mg/kg/day), which rapidly improved the perivascular and other organ lesions in six patients (the other one patient have not yet been evaluated due to the short follow-up).

Conclusion. Perivascular lesions show wide distribution in patients with IgG4-RD. Serum CRP levels are not necessarily elevated in these patients. Steroid therapy is effective in IgG4-RD and results in resolution of lesions.

Keywords

IgG4-related disease, Perivascular lesions, Steroid therapy

History

Received 20 December 2013
Accepted 10 March 2014
Published online 21 April 2014

Introduction

Immunoglobin G4-related disease (IgG4-RD) is a new disease entity characterized by high serum IgG4 concentrations, swelling of organs associated with IgG4-positive plasmacytic infiltration and fibrosis [1,2]. IgG4-RD often involves various organs throughout the body, such as the central nervous system, lacrimal glands, salivary glands, thyroid, lung, liver, pancreas, bile duct, gastrointestinal tract, kidney, prostate, retroperitoneum, arteries, lymph nodes, skin, and breast [1,2].

IgG4-RD that involves the aorta has a predilection for the adventitia and periaortic tissue [3–5]. However, the disease also involves the media, making it by definition an aortitis rather than a periaortitis [4]. Stone et al. [6] proposed the term "IgG4-related aortitis and periaortitis" for these conditions, to reflect the anatomic extent of inflammation. IgG4-related periaortitis may exhibit some overlap with IgG4-related retroperitoneal fibrosis [6]. Moreover, IgG4-RD could involve medium sized arteries, and Stone et al. proposed the term "IgG4-related periarteritis" for this disease [6].

Radiologic examination and serologic analysis of IgG4 concentration are key steps in the diagnosis of IgG4-RD. Autoimmune pancreatitis, a prototype of IgG4-RD, is usually suspected by characteristic radiologic findings and is confirmed by subsequent serologic examination of IgG4 [7]. In this regard, it is important to define the radiologic characteristics of IgG4-RD. Indeed, some reports on the radiologic findings in other organs such as lacrimal and salivary glands, lung, liver, and kidney other than perivascular lesions have already been published [8–11]. Moreover, recently, Inoue et al. [12] reported the radiologic characteristics of periaortitis and periarteritis of IgG4-RD. To our knowledge, however, the clinical features of IgG4-related perivascular lesions (periaortitis and periarteritis) have not been elucidated.

The purpose of this retrospective study was to define the clinical features of IgG4-RD complicated with perivascular lesions.

Patients and methods

Study population

We examined 7 patients, out of 21 all patients with IgG4-RD, complicated with perivascular lesions detected by positron

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Table 1. Clinical background.

Case	Age	Gender	Satisfaction for diagnostic criteria	IgG (mg/dl)	IgG4 (mg/dl)	CRP (mg/dl)	sIL-2R (U/ml)	ANA (times)	Anti-SSA antibody (times)
1	75	M	Definite	4381	>1500*	0.34	2030	–	–
2	66	M	Possible#	2075	323	9.49	822	–	–
3	58	F	Definite	1899	773	<0.10	750	–	–
4	59	M	Definite	3506	1490	<0.10	1140	–	–
5	66	M	Definite	2236	951	<0.10	619	–	–
6	74	M	Definite	1704	216	0.15	1220	–	–
7	70	M	Definite	3804	1280	<0.10	1230	–	–
Mean (± Standard Deviation)	66.9 ± 6.7			2747 ± 1037	933 ± 527	1.42 ± 3.56†	1116 ± 470		

M male, *F* female, *ANA* anti-nuclear antibody; *sIL-2R* soluble interleukin-2 receptor.
*With regard to Case 1, the value of IgG4 was considered 1500. #The tissue biopsy was not performed because the locations of target lesions were deep sites. †As for case 3, 4, 5 and 7, values of CRP were counted as zero.

emission tomography-computed tomography (PET-CT) or contrast-enhanced computed tomography (enhanced CT), who were diagnosed as definite or possible IgG4-RD based on the 2011 comprehensive diagnostic criteria (CDC) for IgG4-RD, proposed by the All Japan IgG4 Team [13] between October 2008 and October 2013 at the University of Tsukuba Hospital (Ibaraki, Japan). This set of CDC includes the following three items; 1) characteristic diffuse/localized swelling or masses in single or multiple organs on clinical examination, 2) high serum IgG4 concentrations (>135 mg/dl), and 3) histopathological examination of the affected organs shows marked lymphocyte and plasmacyte infiltration and fibrosis, plus ratio of infiltrating IgG4-positive/IgG-positive plasma cells of >40%, with >10 IgG4-positive plasma cells/high power field (HPF). According to the above CDC, patients who satisfy all three items are diagnosed as definite IgG4-RD, while patients with 1) and 2) items are diagnosed as possible IgG4-RD.

Data collection and analysis

In this retrospective study, the medical records were checked for clinical background, perivascular involvement, other organ involvement, laboratory data, pathological findings, response to treatment, and prognosis. The results of serological assays, such as serum IgG, IgG4, CRP, soluble interleukin-2 receptor (sIL2-R), anti-nuclear antibody (ANA), and anti-SSA antibody, as well as those of imaging studies, such as PET-CT, enhanced CT, and ultrasonography (US), were collected and analyzed. Pathological examinations were also performed, including hematoxylin and eosin (H&E), immunohistochemical staining for IgG and IgG4 using tissues, such as lacrimal and salivary glands, lymph nodes (LN), nasal mucosa, and perivascular mass lesion. Group data are summarized as mean ± SD.

Assessment of response to treatment

The response to treatment was assessed by the mass volume reduction or improving to normal sizes on imaging studies, such as enhanced CT and US, in addition to physical examinations for mass lesions of lacrimal and salivary glands and LN. Thus, we decided improved or not-improved by treatment depending on all these clinical findings.

Results

Clinical background

Table 1 summarizes the clinical background of the seven enrolled patients. In our hospital, the percentage of patients with perivascular lesions in all patients with IgG4-RD was 33.3% (7/21 cases). Six men and one woman were examined, and their mean age was 66.9 ± 6.7 years. Six out of the seven patients were diagnosed with definite IgG4-RD, while the seventh was considered possible IgG4-RD [13]. In Case 2, no tissue biopsy was performed due to the deep location of the target lesions. Serum IgG4 level at diagnosis was higher than 135 mg/dl in all seven patients (mean, 933 ± 527 mg/dl). Serum CRP levels were elevated in only two out of seven patients (mean, 1.42 ± 3.56 mg/dl). Serum sIL-2R level was elevated in all seven patients (mean, 1116 ± 470 U/ml). ANA and anti-SSA antibody by Ouchterlony assay were negative in all seven patients.

Sites of perivascular lesions

Table 2 lists the sites of perivascular lesions detected by PET-CT or enhanced CT. These were located in the pulmonary artery ($n = 1$), thoracic aorta ($n = 2$), coronary artery ($n = 1$), abdominal aorta ($n = 6$), celiac artery ($n = 1$), superior mesenteric artery ($n = 1$), renal artery ($n = 2$), inferior mesenteric artery ($n = 5$), and iliac artery ($n = 3$). Five lesions were detected in one patient, four lesions in two patients, three lesions in two patients, two lesions in one patient, and one lesion in one patient. Five patients showed the continuity in each perivascular lesion, such as abdominal aorta to inferior mesenteric artery, and thoracic aorta to coronary artery. Figure 1A, C, and E show PET-CT images and Figure 1B, D, and F show enhanced CT images of a representative patient (Case 2), demonstrating perivascular thickening of the thoracic and abdominal parts of the aorta, and iliac arteries. Furthermore, PET-CT clearly showed marked uptake of 18F-fluorodeoxyglucose (18-FDG) in these perivascular lesions, indicating active inflammation in these lesions (Figure 1A, C, and E). The SUV max values (1.0 h) in the PET images were 4.67, 5.38, and 4.42 at the ascending aorta, the aortic arch, and the abdominal aorta, respectively. Aortic aneurysms were not found

Table 2. Sites of perivascular lesions.

Case	Perivascular lesions	Number of lesions
1	Thoracic aorta, [abdominal aorta - IMA - iliac artery]	4
2	[Thoracic aorta - coronary artery], [abdominal aorta - iliac artery]	4
3	Pulmonary artery, abdominal aorta, iliac artery,	3
4	IMA	1
5	[Abdominal aorta - IMA], renal artery	3
6	[Abdominal aorta - celiac artery - SMA - renal artery - IMA]	5
7	[Abdominal aorta - IMA]	2

IMA inferior mesenteric artery, *SMA* superior mesenteric artery.
[-]: the continuity in each perivascular lesion.

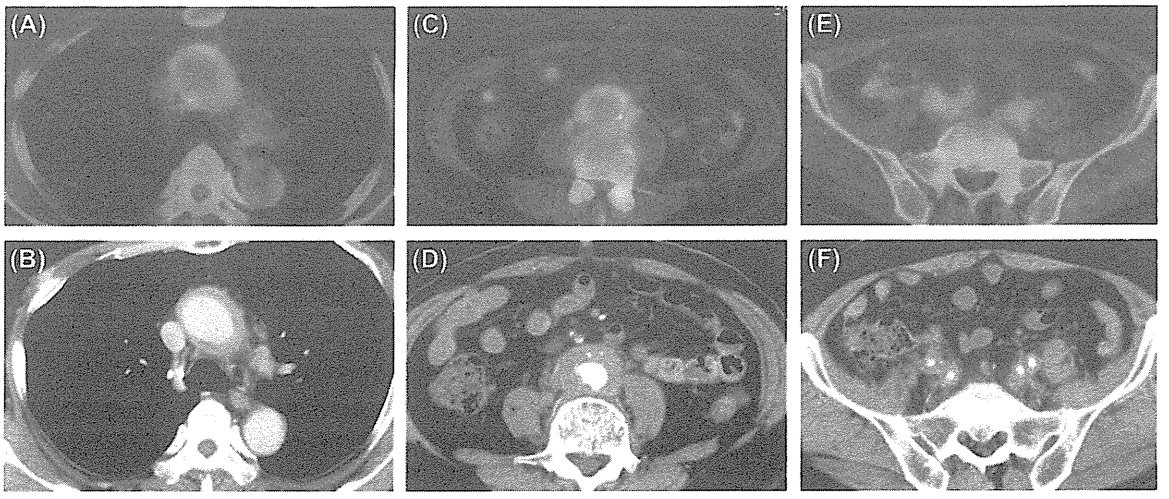

Figure 1. Positron emission tomography-computed tomography and contrast-enhanced computed tomography of perivascular lesions in Case 2. (A and B) Thoracic aorta, (C and D) abdominal aorta, (E and F) Iliac artery. A, C, and E are images obtained by positron emission tomography-computed tomography (PET-CT). B, D, and F are images obtained by contrast-enhanced computed tomography (enhanced CT).

in our seven cases. These findings suggest the wide distribution of perivascular lesions in patients with IgG4-RD. Moreover, perivascular lesions were more common in vessels below the diaphragm ($n = 18$) than above the diaphragm ($n = 4$).

Involvement of other organs

Table 3 summarizes involvements of other than perivascular lesions that were identified in six out of seven patients, including LN ($n = 6$), lacrimal glands ($n = 3$), salivary glands ($n = 2$), interstitial nephritis ($n = 2$), soft tissue mass at renal hilum ($n = 1$), intraorbital inflammation, including infraorbital nerve and inner muscle cone inflammation ($n = 3$), autoimmune pancreatitis ($n = 1$), obturator nerve ($n = 1$), and para-vertebral mass ($n = 1$). Regarding the distributions of LN involvements, mediastinal LNs were involved in five patients, cervical LNs in four patients, and para-aortic LNs in four patients. Although perivascular lesions were the only involvement detected in one patient (Case 6), widespread lesions involving various organs were detected in the other six patients. Moreover, we could divide these seven patients into typical IgG4-RD having Mikulicz's disease (lacrimal and salivary glands) or autoimmune pancreatitis (Case 3, 5, and 7), and non-typical IgG4-RD having only other involvements (Case 1, 2, 4, and 6).

Pathological findings

Table 4 summarizes the pathological findings in perivascular mass lesion and other organs. In Case 6, the biopsy sample obtained by laparotomy from the perivascular mass lesion of the abdominal aorta showed lymphoplasmacytic infiltration and fibrosis as well as IgG4-positive plasamacytic infiltration, which were compatible with the characteristic pathological features of IgG4-RD (Supplementary Figure 1 to be found online at http://informahealthcare.com/doi/abs/10.3109/14397595.2014.903596). In the other six cases, all samples from other organs, such as lacrimal glands, labial salivary glands, nasal mucosa, and LN also showed lymphoplasmacytic infiltration and fibrosis. Immunohistochemistry showed that ratio of IgG4-positive plasmacytes/IgG-positive plasmacytes was >40% in all these samples except those of the labial salivary glands in Case 3. Obliterative phlebitis was found only in the lacrimal grand of Case 3, on the other hand storiform fibrosis was found in all samples other than in the cervical lymph node of Case 7. Importantly, perivascular mass lesion of Case 6 did not show obliterative phlebitis but storiform fibrosis.

Response to treatment and prognosis

Table 5 lists the response to treatment and prognosis. The initial treatment in all seven patients was oral prednisolone at 0.6 mg/kg/day (30–40 mg/day), which was continued for 2–4 weeks. The dose was subsequently tapered by 10% every fortnight, with a selected maintenance dose of 5–10 mg/day. We assessed response to treatment by imaging studies as well as physical examinations. Imaging studies such as CT and US were performed 3–6 months after initiation of prednisolone. Imaging studies confirmed rapid improvement of the perivascular and organ lesions in six patients following such treatment, although such assessment has not yet been performed in the Case 7 because of the short follow-up period (5 months). Serum IgG4 levels decreased in all seven patients.

Table 3. Summary of involvement of other organs.

Case	LN	LG	SG	Renal involvement	Orbital involvement	Pancreas	Others
1	+ (S,C,M,H)	+	–	–	Intraorbital inflammation	–	–
2	+ (C,A,M,P)	–	–	–	–	–	–
3	+ (S,M,P)	+	+	Interstitial nephritis	Infraorbital nerve	–	–
4	+ (C,M,H,P)	–	–	–	Inner muscle cone inflammation	–	Obturator nerve
5	+ (M)	+	+	Soft tissue mass of renal hilum	–	–	Para-vertebral
6	–	–	–	–	–	–	–
7	+ (C,P,I)	–	–	Interstitial nephritis	–	Autoimmune pancreatitis	–

LN lymph nodes, *LG* lacrimal glands, *SG* salivary glands, +: positive, −: negative, *( ) in LN column* the distributions of lymph nodes involvements, *S* submandibular, *C* cervical, *M* mediastinal, *H* hilar, *A* axillary, *P* para-aortic, *I* inguinal.

Table 4. Pathological findings.

Case	Tissue	Lymphoplasmacytic infiltration	Fibrosis	Storiform fibrosis / Obliterative phlebitis	IgG4/IgG
1	Lacrimal gland	+	+	+	85%
				−	
2	Not performed				
3	Lacrimal gland	+	+	+	80%
				+	
	Labial salivary gland	+	+	+	3%
				−	
4	Lacrimal gland	+	+	+	95%
				−	
	Mucosa of nasal cavity	+	+	+	80%
				−	
5	Lacrimal gland	+	+	+	80%
				−	
6	Perivascular mass lesion	+	+	+	80%
				−	
7	Inguinal LN	+	+	+	95%
				−	
	Cervical LN	+	+	−	55%
				−	

IgG4/IgG ratio of IgG4-positive plasmacytes/IgG-positive plasmacytes, *LN* lymph node, +: positive, −: negative.

Discussion

The present study described five important features of IgG4-related perivascular lesions. First, IgG4-related perivascular lesions showed a tendency to be more common in males and individuals with advanced age (male/female; 6/1, mean age; 66.9 years) in this study compared to the data reported for other types of IgG4-RD, such as IgG4-related dacryoadenitis and sialadenitis (male/female; 31/33, mean age; 57.0 years) [14]. Second, the perivascular lesions showed wide distribution in patients with IgG4-RD. Moreover, perivascular lesions tended to be more common in vessels below than above the diaphragm. In this study, neither stenosis nor aneurysm of vessels was detected in the perivascular lesions. Third, involvement of other organs was noted in the majority of patients with IgG4-related perivascular lesions (six out of seven patients). These included the LN, lacrimal glands, salivary glands, kidney, eye, pancreas, cranial nerves, and para-vertebral soft tissue. Recently, we reported that patients with IgG4-RD complicated with orbital involvement often present with involvement of other organs [15]. Another study of 17 patients with IgG4-related periaortitis and periarteritis described the presence of extravascular IgG4-related lesions in 12 of their patients [12]. These findings suggest that IgG4-RD is a systemic multi-organ disease. Interestingly, three of the present cohort (Cases 1, 3, and 4) had intraorbital inflammation, which is less common in IgG4-related ophthalmic disease than dacryoadenitis [16]. Furthermore, no intraorbital inflammation was detected in the 17 patients with IgG4-related pariaortitis and periarteritis described in the above report [12]. Considered together, it seems that intraorbital inflammation is rare in IgG4-RD with perivascular lesions. Fourth, serum CRP levels were not necessarily elevated in patients with IgG4-related perivascular lesions, in contrast to other vasculitis syndromes such as Takayasu's aortitis and giant cell arteritis. Case 2 in this study had both elevated serum CRP level and marked uptake of 18-FDG in perivascular lesions by PET-CT. These findings suggest the presence of active inflammation in patients with IgG4-related perivascular lesions and high CRP levels compared to those with normal CRP levels. Fifth, steroid therapy was effective for both perivascular lesions and extravascular involvements in IgG4-RD. None of our seven patients developed vascular stenosis, occlusion, or aneurysm after treatment.

Although the exact pathogenic mechanism of IgG4-RD remains unclear, few have been proposed in recent years. The latest studies reported higher proportions of type 2 helper T (Th2) cells and regulatory T (Treg) cells and increased production levels of Th2 and Treg cytokines in tissues of IgG4-RD, such as sclerosing pancreatitis and cholangitis [17], sialadenitis [18–20], and tubulointerstitial nephritis [21]. Importantly, it is reported that Th2 cytokines (IL-4 and IL-13) and Treg cytokine (IL-10) can induce IgG4 and IgE-specific class switch recombination [22,23], and that tumor growth factor (TGF) β, a Treg cytokine, can induce tissue fibrosis [24]. Thus, over-production

Table 5. Response to treatment.

Case	Initial PSL dose (mg/day)	Response to treatment: Perivascular lesions (assessed by imaging)	Response to treatment: Other organ involvements (assessed by imaging or physical examination)	IgG4 (mg/dl): Baseline	IgG4 (mg/dl): 1-month later	IgG4 (mg/dl): Last examination	Follow-up period (month)	PSL dose at last follow up (mg/day)
1	30	Improved	Improved	>1500	878	149	9	14
2	40	Improved	Improved	323	160	66.2	7	17.5
3	30	Improved	Improved	773	311	60.3	20	5
4	40	Improved	Improved	1490	407	222	19	8
5	35	Improved	Improved	951	765	55.8	13	10
6	30	Improved	None	216	84.7	53.8	5	10
7	30	Not performed	Improved	1280	967	176	5	14

PSL prednisolone.

of IL-4, IL-13, IL-10, and TGFβ could contribute to the pathogenesis of IgG4-RD, by increasing serum IgG4 level, and enhancing infiltration of IgG4-positive plasmacytes and fibrosis [20]. However, there is only little information on the pathogenesis of perivascular lesions in IgG4-RD. The reason for the lack of thorough analysis of the pathogenesis of perivascular lesions is probably related to the difficulty in obtaining biopsy material from the lesions. Actually, in the present study, only one patient (Case 6) underwent laparotomy biopsy to obtain tissue samples from perivascular mass lesion on the abdominal aorta. Importantly, the pathological findings in that specimen showed lymphoplasmacytic infiltration and fibrosis as well as infiltration of IgG4-positive plasmacytes, findings that were compatible with the characteristic pathological features of IgG4-RD. Therefore, the pathogenesis of perivascular lesions in IgG4-RD could be similar to that of other organ involvements in IgG4-RD. It is noteworthy that blood and imaging findings, such as CRP level, perivascular thickening, and mass-like lesions, are quite different from those encountered in other vasculitis syndromes, e.g., Takayasu's aortitis and giant cell arteritis, indicating the different pathogenic process involved in these two entities.

Clinically, it is important to scan the whole body for systemic involvement including perivascular lesions, using enhanced CT or PET-CT. Further studies are needed to define the pathogenesis, clinical features, treatment options, and prognosis of patients with IgG4-related perivascular lesions.

Conclusions

Wide distribution of perivascular lesions was typically seen in patients with IgG4-RD. Serum CRP levels were not necessarily elevated in all patients. Steroid therapy resulted in clinical improvement based on improvement of perivascular and other organ lesions in IgG4-RD.

Authors' contributions

All authors took part in the design of the study, contributed to data collection, and participated in writing the manuscript. All authors accept equal responsibilities for the accuracy of the contents of this paper.

Acknowledgements

We thank Dr. F. G. Issa for the critical reading of the manuscript.

This work was supported in part by Health and Labour Sciences Research Grants for research on intractable diseases (Research on IgG4-related disease) from the Ministry of Health, Labour and Welfare of Japan.

Conflict of interest

None.

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Supplementary materials available online

Supplementary Figure 1.

MODERN PATHOLOGY (2014) 27, 1126–1136

T helper 2 and regulatory T-cell cytokine production by mast cells: a key factor in the pathogenesis of IgG4-related disease

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IgG4-related disease is a systemic disorder with unique clinicopathological features and uncertain etiological features and is frequently related to allergic disease. T helper 2 and regulatory T-cell cytokines have been reported to be upregulated in the affected tissues; thus, the production of these cytokines by T helper 2 and regulatory T cells has been suggested as an important factor in the pathogenesis of IgG4-related disease. However, it is not yet clear which cells produce these cytokines in IgG4-related disease, and some aspects of the disorder cannot be completely explained by T-cell-related processes. To address this, we analyzed paraffin-embedded sections of tissues from nine cases of IgG4-related submandibular gland disease, five cases of submandibular sialolithiasis, and six cases of normal submandibular gland in order to identify potential key players in the pathogenesis of IgG4-related disease. Real-time polymerase chain reaction analysis confirmed the significant upregulation of interleukin (IL)4, IL10, and transforming growth factor beta 1 (TGFβ1) in IgG4-related disease. Interestingly, immunohistochemical studies indicated the presence of mast cells expressing these cytokines in diseased tissues. In addition, dual immunofluorescence assays identified cells that were double-positive for each cytokine and for KIT, which is expressed by mast cells. In contrast, the distribution of T cells did not correlate with cytokine distribution in affected tissues. We also found that the mast cells were strongly positive for IgE. This observation supports the hypothesis that mast cells are involved in IgG4-related disease, as mast cells are known to be closely related to allergic reactions and are activated in the presence of elevated non-specific IgE levels. In conclusion, our results indicate that mast cells produce T helper 2 and regulatory T-cell cytokines in tissues affected by IgG4-related disease and possibly have an important role in disease pathogenesis.
Modern Pathology (2014) **27,** 1126–1136; doi:10.1038/modpathol.2013.236; published online 3 January 2014

Keywords: cytokine; IgE; IgG4-related disease; mast cells; regulatory T cell

IgG4-related disease has recently been recognized as a clinical entity with unique clinicopathological features that can affect systemic organs.[1–4] Histological examination of IgG4-related disease has shown diffuse lymphoplasmacytic infiltration, interstitial fibrosis, obliterative phlebitis, and eosinophilic infiltration. Additionally, numerous IgG4-positive plasma cells are observed and the IgG4-positive/IgG-positive cell ratio is elevated above 40% in affected tissues. It has been suggested that these distinctive features are caused by the T helper 2 cell cytokines interleukin (IL)4 and IL5 and the regulatory T-cell cytokines IL10 and transforming growth factor beta 1 (TGFβ1).[5,6] These cytokines are

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Received 31 July 2013; revised 3 November 2013; accepted 4 November 2013; published online 3 January 2014

upregulated in the tissues affected by IgG4-related disease, where IL4 and IL10 are thought to stimulate B cells and plasma cells to induce lymphoplasmacytic infiltration and IgE and IgG4 production, whereas TGFβ1 is thought to induce interstitial fibrosis. On the basis of these observations, T helper 2 and regulatory T cells have been considered to have a key role in the pathogenesis of IgG4-related disease. However, it has not been definitively determined whether these T cells are actually responsible for the production of such cytokines in affected tissues. Moreover, the hypothesis that T cells produce the disease-related cytokines does not explain why an anti-CD20 monoclonal antibody, rituximab, would be effective in treating refractory IgG4-related disease patients.[7]

An alternative to the T-cell hypothesis is the involvement of mast cells in IgG4-related disease. Mast cells were first found to release histamine granules in immediate hypersensitivity reactions; however, recent research has revealed that mast cells are involved in a variety of immune responses, including host defense, immune regulation, allergy, chronic inflammation, and autoimmune disease.[8] In response to IgE stimulation, mast cells secrete various mediators, including T helper 2 cytokines and regulatory cytokines.[9] Non-specific IgE alone can induce cytokine secretion independent of any antigen.[10] As IgG4-related disease is frequently complicated with allergic disease and accompanied by elevation of serum IgE levels, we hypothesized that mast cells may be involved in the pathogenesis of IgG4-related disease.

Materials and methods

Samples

Tissue samples from nine cases of submandibular gland IgG4-related disease were obtained. The serum IgG4 levels were elevated in all nine cases. Samples from five cases of submandibular sialolithiasis and six cases of normal submandibular glands which were resected during treatment for oral cancer were obtained and used as disease controls. Formalin-fixed paraffin-embedded specimens were used for immunohistochemistry, dual immunofluorescence, RNA extraction, and real-time polymerase chain reaction (PCR) analysis. All samples were obtained with the approval of the Institutional Review Board at Okayama University.

Real-Time Quantitative PCR

Total RNA was extracted from the paraffin-embedded sections of all samples by using an miRNeasy FFPE Kit (QIAGEN, Valencia, CA, USA). Complementary DNA was prepared by reverse transcription PCR by using a SuperScript VILO MasterMix kit (Invitrogen, Carlsbad, CA, USA). Multiplex real-time PCR was performed for quantitative analysis, according to the standard protocol by using Taqman Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) and a Step One Plus Real-Time PCR System (Applied Biosystems). Specific primers and probes for TGFβ1, IL4, IL5, IL10, and β-actin were obtained from Applied Biosystems. The PCR cycling conditions were as follows: 30 s at 95 °C and 50 cycles of 5 s at 95 °C, and 30 s at 60 °C. The expression of each cytokine was normalized to that of β-actin, which was used as an endogenous control.

Histological Examination and Immunohistochemistry

All of the diseased and normal tissue samples used in this study were surgically resected specimens of submandibular glands. The specimens were fixed in 10% formaldehyde and embedded in paraffin. Serial 4-μm-thick sections were cut from the block of paraffin-embedded tissue and stained with hematoxylin and eosin (H&E). The sections were immunohistochemically stained using an automated Bond Max stainer (Leica Biosystems, Melbourne, Germany). The following primary antibodies were used: TGFβ1 (ab49754; 1:100; Novocastra, Newcastle, UK), IL4 (orb22602; 1:400; Biorbit, Cambridge, UK), IL5 (MAB605; 1:400; R&D, Minneapolis, MN, USA), IL10 (orb22606; 1:100; Biorbit), KIT/CD117 (YR145; 1:100; EPITOMICS, Burlingame, CA, USA), IgG (polyclonal; 1:20 000; Dako, Glostrup, Denmark), IgG4 (HP6025; 1:10000; The Binding Site, Birmingham, UK), forkhead box P3 (FOXP3) (236A/E7; 1:100; Abcam, Cambridge, UK), CD4 (1F6; 1:40; Nichirei, Tokyo, Japan), and IgE (A094; 1:500; Dako).

Following immunostaining, the number of IgG4-positive and IgG-positive cells was estimated in areas with the highest density of IgG4-positive cells. In accordance with the consensus statement on the pathological features of IgG4-related disease published in 2012,[2] three different high-power fields (HPFs) (eyepiece, $\times$10; lens, $\times$40) were examined to calculate the average number of IgG4-positive cells per HPFs and the IgG4-positive/IgG-positive cell ratio. Cells that were positive for each cytokine, KIT, FOXP3, and IgE were counted in the three different fields (eyepiece, $\times$10; lens, $\times$20) determined to have the highest density of positive cells. The average number of positive cells per square millimeter (mm^2) was calculated.

Dual Immunofluorescence Assays

For indirect dual immunofluorescence assays, paraffin sections were stained with the primary antibodies for KIT and TGFβ1, KIT and IL4, KIT and IL5, or KIT and IL10. Fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (Alexa Fluor anti-mouse 555 and Alexa Fluor anti-rabbit 488; both Invitrogen Co, Carlsbad, CA, USA) were used at a dilution of 1:400. The stained specimens

were examined with a conventional immunofluorescence microscope (IX71; Olympus, Tokyo, Japan).

Statistical Analysis

Data are presented as mean ± s.d. values. All statistical analyses were performed using the Mann–Whitney *U*-test with the SPSS software (version 14.0; SPSS Inc., Chicago, IL, USA). A probability of $P<0.05$ was considered to be statistically significant.

Results

Confirmation of Histological Diagnosis in IgG4-Related Disease

We confirmed that the tissue specimens from all nine cases of submandibular gland IgG4-related disease showed typical histological features of IgG4-related disease, such as lymphoplasmacytic infiltration and dense fibrosis. Additionally, in all cases, numerous IgG4-positive cells were observed and the IgG4-positive/IgG-positive cell ratio was >40%.

Histological Findings of Sialolithiasis

The specimens showed lymphoid follicle formation and moderate to severe infiltration of lymphocytes and plasma cells as well as various numbers of neutrophils with various degree of fibrosis. Some specimens included salivary calculus (Figure 1a).

Elevated Expression of Cytokines in IgG4-Related Disease

The expression of T helper 2 cell cytokines (IL4 and IL5) and regulatory T-cell cytokines (IL10 and TGFβ1) was examined in samples from the nine cases of IgG4-related disease, five cases of sialolithiasis, and six cases of normal submandibular gland. The expression of the IL4, IL5, IL10, and TGFβ1 cytokines and the β-actin control in these samples was quantitatively analyzed by real-time PCR. As shown in Figure 1, IgG4-related disease exhibited significantly higher expression ratios of IL4/β-actin (31.9 ± 12.1-fold higher), IL10/β-actin (21.0 ± 15.7-fold higher), and TGFβ1/β-actin (28.6 ± 23.3-fold higher) than sialolithiasis and normal submandibular gland ($P<0.05$). In contrast, no significant difference was observed between the IL5/β-actin ratio in IgG4-related disease (0.606 ± 1.13) and those in sialolithiasis (0.119 ± 0.07) and normal submandibular gland (0.462 ± 0.369) (Figure 1b).

Next, the real-time PCR results were supported via immunostaining of cells by using primary antibodies against IL4, IL5, IL10, and TGFβ1 (Figure 2a). The number of IL4-positive cells was significantly higher in IgG4-related disease (4.04 ± 3.12 cells/mm^2) than in submandibular sialolithiasis (0.136 ± 0.186 cells/mm^2; $P<0.01$) and the normal submandibular gland (0.230 ± 0.356 cells/mm^2; $P<0.01$) (Figure 2b). Similarly, many IL10-positive cells were observed in IgG4-related disease (3.40 ± 1.84 cells/mm^2), whereas the submandibular sialolithiasis and normal submandibular gland contained few IL10-positive cells (0.342 ± 0.484 cells/mm^2, $P<0.01$; 0.226 ± 0.277 cells/mm^2, $P<0.01$, respectively) (Figure 2b). TGFβ1-positive cells were also more abundant in IgG4-related disease (4.29 ± 2.37 cells/mm^2) than in the submandibular sialolithiasis (1.51 ± 1.11 cells/mm^2; $P<0.05$) and normal submandibular gland (0.626 ± 0.548 cells/mm^2; $P<0.01$) (Figure 2b). Furthermore, we observed that TGFβ1-positive cells tended to infiltrate fibrous lesions.

In IgG4-related disease and control groups, the number of IL5-positive cells was much less than that of the other cytokines examined. No significant differences were observed between the number of IL5-positive cells in IgG4-related disease tissue (1.58 ± 1.44 cells/mm^2), sialolithiasis (0.838 ± 0.531 cells/mm^2; $P=0.450$), and normal submandibular gland tissue (0.811 ± 0.290 cells/mm^2; $P=0.332$) (Figure 2b).

Increased Density and Cytokine-Related Distribution of Mast Cells in IgG4-Related Disease

We compared the number of mast cells in the IgG4-related disease and control groups via immunostaining by using an antibody for KIT, which is a marker for mast cells. The number of KIT-positive mast cells was higher in IgG4-related disease (72.2 ± 24.5 cells/mm^2) than in the normal submandibular gland (30.0 ± 11.9 cells/mm^2; $P<0.01$) (Figure 3). However, no significant difference was observed between the number of mast cells in the IgG4-related disease and the submandibular sialolithiasis (177 ± 269 cells/mm^2; $P=0.73$) (Figure 3). Interestingly, the morphological features and distribution of the mast cells were similar to those of the T helper 2 (IL4 and IL5) or regulatory T-cell (IL10 and TGFβ1) cytokine-positive cells. Furthermore, dual immunofluorescence assays showed that KIT-positive mast cells were also positive for each of the IL4, IL5, IL10, and TGFβ1 cytokines (Figure 4). Additionally, although only a small number of IL5-positive cells was detected in the immunohistochemical experiments, these cells also exhibited KIT coexpression (Figure 4).

T-Cell Distribution in IgG4-Related Disease and Control Groups

To assess the number and distribution of T cells, we performed immunostaining assays with an antibody against FOXP3, which is a regulatory T-cell marker.