Table 1: Prevalence of anti-endothelial cell antibodies.

Disease	% of positive sera
Systemic lupus erythematosus	15–85
Rheumatoid arthritis	0–87
Mixed connective tissue disease	33–45
Systemic sclerosis	15–84
Polymyositis/dermatomyositis	44–64
Antiphospholipid syndrome	0–64
Sjögren's syndrome	24-25
Polyarteritis nodosa	50–56
Microscopic polyangiitis	2–60
Granulomatosis with polyangiitis	19–80
Eosinophilic granulomatosis with polyangiitis	50–69
Takayasu arteritis	54–95
Giant-cell arteritis	33–50
Behçet's disease	14–80
Kawasaki disease	65

for customized and specific therapeutic approaches against a highly pathogenic subset of autoantibodies using small molecules have been reported [5].

In 1971, Lindqvist and Osterland first described autoantibodies to vascular endothelium based on indirect immunofluorescence (IIF) experiments [6]. These autoantibodies were called anti-endothelial cell antibodies (AECAs) and were defined as autoantibodies targeting antigens present on the endothelial cell (EC) membrane [7]. As target antigens of AECAs are present on the ECs, which are always in contact with these circulating antibodies, AECAs have the potential to induce vascular lesions directly. Here, we present a review of AECAs and a novel method for identification of cell-surface autoantigens.

2. AECAs

2.1. AECAs and Disease. The presence of AECAs has been reported in patients with a wide variety of diseases, including collagen diseases (Table 1), inflammatory bowel disease, diabetes, thyroid diseases, thrombotic thrombocytopenic purpura, primary sclerosing cholangitis, interstitial lung disease, chronic obstructive lung disease, uveoretinitis, renal transplantation, Susac syndrome, masked hypertension, and atherosclerosis [8–23]. AECAs are correlated to disease activity in some collagen diseases, and are thought to be critical especially for vascular lesions in collagen diseases [23]. In addition, AECAs have been shown to be clinical signs of vasculitis in patients with systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) [24]. AECAs were also reported to play critical roles in several pathophysiological conditions, including pulmonary hypertension, digital ulcers, and gangrene [21, 22].

AECAs are detected even in healthy subjects [25, 26]. These natural autoantibodies interact with living ECs with lower affinity as compared to pathologic AECAs, and their antigens are highly conserved protein families. They contribute to modulate endothelial function with protective anti-inflammatory and anti-thrombotic functions [26].

2.2. Detection and Identification of AECAs. Methods for detection of AECAs have not been standardized, and a number of methods have been reported, including IIF, cell-based-enzyme linked immunosorbent assay (ELISA), flow cytometry, radioimmunoassay, western blotting (WB), and immunoprecipitation [22, 23]. As these each of methods have advantages and disadvantages, use of different technical approaches to obtain more robust data is recommended [7].

Human umbilical vein endothelial cells (HUVECs) are commonly used as a substrate, but antigen patterns of ECs differ among other ECs, passage numbers, and culture conditions [27]. It is also important whether ECs are fixed or not because fixation induces permeabilization of the EC membrane, and intracellular antigens become accessible to antibodies [22]. The results of AECA positivity were therefore not considered in the same light, and the prevalence of AECAs differed among studies (Table 1). Miura et al. recently reported a novel solubilized cell-surface protein capture ELISA for detection of AECAs [28], and further evaluation and standardization are needed.

2.3. Pathogenicity of AECAs. An experimental animal model for pathogenicity of AECAs was reported by Damianovich et al. [29]. In their experiment, BALB/c mice were actively immunized with the purified AECAs from a patient with granulomatosis with polyangiitis. Three months after a booster injection with human AECAs, mice developed endogenous AECAs, and histological examination of lungs and kidneys revealed both lymphoid cell infiltration surrounding arterioles and venules.

AECAs have been shown to be correlated with disease activities, and have the potential to induce vascular lesions because their targets are expressed on ECs that are readily accessible to these circulating antibodies. AECAs are also considered to play roles in the development of pathological lesions by a number of methods as described below [22, 23, 30–32].

The first is the cytotoxicity of ECs through complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC). CDC activity of AECAs was reported in patients with SLE, Takayasu arteritis, hemolytic-uremic syndrome, and Kawasaki disease [7, 24, 33–35]. Recently, we confirmed that fibronectin leucine-rich transmembrane protein 2 (FLRT2) is a novel target antigen of AECAs in SLE, which exerts direct cytotoxic effects through CDC [9].

The second is the induction of coagulation. AECAs may exhibit procoagulant effects by the production of tissue factor in SLE and the release of heparin sulfate in systemic sclerosis (SSc) [36, 37].

The third is the induction of apoptosis. AECAs may induce EC apoptosis through CD95 or cross-reaction with anti-phospholipid antibodies [38–40]. Dieudé et al. reported that heat-shock protein (Hsp60) bound to ECs and induced phosphatidylserine exposure and then apoptosis [41].

Margutti et al. identified antibodies to the C-terminus of Ral-binding protein 1 (RLIP76), and these autoantibodies induced oxidative stress-mediated EC apoptosis [42].

The fourth is the activation of ECs. AECAs were reported to induce the secretion of interleukin (IL)-1β, IL-6, IL-8, and monocyte chemotactic protein-1, (MCP-1), and the expression of adhesion molecules such as E-selectin, intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1) [8, 24, 31], which cause leukocyte recruitment and adhesion.

Alard et al. reported that recognition of cell-surface adenosine triphosphate (ATP) synthase in the low pH microenvironment contributes to intracellular acidification of ECs, which may induce cell death and trigger inflammation [43].

As described above, there is a great deal of evidence that AECAs play pathogenic roles in collagen diseases. Identification of targets of AECAs is required because (a) antigen-specific detection systems are important for establishing diagnostic tools and standardization of AECAs measurement, (b) identification will enable thorough analysis of the pathogenicity of AECAs, and (c) AECA-autoantigen interactions may be good targets for specific therapeutic approaches against highly pathogenic autoantibodies.

3. Technologies for Identification of Autoantigens for AECAs

The prevalence of AECAs varies according to the type of ECs used for detection [44]. It was demonstrated that AECAs cross-react with human fibroblasts [45], and partial inhibition of AECA activity was documented by absorption of the AECA-containing sera with mononuclear cells [8]. It was also reported that a structure shared by platelets and ECs was recognized by a subset of AECAs [46]. These data suggested that the target antigens of AECAs may include not only EC-specific but also non-EC-specific molecules.

Target antigens of AECAs have been investigated intensively, but they are heterogeneous, and the following classification of target antigens was proposed: membrane component, ligand-receptor complex, and molecule adhering to the plasma membrane [8]. The EC autoantigens may be either constitutively expressed or translocated from intracellular compartment to membrane by cytokines, such as IL-1 and tumor necrosis factor α (TNFα), or physical effects [8, 47]. The reported autoantigens and their pathogenicities are summarized in Table 2 [7, 9, 22–24, 42, 43, 47–56].

Several molecules can bind to ECs and are called "planted antigens" for AECA presumably via charge-mediated mechanisms, a DNA-histone bridge, or a specific receptor. Myeloperoxidase, DNA, and β2-glycoprotein I (β2-GPI) are thought to adhere to ECs during incubation of ECs with sera from patients. Extracellular matrix components, such as vimentin, may also be target antigens for AECAs [57]. Proteinase 3 (PR3) could represent another potential cryptic target antigen [58]. PR3 has been maintained to migrate to the plasma membrane of ECs, following stimulation [8].

As methods for identification of target antigens of AECAs, immunoprecipitation and WB of glycoproteins from the EC membrane with AECA-positive sera have been used [8, 23]. Although numerous protein bands were reported as candidates for target antigens by this method, some of the bands were considered to be artifacts [8], and further identification of given bands was also sometimes difficult.

Alternative methods have been developed, such as proteomics analysis using two-dimensional electrophoresis followed by matrix-assisted laser desorption ionization time of flight mass spectrometry [8, 23] and expression libraries [8, 42, 56].

Proteomics analysis identified vimentin, Hsp60, voltage-dependent anion-selective channel 1 (VDAC-1), peroxiredoxin 2, and ATP synthase as targets for AECAs [41, 43, 48–50]. Expression libraries also identified tropomyosin, T-plastin, and RLIP76 [42, 56], and these technologies are therefore promising. The problem is that most of the molecules reported to date as targets for AECAs are intracellular proteins (Table 2) although AECAs must be directed against the cell surface. These two methods are not specific for detecting cell-surface molecules rather than intracellular molecules. In addition, extraction of some membrane proteins has been reported to be difficult in proteomics analysis, and this may make it difficult to identify such proteins as AECA targets [7].

To overcome this problem, we constructed a novel expression cloning system for specific identification of cell-surface antigens [9], which we call serological identification system for autoantigens using a retroviral vector and flow cytometry (SARF) (Figure 1), and we have confirmed that this system is useful to identify autoantigens expressed on the EC surface [9].

4. Strategy for Identification of Cell-Surface Autoantigens: SARF

4.1. Generation of HUVEC cDNA-Expressing Cells (Figure 1(a)). Our strategy to identify AECA target molecules involves use of a retroviral vector system and flow cytometry [9]. As described previously, antigen patterns of ECs differ among other ECs [27]. Because we used HUVECs as a substrate for AECAs measurement, we generated a HUVEC cDNA library using HUVECs grown in the same conditions as for AECAs measurement and ligated it into the retroviral vector, pMX [59]. Then, the HUVEC cDNA library in pMX was retrovirally transfected into the YB2/0 rat myeloma cell line [60]. As the localization of cellular molecules depends on their structures, only cell-surface molecules are expressed on the surface of YB2/0 cells transfected with the HUVEC cDNA library.

4.2. Sorting of Cells Expressing Cell-Surface Autoantigens (Figure 1(b)). AECAs can bind only to cell-surface molecules in flow cytometry. Therefore, sorting of IgG-binding cells can concentrate and isolate cells expressing target molecules for AECAs on the cell surface. After staining of HUVEC cDNA-expressing YB2/0 cells with AECA IgG and secondary antibody, cells with strong fluorescent signals are sorted by flow cytometry. This step of sorting is repeated for several rounds to concentrate AECA IgG-binding cells. After concentration,

Table 2: Reported target antigens of anti-endothelial cell antibodies.

Disease	Target antigen	Pathogenicity
Systemic lupus erythematosus	DNA-DNA-histone	
	Ribosomal P protein PO	
	Ribosomal protein L6	
	Elongation factor 1-alpha	
	Adenylyl cyclase-associated protein	
	Profilin 2	
	Plasminogen activator inhibitor	
	Fibronectin	
	Heparan sulfate	
	β2-glycoprotein I	
	Heat-shock protein 60 (Hsp 60)	Apoptosis
	Heat-shock protein 70 (Hsp 70)	
	Fibronectin leucine-rich transmembrane protein 2 (FLRT2)	Complement-dependent cytotoxicity
Mixed connective tissue disease	Voltage-dependent anion-selective channel 1 (VDAC-1)	
Systemic sclerosis	Topoisomerase I	
	Centromere protein B (CENP-B)	
Vasculitis	Proteinase 3	
	Myeloperoxidase	
	Peroxiredoxin 2	Cytokine secretion
	Adenosine triphosphate (ATP) synthase	Intracellular acidification
Microscopic polyangiitis	Human lysosomal-associated membrane protein 2	
Behçet's disease	Alpha-enolase	
	C-terminus of Ral-binding protein 1 (RLIP76)	Apoptosis
Kawasaki disease	Tropomyosin	
	T-plastin	
Transplantation	Vimentin	
	Keratin-like protein	
Thrombotic thrombocytopenic purpura	Glycoprotein CD36	
Heparin-induced thrombocytopenia	Platelet factor 4 (PF4)	
	Heparin sulfate	

several cell clones can be established from the AECA IgG-binding cell population by the limiting dilution method.

4.3. Identification of Novel Cell-Surface Autoantigens. After polymerase chain reaction (PCR) amplification and cloning of HUVEC cDNA inserted into the genomic DNA of cloned cells, DNA sequencing can be performed followed by BLAST analysis, which enables the identification of the inserted cDNA. In this step, microarray analysis is an alternative method to identify the inserted cDNA. Next, an expression vector of the identified cDNA is generated and transfected into a cell line that does not express the identified protein. Finally, it is necessary to confirm that AECA IgG shows binding activity to 7-amino-actinomycin D-(7-AAD-) negative identified protein-expressing cells. If the binding activity is confirmed, it can be concluded that the identified protein is a novel autoantigen.

5. Novel Autoantigens Identified by SARF

5.1. FLRT2. We reported the membrane protein FLRT2 as a novel autoantigen of AECAs in patients with SLE based on results obtained using SARF [9]. FLRT2 is type I transmembrane protein located on the plasma membrane [61]. FLRT2 was shown to be expressed in the pancreas, skeletal muscle, brain, and heart with Northern blotting [61], and we confirmed the expression of FLRT2 on HUVECs and other ECs by flow cytometry and IIF [9]. Anti-FLRT2 antibody activity accounted for 21.4% of AECAs in SLE, and anti-FLRT2 activity was significantly correlated with low levels of complement C3, C4, and CH50 [9]. Anti-FLRT2 antibody induced CDC against FLRT2-expressing cells including ECs, indicating that anti-FLRT2 autoantibody may exhibit direct pathogenicity [9].

5.2. ICAM-1. As AECAs can be detected in patients with collagen diseases, especially SLE, RA, and Takayasu arteritis [9], we further attempted to identify the autoantigens using SARF. One sample (X10-3) from an RA patient showed strong AECA activity (Figure 2(a)), and we selected this serum sample as the prototype of AECA for subsequent cell sorting. Using SARF, HUVEC cDNA-expressing YB2/0 cells were stained with X10-3 IgG and fluorescein isothiocyanate-(FITC-) conjugated secondary antibody, and cells with strong

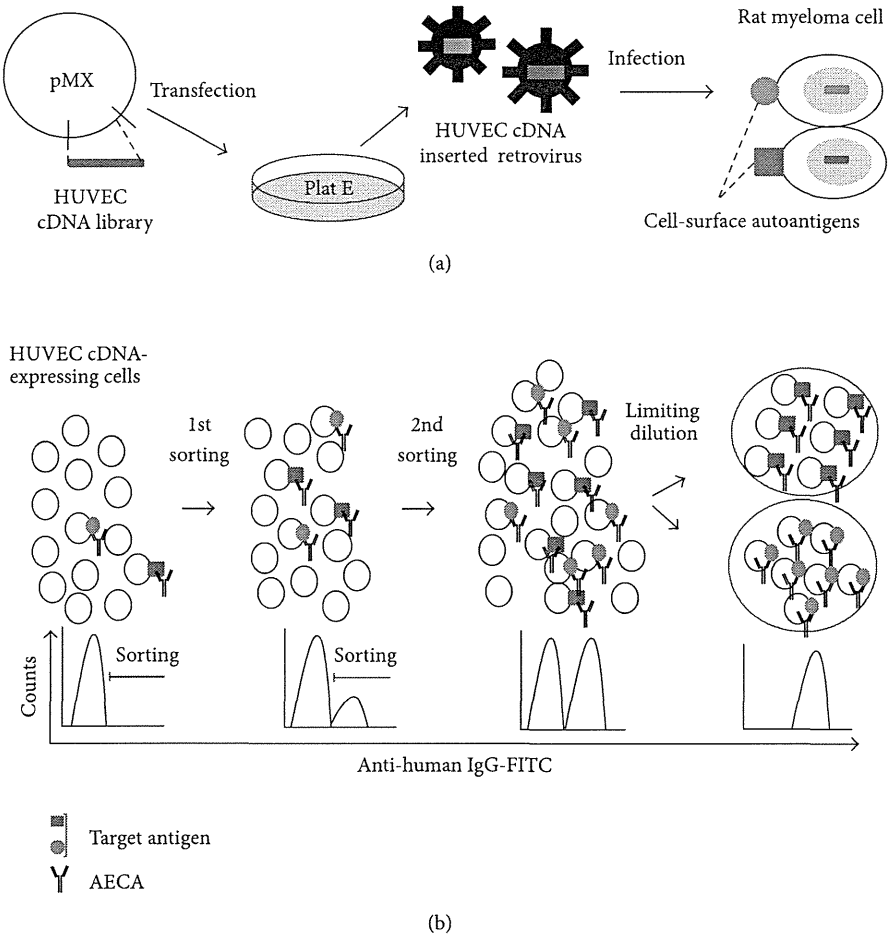

FIGURE 1: Serological identification system for autoantigens using a retroviral vector and flow cytometry (SARF). (a) Generation of human umbilical vein endothelial cell (HUVEC) cDNA-expressing cells. (b) Sorting of cells expressing cell-surface autoantigens.

FITC signals were sorted by flow cytometry (Figure 2(b)). After the 4th sorting, cells bound to X10-3 IgG were markedly increased (Figure 2(c), left), and the C5 clone was established from the X10-3 IgG-binding cell population by the limiting dilution method (Figure 2(c), right). Microarray analysis revealed that the signal of ICAM-1 was significantly increased ($2^{6.16}$-fold), and we confirmed that the ICAM-1 cDNA was inserted into the genomic DNA of X10-3-C5 clone (Figure 2(d)). We also confirmed the expression of ICAM-1 on the X10-3-C5 clone (Figure 2(e)). Next, we generated an expression vector for ICAM-1, which was transfected into YB2/0 cells. X10-3 IgG showed significant binding activity to 7-AAD-negative ICAM-1-expressing YB2/0 cells (Figure 2(f)), indicating that X10-3 IgG has anti-ICAM-1 activity. Thus, the membrane protein ICAM-1 was identified as a novel autoantigen of AECA in RA. ICAM-1 is an immunoglobulin-(Ig-) like cell adhesion molecule expressed by several cell types, including leukocytes and ECs. ICAM-1 plays an important role in both innate and adaptive immune responses. It is involved in the transendothelial migration of leukocytes to sites of inflammation, as well as in interactions between antigen presenting cells (APC) and T cells (immunological synapse formation) [62].

ICAM-1 was also confirmed to transduce signals "outside in" [63, 64]. The cross-linking of ICAM-1 with monoclonal antibodies was reported to activate the mitogen-activated protein kinase (MAPK) kinases ERK-1/2 and/or JNK [65–67]. The activation of ERK-1 lead to AP-1 activation [66], the ERK-dependent production and secretion of IL-8 and RANTES [67], and upregulation of VCAM-1 on the cell surface [66, 68]. ICAM-1 cross-linking can also upregulate tissue factor production [69] and proinflammatory cytokines, including IL-1 [70]. Lawson et al. reported production of anti-ICAM-1 IgM after cardiac transplantation, and the antibody induced robust activation of the ERK-2 MAPK pathway [71]. The use of anti-ICAM-1 antibody was examined for the treatment of RA, but the second course of therapy was associated with adverse effects suggestive of immune complex formation [72]. Identification of anti-ICAM-1 antibody in a patient with RA suggested that this autoantibody may exhibit such pathogenic roles.

5.3. Pk (Gb3/CD77). Using serum from an SLE patient who showed hemolytic anemia, SARF revealed that cDNA inserted into the cloned cells that were sorted with this AECA-IgG was alpha 1,4-galactosyltransferase (A4GALT).

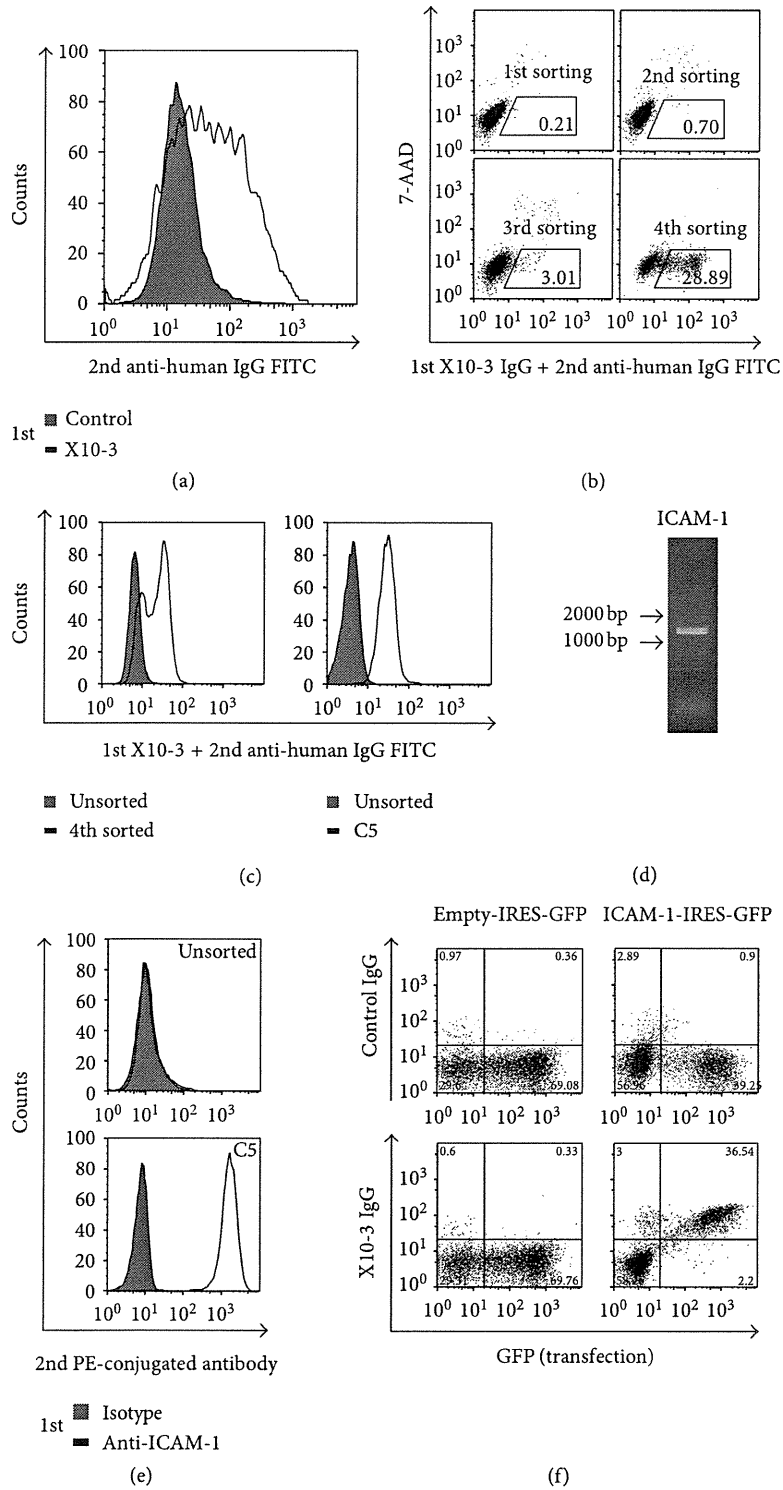

FIGURE 2: Identification of intercellular adhesion molecule 1 (ICAM-1) as a target antigen of anti-endothelial cell antibodies (AECAs). (a) Nonpermeabilized HUVECs were stained with 0.5 mg/mL of IgG of control or X10-3 from a patient with rheumatoid arthritis followed by secondary antibody and analyzed by flow cytometry. (b) HUVEC cDNA-expressing cells were stained with 0.5 mg/mL of X10-3 IgG followed by secondary antibody, and cells in the positive fraction were sorted (black box). (c) Unsorted and 4th sorted cells (left) and unsorted and cloned cells from 4th sorted cells, C5 (right), were stained with 0.5 mg/mL of X10-3 IgG followed by secondary antibody and analyzed by flow cytometry. (d) ICAM-1 cDNA fragments inserted into the genomic DNA of C5 were amplified, and PCR products were electrophoresed on an 0.8% agarose gel. (e) Unsorted and C5 were stained with isotype control or anti-ICAM-1 antibody, followed by secondary antibody and analyzed by flow cytometry. (f) Expression vector, empty-IRES-GFP, or ICAM-1-IRES-GFP were transfected into YB 2/0 cells, and these cells were stained with 0.5 mg/mL of control IgG or X10-3 IgG, followed by secondary antibody and analyzed by flow cytometry.

This AECA showed significant binding activity to 7-AAD-negative A4GALT-overexpressing YB2/0 cells. The A4GALT locus encodes a glycosyltransferase that synthesizes the terminal Galα1-4Gal of Pk (Gb3/CD77) glycosphingolipid [73, 74]. This means that synthesis of the terminal Galα1-4Gal is needed for the binding of this AECA-IgG.

Gb3 is the Pk blood group antigen and has been designated CD77 [74]. Monoclonal antibodies against Pk (Gb3/CD77) are used as markers for Burkitt's B-cell lymphoma and are able to initiate apoptosis [75]. Pk (Gb3/CD77) plays a direct role in the entry of Shiga toxin into the cell [76], and the presence of Pk (Gb3/CD77) in the ECs of the kidney accounts for the development of hemolytic uremic syndrome during bacterial infection with Shigella species that produce verotoxin [77]. The anti-Pk (Gb3/CD77) antibody was reported to cause acute intravascular hemolytic transfusion reactions and recurrent spontaneous abortions due to damage to the placenta [73, 78]. These data suggested that Pk (Gb3/CD77) is one of the target antigens of AECAs in SLE patients manifesting hemolytic anemia, and that anti-Pk (Gb3/CD77) antibody may exhibit some pathogenic roles.

Identification of A4GALT indicated the usefulness of SARF, which can be used to identify genes that encode not only the membrane protein itself, but also the transferase(s) responsible for modifying the membrane protein.

As described above, this system is very useful for identification of cell-surface autoantigens. Although this system seems to present difficulties in sorting cells at very low frequency, we could isolate and clone autoantigen-expressing cells by repeated sorting.

As AECAs are a heterogeneous group of autoantibodies that target ECs, it is predicted that there are different autoantigens. Thus, it is important to determine the clinical significance and potential pathogenicity of identified autoantibodies. If an autoantibody is specific for a disease or pathophysiology, it could be used as a marker for diagnosis or classification according to the underlying pathophysiology. At the same time, the pathogenic potential of the autoantibody should also be examined. Along with in vitro studies mentioned previously, experimental animal models of identified autoantibody should be constructed to determine the pathogenetic reactions in vivo.

6. Summary

AECAs are considered to be critical, especially for vascular lesions in collagen diseases, but most are directed against molecules localized within the cell and not expressed on the cell surface. In addition to conventional immunoprecipitation and WB, proteomics and expression library analyses have been performed to identify the targets for AECAs with some success. SARF was developed to identify autoantigens expressed on the EC surface with greater sensitivity. Using SARF, we successfully identified three different membrane proteins as targets for AECAs: FLRT2 from patients with SLE, ICAM-1 from a patient with RA, and Pk (Gb3/CD77) from an SLE patient with hemolytic anemia. Using this technology, it may be possible to determine cell-surface autoantigens of AECAs and achieve a comprehensive understanding of AECA-mediated vascular injury. Furthermore, SARF can be used when autoantibodies against cell-surface molecules are considered to take part in autoimmune diseases. The identification of such pathogenic autoantibodies may enable the development of more specific intervention strategies in autoimmune diseases.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Original article

Anti-MDA5 antibody, ferritin and IL-18 are useful for the evaluation of response to treatment in interstitial lung disease with anti-MDA5 antibody-positive dermatomyositis

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Abstract

Objective. The aim of this study was to investigate the precise clinical characteristics and to analyse the association between the anti-MDA5 antibody (anti-MDA5ab) titre and disease status in patients with anti-MDA5ab-positive DM.

Methods. Twenty-seven patients who presented with DM and were positive for the anti-MDA5ab were enrolled. The association between the clinical manifestations and the clinical parameters, including the anti-MDA5ab, was analysed.

Results. The complication of rapidly progressive interstitial lung disease (RP-ILD) occurred in 20 (74%) patients. The frequencies of fatal outcome, relapse and malignancy were 33, 4 and 4%, respectively. Remarkably, a fatal outcome occurred within the first 6 months. Compared with six non-RP-ILD patients, elderly age at onset, severely involved pulmonary function and high levels of serum ferritin were present in 20 RP-ILD patients with anti-MDA5ab. Alveolar–arterial oxygen difference ($AaDO_2$) $\geqslant$32 mmHg and ferritin $\geqslant$828 ng/ml at admission were poor prognostic factors in RP-ILD patients with anti-MDA5ab-positive DM. The median value of the anti-MDA5ab titre on admission was higher in patients who later died than in those who survived. The efficacy of treatment was indicated by the anti-MDA5ab, ferritin and IL-18 concentrations. The decline index of the anti-MDA5ab titre after treatment was lower in the subset of patients who died than in the subset of patients who lived. Sustained high levels of anti-MDA5ab, ferritin and IL-18 were present in the patients who died.

Conclusion. Anti-MDA5ab titre and ferritin and IL-18 concentrations are useful for the evaluation of the response to treatment and the status of ILD in patients with anti-MAD5ab-positive DM.

Key words: dermatomyositis, interstitial lung disease, anti-MDA5 antibody, ferritin, interleukin-18.

Introduction

DM is characterized by inflammation of the skin and muscles [1]. Rapidly progressive interstitial lung disease (RP-ILD) in particular is of prime importance in the clinical management of patients with DM because it is an intractable and life-threatening complication [2–5]. Clinically amyopathic DM (CADM) includes typical skin lesions with amyopathy or hypomyopathy [6]. CADM patients with the anti-MDA5 antibody (anti-MDA5ab) frequently develop the complication of RP-ILD, especially in Japan [7–10]. Sato *et al.* [7] identified melanoma differentiation-associated gene 5 (MDA5) as the CADM-140 antigen. The MDA5 protein plays a role in the innate immune response. MDA5 initially recognizes picornaviruses and evokes antiviral responses by eliciting the production of type I IFNs

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and TNF-α [11]. We previously reported that high levels of ferritin are associated with the development and prognosis of RP-ILD with DM [9, 12]. In addition, IL-18 is a potential contributor to ILD with DM [13]. High levels of ferritin and IL-18 are also implicated in macrophage activation syndrome (MAS) [14, 15]. Although a cytokine storm may contribute to the pathogenesis of RP-ILD with anti-MDA5ab-positive DM, especially in the skin and lungs, the precise pathogenesis remains unknown. Moreover, long-term prognosis, frequency of recurrence, complication with malignancy and the association between the anti-MDA5ab titre and the clinical course remain unclear in anti-MDA5ab-positive DM.

Thus we investigated the clinical characteristics and the correlation between the anti-MDA5ab titre and clinical parameters, such as ferritin and IL-18 levels, in patients with anti-MDA5ab-positive DM. In addition, we analysed the association between the anti-MDA5ab titre and the clinical course in these patients.

Patients and methods

Patients

The present retrospective study included patients with idiopathic inflammatory myopathy who were admitted to the Tokyo Women's Medical University Aoyama Hospital or Keio University Hospital from August 1992 to December 2009. All of the enrolled patients suffered from skin rash, myopathy or respiratory symptoms (or a combination thereof) at admission. These patients were diagnosed with DM or CADM based on the criteria of Bohan and Peter [16] or Sontheimer [17], respectively. In general, CADM presents with typical skin lesions and either amyopathy or hypomyopathy for >6 months. A subset of the CADM group included patients who developed fatal ILD within the first 6 months of this study. Medical records were obtained from 142 and 53 patients who were diagnosed with DM and CADM, respectively. In the present study, 5 DM patients and 22 CADM patients who were positive for the anti-MDA5ab were enrolled. The frequencies of anti-MDA5ab positivity were 4 and 42% in the DM patients and in the CADM patients, respectively. Clinical data were obtained from medical records on admission. The study was approved by the ethical committee of the Institute of Rheumatology, Tokyo Women's Medical University, and the study complied with the Declaration of Helsinki guidelines. Disease duration was defined as the time between the appearance of symptoms, such as skin rash, myopathy or respiratory symptoms, and the initiation of treatment.

Evaluation of clinical laboratory parameters and the anti-MDA5ab

Blood tests evaluated creatine kinase (CK), lactate dehydrogenase (LD), KL-6, CRP, ferritin and ANA. Serum IL-18 was measured with an ELISA (R&D Systems, Minneapolis, MN, USA). The median level (range) of IL-18 was 50.5 (18–121) pg/ml in 30 healthy controls. Anti-MDA5ab was detected with an ELISA using recombinant MDA-5 as an antigen, as described previously [7]. The normal value for the anti-MDA5ab titre was ⩽8 U/ml.

Evaluation of pulmonary function and classification of ILD

The PaO_2/F_iO_2 (P/F ratio), pulse oximeter oxygen saturation/FiO_2 (S/F ratio), alveolar–arterial oxygen difference ($AaDO_2$), forced expiratory volume in 1 s (FEV1)/forced vital capacity (FVC) ratio, VC percentage (%VC) and diffusing capacity of the lung for carbon monoxide (DLco) were used to evaluate pulmonary function. The normal values are defined as >380 for the P/F ratio, >450 for the S/F ratio, <10 mmHg for $AaDO_2$, >70% for the FEV1/FVC ratio, >80% for %VC and >20 ml/min/mmHg for the DLco. The ILD was assessed with chest radiography and CT or high-resolution CT of the chest. RP-ILD is defined as a progressive ILD within 3 months of the onset of respiratory symptoms. Chronic ILD is defined as an asymptomatic, non-rapidly progressive ILD or slowly progressive ILD over 3 months by the International Consensus Statement of Idiopathic Pulmonary Fibrosis of the American Thoracic Society and the European Respiratory Society [18].

Statistical analysis

Statistical analyses were performed using the Student's *t*-test to compare mean values, the Mann–Whitney U-test to compare median values and Fisher's exact test to compare frequencies. Correlation coefficients were established by employing Spearman's correlation coefficients. The cumulative survival rate was calculated using the Kaplan–Meier test. The Wilcoxon signed-rank test was performed when comparing clinical parameters upon admission with those parameters after treatment in each patient. The data were analysed using JMP® software (SAS Institute, Cary, NC, USA). A value of $P < 0.05$ indicated statistical significance.

Results

Clinical characteristics in patients with anti-MDA5ab-positive DM

The clinical characteristics of 27 patients with anti-MDA5ab-positive DM are shown in Table 1. The laboratory data were obtained at the first examination upon admission. The frequency of CADM was 81%. The median value of CK was 92 IU/l (interquartile range: 67–271). The complication of RP-ILD was present in 20 (74%) patients. Six additional patients had the complication of chronic ILD, and one patient had neither complication. Although the values of FEV1/FVC ratio and %VC were normal in almost all patients, the DLco levels were decreased. The median values of KL-6, CRP, ferritin and IL-18 were high. ANA positivity was found in four patients (homogeneous and speckled pattern in two patients, homogeneous pattern in one patient and nucleolar pattern in one patient). In 9 of the other 23 patients without ANA positivity, a cytoplasmic pattern was revealed. The frequencies of the fatal

Table 1 Clinical characteristics of patients with anti-MDA5ab-positive DM ($n = 27$)

Characteristic	Value
Age, years	48 (13)
Female, *n* (%)	20 (74)
Disease duration, weeks	6 (3–8)
CADM, *n* (%)	22 (81)
RP-ILD, *n* (%)	20 (74)
P/F ratio	348 (324–438)
$AaDO_2$, mmHg	26.2 (10.2–41.5)
FEV1/FVC ratio ($n = 18$)	82 (78–89)
%VC ($n = 22$)	76 (71–84)
DLco, ml/min/mmHg ($n = 9$)	10 (9.1–13.6)
LD, IU/l	382 (253–512)
KL-6, U/ml (normal value $\leqslant 500$) ($n = 23$)	735 (570–985)
CRP, mg/dl	0.63 (0.13–1.37)
Ferritin, ng/ml	642 (217–1120)
IL-18, pg/ml (normal range 18–121) ($n = 21$)	550 (328–746)
ANA $\geqslant 160\times$, *n* (%)	4 (15)
Fatal outcome, *n* (%)	9 (33)
Relapse, *n* (%)	1 (4)
Malignancy, *n* (%)	1 (4)

The values of age indicate the mean (S.D.), and the laboratory markers and pulmonary function tests are presented as the median (interquartile range).

outcome, relapse and malignancy were 33, 4 and 4%, respectively.

Comparison of clinical manifestations between patients with anti-MDA5ab-positive DM with and without RP-ILD

Clinical manifestations were compared between patients who had anti-MDA5ab-positive DM with and without RP-ILD (Table 2). The following information indicates the significant results for the patients with RP-ILD: elderly age at onset ($P = 0.0021$), decreased P/F ratio ($P = 0.0079$), increased $AaDO_2$ ($P = 0.0031$), increased ferritin ($P = 0.036$) and high frequency of fatal outcome ($P = 0.036$). The median values of %VC and DLco were lower in patients with RP-ILD than in those without RP-ILD, although the difference was not statistically significant. The ferritin level was significantly higher in the patients with RP-ILD. The frequency of fatal outcome was high: 45% in the patients with RP-ILD. The cut-off value as a predictor for RP-ILD was estimated by a receiver operating characteristic (ROC) curve of age at onset, P/F ratio, $AaDO_2$ and ferritin. The following parameters can be used as cut-off values (odds ratio, *P*-value): age $\geqslant 46$ years (14, 0.011), P/F ratio <438 torr (23, 0.0047), Aa $DO_2 \geqslant 22$ mmHg (34, 0.0017) and ferritin $\geqslant 217$ ng/ml (48, 0.0014).

No association between anti-MDA5ab titre and clinical parameters

Correlation coefficients between the anti-MDA5ab titre and clinical parameters were established in patients with anti-MDA5ab-positive DM. The clinical parameters included $AaDO_2$, %VC and laboratory markers (KL-6, CRP, ferritin and IL-18). All of these clinical parameters were obtained from 18 patients at the first examination upon admission. There was no significant correlation between anti-MDA5ab titre and other clinical parameters. Significant correlations were only found between $AaDO_2$ and ferritin ($r_s = 0.47$, $P = 0.014$) in patients with anti-MDA5ab-positive DM.

Comparison of clinical manifestations in living patients and patients who died with RP-ILD with anti-MDA5ab-positive DM

We analysed the clinical manifestations of the patients who had anti-MDA5ab-positive DM and died, and compared them with the manifestations of the surviving anti-MDA5ab-positive DM patients with RP-ILD (Table 3). The P/F ratio and $AaDO_2$ on admission were significantly worse and the ferritin levels were significantly higher ($P = 0.017$) in the patients who died. The median anti-MDA5ab titre was higher, although not significantly ($P = 0.099$), in patients who died than in those who survived. RP-ILD was refractory and progressive in the patients who died, although almost all of these patients received combination therapy, including prednisolone (PSL), i.v. CYC therapy (IVCY) and calcineurin inhibitor (CNI). The cut-off values as a predictor of fatal outcome in RP-ILD were estimated by the ROC curve of the P/F ratio, $AaDO_2$ and ferritin and are as follows (odds ratio, *P*-value): P/F ratio <324 torr (9.3, 0.035), Aa $DO_2 \geqslant 32$ mmHg (9.3, 0.035) and ferritin $\geqslant 828$ ng/ml (14, 0.025).

Survival rates of patients with anti-MDA5ab-positive DM

The cumulative 100-month survival rate was 66% for the entire anti-MDA5ab-positive DM patient group (Fig. 1A). Fatal outcome occurred remarkably often within the first 6 months. The median survival duration was 2 months in the nine patients who died. In contrast, the median survival duration was 29 months in the 18 surviving patients. Next, the patients with anti-MDA5ab-positive DM were divided into an RP-ILD subset and a non-RP-ILD subset. As shown in Fig. 1B, the cumulative 100-month survival rates were significantly lower in the RP-ILD subset than in the non-RP-ILD subset (log-rank test, $P = 0.039$).

Association between the anti-MDA5ab titre and the clinical course in patients with anti-MDA5ab-positive DM

We investigated the association between the clinical parameters and the clinical course. Clinical parameters included the anti-MDA5ab titre, the S/F ratio, KL-6, ferritin and IL-18 concentrations. Seventeen patients with anti-MDA5ab-positive DM, including 15 patients with RP-ILD and 2 patients with chronic ILD, were enrolled. Eleven patients were categorized as the living subset and the remaining six patients formed the dead subset. All six patients in the dead subset had the complication of

Table 2 Comparison of the clinical manifestations between patients with anti-MDA5ab-positive DM with and without RP-ILD

Variable	RP-ILD (–) ($n=7$)	RP-ILD (+) ($n=20$)	*P*
Age, years	35 (4)	52 (2)	0.0021
Female, *n* (%)	6 (86)	14 (70)	0.63
Disease duration, weeks	8 (6–16)	4 (2–8)	0.098
CADM, *n* (%)	6 (86)	16 (80)	1
CK, IU/l	165 (84–271)	85 (47–345)	0.36
LD, IU/l	472 (221–643)	373 (267–500)	0.51
P/F ratio	448 (348–522)	339 (308–388)	0.0079
$AaDO_2$, mmHg	4 (0–18)	30 (24–54)	0.0031
%VC	82 (74–98) ($n=6$)	76 (67–82) ($n=16$)	0.21
DLco, ml/min/mmHg	14.7 (12.5–16.9) ($n=2$)	9.6 (8.9–12.7) ($n=7$)	–
KL-6, U/ml (normal value $\leqslant$500)	346 (278–1104) ($n=5$)	801 (675–1009) ($n=18$)	0.1
CRP, mg/dl	0.46 (0.02–0.80)	0.72 (0.15–1.85)	0.22
Ferritin, ng/ml	186 (120–626)	835 (285–1480)	0.036
IL-18, pg/ml (normal range 18–121)	550 (216–736) ($n=5$)	552 (243–765) ($n=16$)	0.65
Anti-MDA5ab, U/ml	258.8 (217.1–542.7)	152.3 (56.7–376.8)	0.21
Fatal outcome, *n* (%)	0 (0)	9 (45)	0.036

The values of age indicate the mean (S.D.), and laboratory markers and pulmonary function tests are presented as the median (interquartile range).

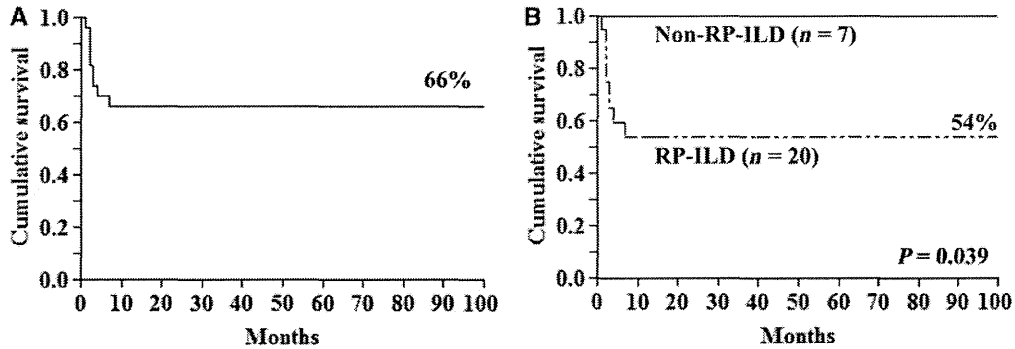
Table 3 Comparison of the clinical manifestations between living patients (alive) and patients who died (dead) with RP-ILD and anti-MDA5ab-positive DM upon admission

Variable	Alive ($n=11$)	Dead ($n=9$)	*P*
Age, years	50 (3)	54 (3)	0.29
Female, *n* (%)	9 (82)	5 (56)	0.34
Disease duration, weeks	8 (2–12)	4 (3–7)	0.76
CADM, *n* (%)	9 (82)	7 (78)	1
CK, IU/l	95 (38–383)	77 (62–324)	0.91
P/F ratio	369 (331–403)	319 (246–352)	0.03
$AaDO_2$, mmHg	26 (22–34)	41 (30–102)	0.044
%VC	76 (71–90) ($n=10$)	71 (62–78) ($n=6$)	0.25
DLco, ml/min/mmHg	9.5 (7.8–13.2) ($n=4$)	10 (8.9–12.7) ($n=3$)	–
LD, IU/l	364 (243–488)	460 (308–518)	0.3
KL-6, U/ml (normal value $\leqslant$500)	842 (678–1009) ($n=10$)	731 (602–1099) ($n=8$)	0.59
CRP, mg/dl	0.63 (0.10–1.96)	1.06 (0.17–2.16)	0.7
Ferritin, ng/ml	409 (248–843)	1600 (835–1935)	0.017
IL-18, pg/ml (normal range 18–121)	503 (343–727) ($n=10$)	540 (338–798) ($n=7$)	0.70
Anti-MDA5ab, U/ml	129.3 (44.6–254.0)	332.1 (92.0–599.8)	0.099
Treatment			
PSL + IVCY + CNI	5 (46)	7 (78)	0.2
PSL ± IVCY or CNI	6 (54)	2 (22)	
Improvement of ILD	11 (100)	0 (0)	<0.0001

The age values are presented as the mean (S.D.), and laboratory markers and pulmonary function tests are presented as the median (interquartile range).

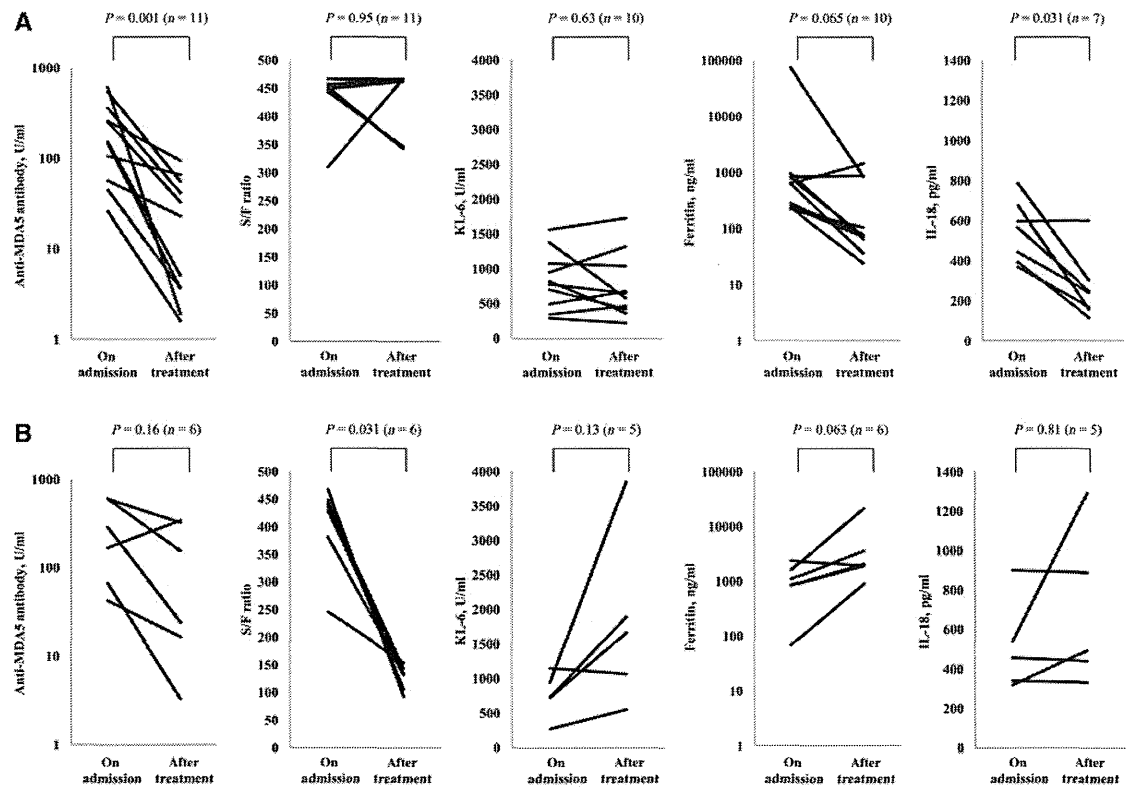
refractory RP-ILD and died within 6 months of treatment because of respiratory failure resulting from RP-ILD. We compared the clinical parameters upon admission with the parameters after treatment in each subset (Fig. 2). The median duration of evaluation after treatment was 3 months in the living subset and 2 months in the dead subset. There was no significant difference ($P=0.21$) between the two subsets in terms of the duration of evaluation after treatment. The data for each clinical parameter could only be partially obtained in some patients. The number of patients for whom data were obtained is indicated in each figure panel.

FIG. 1 Cumulative 100-month survival rates for all patients with anti-MDA5ab-positive DM (**A**), and the RP-ILD and non-RP-ILD subsets of anti-MDA5ab-positive DM patients (**B**).



The cumulative 100-month survival rates were calculated using the Kaplan–Meier test. The log-rank test was also used to compare survival rates. Survival rates and *P*-values are indicated in each figure panel.

FIG. 2 Comparison between clinical parameters upon admission and after treatment in patients with anti-MDA5ab-positive DM.



Based on patient survival, we analysed clinical parameters in two subsets: the living subset and the dead subset (**A** and **B**). The number of patients for whom data were obtained is indicated in each figure panel. Statistical analyses were performed with the Wilcoxon signed-rank test for comparisons of median values.

Anti-MDA5ab titre was significantly lower ($P = 0.001$) after treatment than on admission in the living subset (Fig. 2A). Anti-MDA5ab disappeared after treatment in 5 (45%) of the 11 living patients. On the other hand, there was no statistically significant difference ($P = 0.16$) in the dead subset between the anti-MDA5ab titre upon admission compared with the antibody titre after treatment (Fig. 2B). Anti-MDA5ab was still present after treatment in all dead patients except one. Moreover, the decline index of the anti-MDA5ab titre after treatment was analysed and compared among each subset. The decline index of the anti-MDA5ab was calculated as follows: (the antibody titre after treatment—the antibody titre upon admission) × 100/(the antibody titre upon admission). The median decline indices of the anti-MDA5ab titre (interquartile range) were 90% (63–97%) and 68% (9–92%) in the living and dead subsets, respectively.

In the dead subset, the S/F ratio was significantly lower after treatment ($P = 0.031$). The levels of KL-6 tended to decrease in the living subset and increase in the dead subset. On the other hand, the levels of ferritin more sensitively reflected the response to treatment than the levels of KL-6. The median values of ferritin after treatment were 76 ng/ml and 1987 ng/ml in the living and dead subsets, respectively ($P = 0.0017$). Moreover, the levels of IL-18 were significantly lower ($P = 0.031$) after treatment in the living subset. In the dead subset, the levels of IL-18 were not significantly lower after treatment.

Discussion

We have measured the clinical characteristics of disease and have demonstrated an association between clinical parameters and clinical course in patients with anti-MDA5ab-positive DM. The clinical manifestations of anti-MDA5ab-positive DM have been reported, mainly in Japanese studies [7–10]. Two different subsets of ILD with CADM patients are those with RP-ILD or with chronic ILD [19]. Fathi *et al.* [20] have reported that patients with inflammatory myopathy with ILD require careful evaluation of their clinical features because the course of ILD cannot be predicted at the first examination. However, we determined that investigation of both the anti-MDA5ab and the serum ferritin concentration are useful for predicting the onset of RP-ILD in DM [7, 21]. On the other hand, the serum ferritin level was <500 ng/ml in some patients with DM-associated RP-ILD [12]. These patients with RP-ILD were occasionally positive for the anti-aminoacyl-tRNA synthetase antibody and appeared to be well controlled with CSs and immunosuppressant agents compared with patients with DM-associated RP-ILD having anti-MDA5ab and/or hyperferritinaemia. This distinction in response to treatments might be responsible for the cellular phenotypes affecting the pathogenesis of ILD. Taken together, if the serum ferritin level is high in patients with DM, it should be considered that these patients may have anti-MDA5ab, and their clinical course may be complicated by RP-ILD.

CADM with RP-ILD showed a rapidly progressive pattern with a 6-month survival rate of 40.8–45%, which corresponded to the results in our study [22, 23]. In our study, $AaDO_2$ levels ⩾32 mmHg and ferritin levels ⩾828 ng/ml on admission were poor prognosis factors for RP-ILD with anti-MDA5ab-positive DM. The median anti-MDA5ab titre on admission was higher in the patients who died than in the living patients, although the difference between the two subsets was not statistically significant. However, the median anti-MDA5ab titre on admission was higher in the patients without RP-ILD than in those with RP-ILD. The anti-MDA5ab titre before treatment was not predictive of the prognosis of RP-ILD in anti-MDA5ab-positive DM. Measuring levels of serum ferritin and $AaDO_2$ before treatment is useful for predicting the prognosis of RP-ILD in DM.

We analysed the association between the anti-MDA5ab titre and the clinical course. We confirmed that the anti-MDA5ab titre has disappeared in improving surviving patients in our longitudinal observation (data not shown). Relapse has not occurred in any of the improving surviving patients except one. In the future we will investigate whether the anti-MDA5ab titre is increased again in either a pulmonary flare or skin exacerbation. Moreover, we have analysed several patients, in whom the serum ferritin level and IL-18 level were high, and were correlated with the clinical course in patients with RP-ILD with DM [24]. Immunosuppressive therapy had some effect on clinical parameters such as cytokines and antibodies regardless of clinical course. In the present study, however, immunosuppressive therapy was received more intensively in the dead subset than in the living subset of patients with anti-MDA5ab-positive DM. The frequency of receiving PSL + CNI + IVCY was higher in the dead subset than in the living subset. Moreover, there was no significant difference between the two subsets in terms of the duration of evaluation after treatment. Taken together, the sustained high levels of anti-MDA5ab, ferritin and IL-18 could be attributed to the poor response to treatment in the dead subset. Investigations of the anti-MDA5ab titre, ferritin level and IL-18 level after treatment are useful for predicting the clinical course and evaluating the response to treatment in patients with ILD with anti-MDA5ab-positive DM.

The levels of serum ferritin and IL-18 were associated with the status of ILD with anti-MDA5ab in the present study, as shown in previous reports [9, 12, 24]. Serum ferritin is an important laboratory finding of MAS [14]. MAS is now an accepted term that is used to refer to a form of secondary haemophagocytic lymphohistiocytosis observed in the context of rheumatic disorders [14, 15]. The pathophysiology of MAS involves a lack of T lymphocyte regulation and the excessive production of cytokines, such as TNF-α, IL-1β, IL-6 and IL-18, resulting in the activation of macrophages [15, 25]. The mRNA for IL-18 and IL-12 is readily detected in Kupffer cells and activated macrophages, and dendritic cells produce IL-18 in active inflammatory myopathies [26, 27]. We also reported that IL-18 is a key mediator in ILD with DM [13]. Moreover,

alveolar macrophages activated by some antigens, microbes and autoimmune stimuli are induced to produce leukotriene B4 and IL-8. These factors stimulate neutrophils to induce fibrosis in the lungs [28]. The MDA5 protein initially recognizes picornaviruses, such as the Coxsackie virus, and evokes antiviral responses by producing type I IFNs and TNF-α [11]. Previously, Coxsackie virus infection was reported to be one of the contributing factors in the pathogenesis of JDM [29]. Anti-MDA5ab-positive DM may be a type of MAS mainly in the skin and lungs that contributes to infections such as those caused by the Coxsackie virus. In conclusion, anti-MDA5ab titre, serum ferritin and IL-18 are useful for the evaluation of the response to treatment of RP-ILD with anti-MDA5ab-positive DM.

Rheumatology key messages

- Anti-MDA5ab is a disease-specific marker in DM with RP-ILD.
- Anti-MDA5ab titre, ferritin and IL-18 are useful for evaluation of response to treatment in DM with RP-ILD.

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Frequency of Class III and IV Nephritis in Systemic Lupus Erythematosus Without Clinical Renal Involvement: An Analysis of Predictive Measures

DAISUKE WAKASUGI, TAKAHISA GONO, YASUSHI KAWAGUCHI, MASAKO HARA, YUMI KOSEKI, YASUHIRO KATSUMATA, MASANORI HANAOKA and HISASHI YAMANAKA

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Frequency of Class III and IV Nephritis in Systemic Lupus Erythematosus Without Clinical Renal Involvement: An Analysis of Predictive Measures

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ABSTRACT. ***Objective.*** To determine the frequency of International Society of Nephrology/Renal Pathology Society (ISN/RPS) class III or IV lupus nephritis in patients with systemic lupus erythematosus (SLE) without clinical renal involvement.

Methods. We investigated the renal pathology of 195 patients with SLE, including 86 patients without clinical renal involvement.

Results. Lupus nephritis other than class I was found in 58% of the patients without clinical renal involvement, and class III and IV nephritis was found in 15% of these patients. To reveal the predictive measures involved in class III or IV lupus nephritis, we explored the clinical measures in patients with SLE who did not have clinical renal involvement. Anti-dsDNA antibody titers were significantly higher ($p = 0.0266$) and C3 values were significantly lower ($p = 0.0073$) in patients with class III or IV lupus nephritis than in patients without class III or IV lupus nephritis. The sensitivity and specificity values were 77% and 73%, respectively, for cutoff levels of both 40 IU/ml for anti-dsDNA antibodies and 55 mg/dl for C3 (OR 8.8, $p = 0.0011$).

Conclusion. The frequency of nephritis, including ISN/RPS class III and IV, was unexpectedly high in SLE patients without clinical renal involvement. ISN/RPS class III or IV lupus nephritis could be hidden in patients with SLE who present both a high titer of anti-dsDNA antibody and a low concentration of C3, even when they have clinically normal urinary findings and renal function. (First Release Nov 15 2011; J Rheumatol 2012;39:79–85; doi:10.3899/jrheum.110532)

Key Indexing Terms:
SYSTEMIC LUPUS ERYTHEMATOSUS
COMPLEMENT
SILENT LUPUS NEPHRITIS
ANTI-dsDNA ANTIBODY

Systemic lupus erythematosus (SLE) is an autoimmune disease with multiple organ manifestations, including skin lesions, arthritis, serositis, nephritis, and neuropsychiatric and hematological disorders. In the 1950s, the 5-year survival rate in patients with SLE who had World Health Organization (WHO) class IV nephritis was 17%; more recently, however, therapy with corticosteroids and immunosuppressive agents (IA) has improved the prognosis of patients with SLE. The 5-year survival rate increased to 82% in the 1990s[1]. However, WHO class IV lupus nephritis is one of the most common manifestations that contribute to endstage renal failure (ESRF). The frequency of ESRF was 40.9% in patients with WHO class IV nephritis, higher than the 2.6% frequency in those with non-class IV lupus nephritis[2]. In general, combination therapy with corticosteroids and IA, such as cyclophosphamide and mycophenolate mofetil, should be recommended in active lupus nephritis with International Society of Nephrology/Renal Pathology Society (ISN/RPS) class III or IV. The early diagnosis and treatment of ISN/RPS class III or IV lupus nephritis is important to improve renal and overall survival in patients with SLE.

The renal manifestations of SLE range from asymptomatic urinary findings, such as microhematuria and proteinuria, to nephrotic syndrome or progressive renal impairment[3]; these manifestations are observed in 31% to 65% of patients with SLE[4]. Although renal biopsy is the "gold standard" for diagnosing and classifying lupus nephritis, it is invasive and has potential complications. Renal biopsy is not always performed on patients with SLE because some of them have normal renal findings or severe manifestations, such as thrombocytopenia, infections, or neuropsychiatric involvement. Thus, it would be beneficial if noninvasive examination could predict the development and severity of lupus nephritis when renal biopsy cannot be performed. Some markers, such as ß-1 integrin in peripheral blood T cells, urinary chemokine, and growth fac-

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tor, have been reported to predict active lupus nephritis, such as ISN/RPS class IV[5,6], although these markers are not available in clinical practice. Clinical measures such as urine sediment, proteinuria, serum complements, and anti-dsDNA antibody are considered conventional and useful predictors for the disease activity of lupus nephritis[7,8,9]. However, some patients show renal histological changes despite normal urinary findings and renal function. This condition is called silent lupus nephritis (SLN)[10,11]. Although most patients with SLN show mild lupus nephritis (i.e., ISN/RPS class II), it is believed that ISN/RPS class III or IV lupus nephritis is rare in patients with SLN[10,11]. There is a notable difference between the therapeutic strategies used for patients with SLE with or without ISN/RPS class III or IV lupus nephritis. However, the characteristics and predictive factors of ISN/RPS class III or IV lupus nephritis have not been revealed in the literature because previous studies have described only a small number of patients with SLN.

We investigated the frequency and predictive factors of ISN/RPS class III or IV lupus nephritis in SLE patients without clinical renal involvement. We analyzed the association between pathohistological renal changes and conventional clinical measures among 195 patients with SLE. We also compared patients with ISN/RPS class III or IV lupus nephritis with those with other ISN/RPS classes (I, II, or V) of lupus nephritis in patients with SLE who did not have clinical renal involvement.

MATERIALS AND METHODS

Patients. We studied 467 consecutive patients who were hospitalized at our institution between 1994 and 2005. These patients were diagnosed with SLE based on the American College of Rheumatology classification criteria[12]. Of 467 patients, 296 (63%) had a renal biopsy (276 women, 20 men). To clarify precisely the degree of pathohistological renal involvement and disease activity in SLE, renal biopsy was performed in both patients with and those without clinical renal involvement. Written informed consent was obtained from each patient. Renal biopsies could not be performed in 171 of 467 patients who did not consent to a renal biopsy or who had a poor condition for examination. The renal biopsies of 31 patients could not be confirmed based on their clinical records; these patients were excluded. We also excluded 57 patients whose renal specimens contained fewer than 10 glomeruli because they were not diagnosed accurately[13]. Other patients excluded were 1 patient with diabetic nephropathy, 1 with IgA nephropathy, 6 with antiphospholipid antibody-related microangiopathy, and 5 with interstitial nephritis. Ultimately, 195 patients were enrolled. In addition, 7 patients were counted twice because re-biopsies were performed among 195 patients. All patients were Japanese except 3, including 2 non-Japanese Asians and 1 African Canadian. The ethics committee of our institution, in accord with the Declaration of Helsinki, approved our study.

Evaluation of clinical measures. Urinary tests, including proteinuria and hematuria on a dipstick, urinary sediment and quantitative proteinuria measured by 24-h urine, serum creatinine, complement hemolytic activity (CH50), complement components (C3 and C4), and anti-dsDNA antibody, were evaluated upon admission before renal biopsy. CH50, C3, and C4 were measured by the standard method. Anti-dsDNA antibody was detected by radioimmunoassay (normal value < 6 IU/ml). The estimated glomerular filtration rate (eGFR) was calculated according to the described method using variables that included serum creatinine, age, and sex[14].

Evaluation of renal pathohistology. Renal pathohistology was classified according to the 2003 ISN/RPS classification[13]. Biopsy results obtained prior to 2003 were reviewed and reclassified according to the 2003 ISN/RPS classification. Immunohistological pathology was tested by direct immunofluorescence and/or the enzyme-labeled antibody method (streptavidin-biotin). Positive results for glomerular immune deposits were defined as (1+) or more. Cases with minor glomerular abnormalities observed by light microscopy and no evidence of immune deposits were classified as "Nil" because they could not be classified as lupus nephritis according to the 2003 ISN/RPS classification[13,15].

Definition of clinical renal involvement. Clinical renal involvement was indicated for patients when 1 or more of the following criteria were satisfied: (1) proteinuria > 400 mg per day; (2) presence of active urinary sediments (> 5 red blood cells and/or 5 white blood cells per high power field and/or cellular cast); or (3) eGFR < 67 ml/min per 1.73 m^2. We determined these cutoff levels using a receiver-operating characteristic curve to predict class III or IV among our 195 patients with SLE. Our definitions were similar to those described in other reports[10,15].

Statistical analysis. Statistical analyses was performed using the chi-square test to compare frequencies, the t test to compare mean values, and the Mann-Whitney U test to compare median values. The data were analyzed using JMP software (SAS Institute, Cary, NC, USA). P values < 0.05 indicated statistical significance.

RESULTS

Clinical features of 195 patients with SLE. The laboratory and pathohistological features of 195 patients with SLE enrolled in our study are summarized in Tables 1 and 2. The 195 patients enrolled included 109 patients with clinical renal involvement (overt subset) and 86 patients without clinical renal involvement (silent subset). Fifteen patients (8%) had no evidence of lupus nephritis as determined by light microscopy and immunofluorescence (Nil). The remaining 180 patients were classified based on the 2003 ISN/RPS classification. As shown in Table 2, the frequencies of ISN/RPS class I-V lupus nephritis were 28 (14%), 44 (23%), 36 (19%), 47 (24%), and 25 (13%), respectively. There were no patients with class VI lupus nephritis. Of the 180 patients excluded as Nil, immunohistological findings could be assessed in 169 patients. The positive frequencies of glomerular immune deposits with IgG, IgM, IgA, C3, and C1q were 131 (77.5%), 137 (81.1%), 122 (72.2%), 144 (85.2%), and 144 (85.2%), respectively.

Comparison of clinical features and ISN/RPS classification between patients with and without clinical renal involvement. As shown in Table 1, we compared the overt subset with the silent subset. The disease duration after SLE diagnosis was significantly shorter ($p = 0.008$) in the silent subset than in the overt subset. No significant differences were found in the frequency of treatment with and dosage of prednisolone (PSL) between the 2 subsets, although the frequency of treatment with IA was higher in the overt subset. Cyclophosphamide was administered by intravenous pulse therapy in only 2 patients of the overt subset. The remaining 3 patients were given a daily dose of cyclophosphamide orally. As expected, proteinuria was significantly increased ($p < 0.0001$) and serum creatinine was significantly higher ($p < 0.0001$) in the overt subset than in the silent subset. Although there was no significant difference between the 2 subsets in terms of anti-dsDNA antibody titer and C4, a slight difference was