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Review Article

An Innovative Method to Identify Autoantigens Expressed on the Endothelial Cell Surface: Serological Identification System for Autoantigens Using a Retroviral Vector and Flow Cytometry (SARF)

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Autoantibodies against integral membrane proteins are usually pathogenic. Although anti-endothelial cell antibodies (AECAs) are considered to be critical, especially for vascular lesions in collagen diseases, most molecules identified as autoantigens for AECAs are localized within the cell and not expressed on the cell surface. For identification of autoantigens, proteomics and expression library analyses have been performed for many years with some success. To specifically target cell-surface molecules in identification of autoantigens, we constructed a serological identification system for autoantigens using a retroviral vector and flow cytometry (SARF). Here, we present an overview of recent research in AECAs and their target molecules and discuss the principle and the application of SARF. Using SARF, we successfully identified three different membrane proteins: fibronectin leucine-rich transmembrane protein 2 (FLRT2) from patients with systemic lupus erythematosus (SLE), intercellular adhesion molecule 1 (ICAM-1) from a patient with rheumatoid arthritis, and Pk (Gb3/CD77) from an SLE patient with hemolytic anemia, as targets for AECAs. SARF is useful for specific identification of autoantigens expressed on the cell surface, and identification of such interactions of the cell-surface autoantigens and pathogenic autoantibodies may enable the development of more specific intervention strategies in autoimmune diseases.

1. Introduction

Inappropriate humoral and cellular immune responses mediate the tissue damage in autoimmune diseases, and the outcome of an autoimmune disease is influenced mainly by the tissue distribution of target self antigens [1]. The pathogenesis of most autoimmune diseases is highly complex and involves multiple cellular and humoral pathways. One part of the humoral arm of the immune assault is caused by autoantibodies, and the mechanisms of autoimmune damage mediated by many autoantibodies have been studied [2]. Clinically, specific autoantibodies are critical for the diagnosis, classification, and monitoring of autoimmune diseases [2].

Autoantibodies cause damage through a number of mechanisms, including the formation of immune complexes, cytolysis or phagocytosis of target cells, and interference with cellular physiology [3]. The cellular localization of the target antigen is believed to play a critical role in the pathogenetic potential of autoantibodies [4]. Intracellular proteins are preferential targets of autoantibodies in autoimmune diseases, but many questions remain unanswered regarding how autoantibodies against intracellular proteins play pathogenic roles. In contrast, it is generally accepted that autoantibodies against integral membrane proteins are usually pathogenic [1]. Some autoantibodies have been clearly confirmed to be pathogenic in several autoimmune diseases, and a model

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

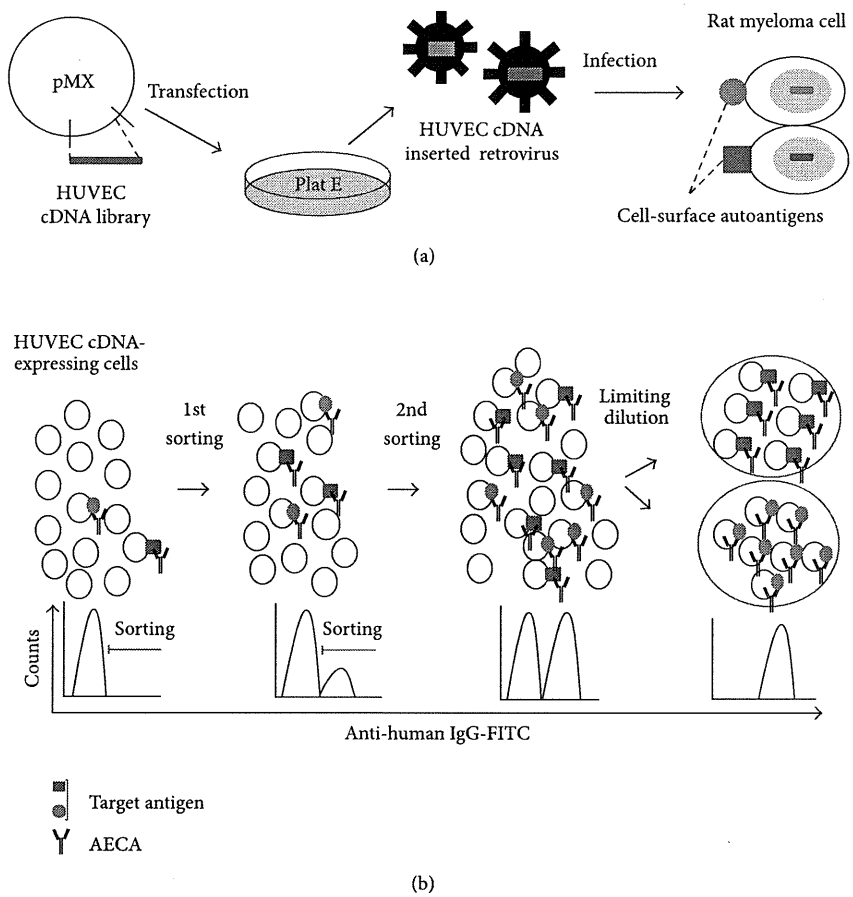

FIGURE 1: Serological identification system for autoantigens using a retroviral vector and flow cytometry (SARF). (a) Generation of human umbilical vein endothelial cell (HUVEC) cDNA-expressing cells. (b) Sorting of cells expressing cell-surface autoantigens.

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).

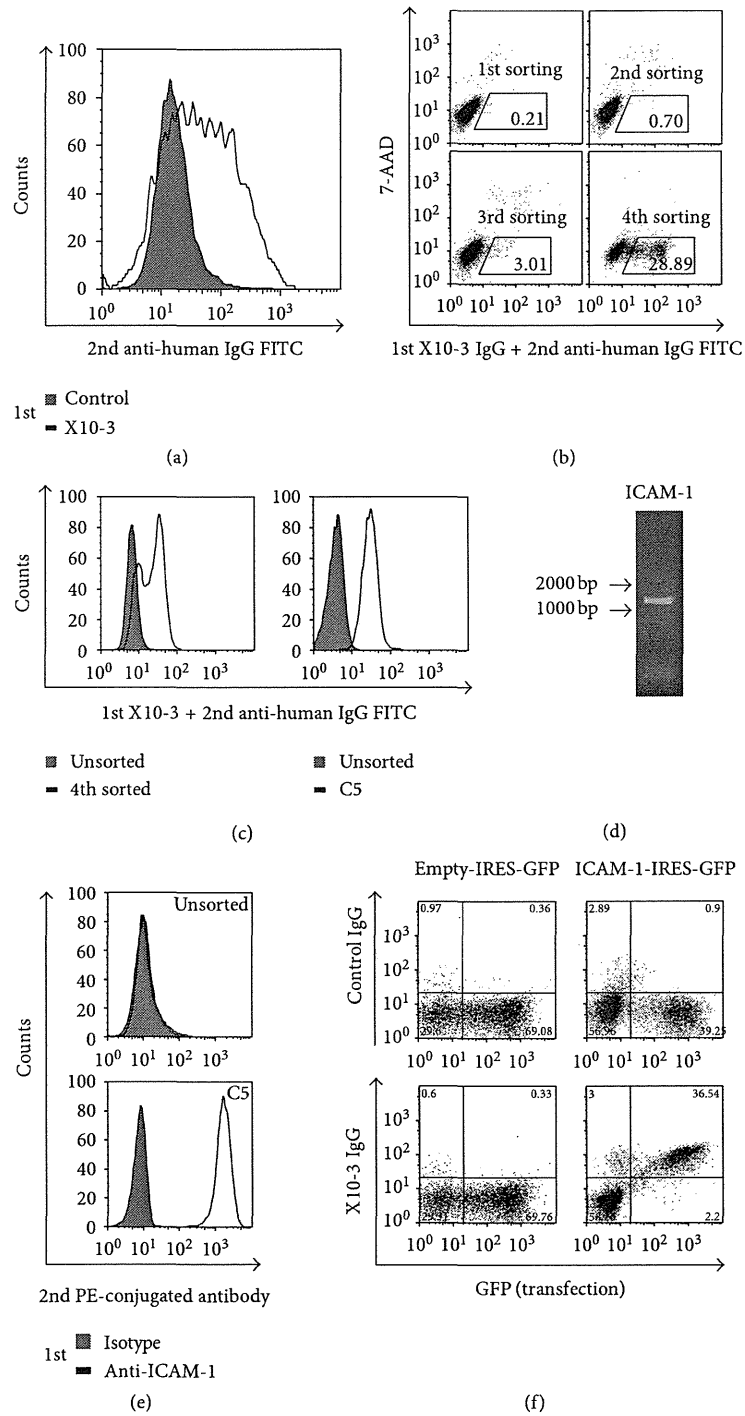

FIGURE 2: Identification of intercellular adhesion molecule 1 (ICAM-1) as a target antigen of anti-endothelial cell antibodies (AECAs). (a) Nonpermeabilized HUVECs were stained with 0.5 mg/mL of IgG of control or X10-3 from a patient with rheumatoid arthritis followed by secondary antibody and analyzed 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

Prevalence and time course of hepatitis B virus infection in patients with systemic lupus erythematosus under immunosuppressive therapy

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Abstract
Objective To clarify the prevalence and time course of hepatitis B virus (HBV) infection in patients with systemic lupus erythematosus under immunosuppressive therapy.
Methods We performed serological examination of 248 lupus patients to determine the presence of HBV, including hepatitis B surface antigen (HBsAg), hepatitis B surface antibody (anti-HBs), and hepatitis B core antibody (anti-HBc). Serum HBV DNA levels were measured in HBsAg-positive patients or resolved HBV carriers (HBsAg-negative, anti-HBs-positive, and/or anti-HBc-positive). If possible, we repeatedly performed examination of markers of HBV infection in resolved carriers.
Results Two (0.8 %) patients were positive for HBsAg. Among 41 (16.5 %) patients who were considered as resolved HBV carriers, 1 (2.4 %) showed serum HBV DNA, which indicated occult HBV infection. The mean age and positive rate of anti-double stranded DNA antibody were significantly higher in resolved carriers than in anti-HBs- and anti-HBc-negative patients. Repeated examination showed that the anti-HBs and anti-HBc titer decreased below the threshold in 4 resolved carriers.
Conclusions The prevalence of resolved HBV carriers in Japanese lupus patients was 16.5 %. Among them, occult HBV infection and decrease in anti-HBs and anti-HBc titer were observed. These findings indicated that all lupus patients should undergo serological examination for HBV before treatment. If patients have already been treated, we must carefully monitor their liver function, even when all HBV markers are negative.

Keywords Hepatitis B virus · Immunosuppressive therapy · Resolved HBV carrier · Systemic lupus erythematosus

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Introduction

Hepatitis B virus (HBV) infection is one of the most common infectious diseases in the world. Of the 6 billion people in the world, one-third (2 billion) have been exposed to HBV [1, 2]. 350 million people are estimated to have chronic HBV infection, and 75 % of these people are from Southeast Asia and the Western Pacific countries [1]. HBV infection is the leading cause of chronic hepatitis, and up to 25 % of patients with chronic hepatitis eventually die of liver cirrhosis and its complications, including hepatocellular carcinoma [2].

Until recently, hepatitis B surface antigen (HBsAg)-negative, hepatitis B surface antibody (anti-HBs)-positive, and/or hepatitis B core antibody (anti-HBc)-positive patients were thought to be "resolved" (hereafter, we refer to as resolved HBV carriers). However, once HBV is internalized into hepatocytes, their DNA genomes are converted to a covalently closed circular form (cccDNA) in the nucleus, which serves as the transcriptional template and maintains a stable intranuclear pool. Therefore, low levels of HBV DNA persist for decades in the liver and peripheral blood mononuclear cells [3].

For the past few years, reactivation of HBV has been reported after organ transplantation, hematopoietic stem cell transplantation, chemotherapy for malignancies, and immunosuppressive therapy in resolved HBV carriers [4].

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This can cause fulminant or fatal hepatitis, which is known as de novo hepatitis B, with an extremely high mortality [4]. Recently published data suggests that tumor necrosis factor blocker (anti-TNF), leflunomide, and corticosteroids have the highest risk of reactivation in patients with rheumatoid arthritis (RA) [5]. However, few studies have reported the prevalence of HBV infection in patients with systemic lupus erythematosus (SLE). Further, the safety of immunosuppressive therapy for resolved HBV carriers with SLE is unclear. Thus, the aim of this study is to clarify the prevalence and time course of HBV infection in patients with SLE under immunosuppressive therapy.

Materials and methods

Patients

Between January 2008 to April 2010, we enrolled 248 lupus patients who were receiving or planned to receive immunosuppressive therapy at the Department of Hematology and Rheumatology in Tohoku University hospital. All patients fulfilled at least 4 of the 1997 American College of Rheumatology criteria (ACR criteria) for lupus classification [6], and were tested for HBV markers, including HBsAg, anti-HBs, and anti-HBc. Serum HBV DNA levels were measured if patients were positive for HBsAg or were resolved HBV carriers. Markers of HBV infection were examined repeatedly, if possible, in resolved HBV carriers. We also retrospectively reviewed the medical records and obtained data about age, sex, liver function, disease activity index using the 'safety of estrogens in lupus erythematosus national assessment—systemic lupus erythematosus disease activity index' ((SELENA-SLEDAI) [7], previous and current medication, clinical features, and autoantibodies at diagnosis or during disease course, which was listed in the ACR criteria. The study protocol was approved by the ethics committees of Tohoku University Graduate School of Medicine and performed in accordance with the Declaration of Helsinki.

Serological examination of HBV and quantification of HBV DNA

All HBV markers (HBsAg, anti-HBs, and anti-HBc) were tested using chemiluminescent immunoassay ([CLIA] ARCHITECT; Abbott Japan, Tokyo, Japan). An anti-HBs titer of less than 10 IU/l, which we qualitatively analyzed, was considered negative. Anti-HBc was quantitatively measured, and the cut-off point was 1.0 S/CO. HBV DNA quantification was performed at BML (Kawagoe, Saitama, Japan), using real-time polymerase chain reaction assay (PCR). The detection threshold was 2.1 log copies/ml.

Statistical analysis

The Chi-square test, or Fisher's exact test when appropriate, and Student's t test were used. A p-value <0.05 was considered statistically significant. All analyses were performed using JMP version 9.0 software (JMP Japan, Tokyo, Japan).

Results

Serological examination of HBV (Fig. 1)

Of the 248 lupus patients, 2 (0.8 %) were HBsAg-positive and had already been treated with entecavir in this study. Of the remaining 246 HBsAg-negative patients, 205 (82.7 %) were negative for both anti-HBs and anti-HBc. We recognized 41 (16.5 %) patients as resolved HBV carriers, including 7 (2.8 %) anti-HBs-positive/anti-HBc-negative patients, 18 (7.2 %) anti-HBs-positive/anti-HBc-positive patients, and 16 (6.4 %) anti-HBs-negative/anti-HBc-positive patients. Among the anti-HB-positive/anti-HBc-negative patients, 1 had undergone HBV vaccination.

Clinical characteristics of resolved HBV carriers

We compared the characteristics of resolved HBV carriers with those of anti-HBs- and anti-HBc-negative patients. Although the female-to-male ratio, current liver function, the rate of abnormal liver function regardless of cause, and SLEDAI did not differ significantly between the 2 groups, mean age of resolved HBV carriers was significantly higher than that of anti-HBs- and anti-HBc-negative patients (50.3 ± 15.0 vs. 43.2 ± 14.7 years, $p = 0.006$; Table 1). Age-based distribution of all patients indicated that most of the patients were young (Fig. 2a). On the other hand, age-based prevalence of resolved patterns (age-based number of

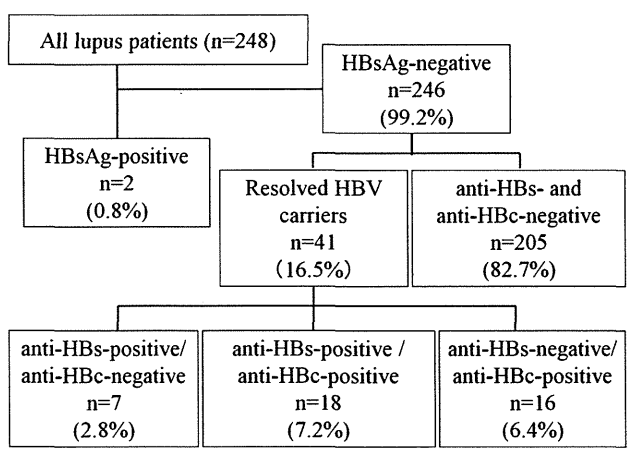

Fig. 1 HBV serology in 248 lupus patients

resolved HBV carriers/age-based number of total patients) was as shown in Fig. 2b. This suggested that older patients had a higher frequency of resolved pattern, especially in their 60s (34.5 %). We then compared the clinical characteristics and autoantibodies listed in the ACR criteria between the 2 groups (Tables 2 and 3). Clinical features did not differ significantly; however, the positive rate of anti-double stranded DNA antibody (anti-dsDNA) that emerged at diagnosis or during disease course was significantly higher in resolved HBV carriers (92.7 vs. 76.6 %, $p = 0.02$, respectively).

Previous and current medication in resolved HBV carriers and quantification of HBV DNA (Table 4)

Although almost half of the resolved HBV carriers had previously undergone administration of prednisolone (PSL) higher than 40 mg/day and any immunosuppressant, the

Table 1 Comparison of age, female-to-male ratio, liver function, and disease activity between resolved HBV carriers and anti-HBs- and anti-HBc-negative patients

	Resolved HBV carriers	Anti-HBs- and anti-HBc-negative patients	Total	*p*
Patients (*n*)	41	205	246	
Female/male ratio (female %)	36/5 (87.8 %)	186/19 (90.7 %)	222/24 (90.2 %)	0.57
Age (years, mean ± SD)	50.3 ± 15.0	43.2 ± 14.7	44.4 ± 15.0	<0.01
AST (IU/l, mean ± SD)	23.5 ± 14.9	21.5 ± 10.2	21.9 ± 11.1	0.43
ALT (IU/l, mean ± SD)	19.7 ± 13.7	21.0 ± 21.5	20.7 ± 20.4	0.63
Alb (g/dl, mean ± SD)	3.83 ± 0.48	3.90 ± 0.39	3.89 ± 0.48	0.37
Abnormal liver function (%)	7/41 (17.1 %)	23/205 (11.2 %)	30/246 (12.2 %)	0.30
SLEDAI (score, mean ± SD)	5.81 ± 4.52	6.00 ± 5.39	5.97 ± 5.24	0.81

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *Alb* Albumin, SLEDAI systemic lupus erythematosus disease activity index

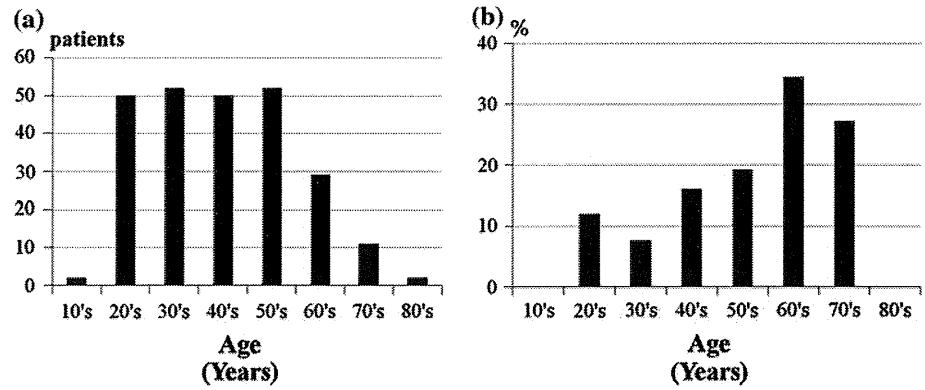

Fig. 2 **a** Age-based distribution of all patients. **b** Age-based prevalence of resolved patterns (age-based number of resolved HBV carriers/age-based total number of patients)

Table 2 Comparison of clinical characteristics between resolved HBV carriers and anti-HBs- and anti-HBc-negative patients

	Resolved HBV carriers	Anti-HBs- and anti-HBc-negative patients	Total	*p*
Malar rash	29/41 (70.7 %)	135/205 (65.9 %)	164/246 (66.6 %)	0.59
Discoid rash	14/41 (34.1 %)	75/205 (36.6 %)	89/246 (36.2 %)	0.86
Photosensitivity	23/41 (56.1 %)	134/205 (65.4 %)	157/246 (63.8 %)	0.29
Oral ulcers	12/41 (29.3 %)	79/205 (38.5 %)	91/246 (37.0 %)	0.29
Neurologic disorders	9/41 (22.0 %)	35/205 (17.1 %)	44/246 (17.9 %)	0.50
Serogitis	7/41 (17.1 %)	55/205 (26.8 %)	62/246 (25.2 %)	0.24
Nephropathy	20/41 (48.8 %)	117/205 (57.1 %)	137/246 (55.7 %)	0.39
Arthritis	34/41 (82.9 %)	165/205 (80.5 %)	199/246 (80.9 %)	0.83
Hemolytic anemia	5/41 (12.2 %)	24/205 (11.7 %)	29/246 (11.8 %)	0.79
Lymphopenia	33/41 (80.5 %)	153/205 (64.6 %)	186/246 (75.6 %)	0.32
Thrombocytopenia	11/41 (26.8 %)	75/205 (36.6 %)	86/246 (35.0 %)	0.28

Table 3 Comparison of autoantibodies between resolved HBV carriers and anti-HBs- and anti-HBc-negative patients

	Resolved HBV carriers	Anti-HBs- and anti-HBc-negative patients	Total	*p*
Antinuclear antibody	41/41 (100 %)	197/205 (96.1 %)	238/246 (97.1 %)	0.36
Anti-dsDNA antibody	38/41 (92.7 %)	157/205 (76.6 %)	195/246 (79.3 %)	0.02
Anti-Smith antibody	16/41 (39.0 %)	97/205 (47.3 %)	113/246 (45.9 %)	0.39
Anti-UIRNP antibody	18/41 (44.0 %)	91/205 (44.4 %)	109/246 (44.3 %)	1.0
Anti-cardiolipin antibody	9/41 (22.0 %)	73/205 (35.6 %)	82/246 (33.3 %)	0.10

Table 4 Previous and current medication in resolved HBV carriers and quantification of HBV DNA

		Total
Patients (*n*)		41
Age (years, mean ± SD)		50.3 ± 15.0
Previous medication	Pulse therapy	8/41 (19.5 %)
(maximum prednisolone dose, mg/day)	40 < PSL ≤ 60	10/41 (24.4 %)
	20 < PSL ≤ 40	14/41 (34.1 %)
	0 ≤ PSL ≤ 20	9/41 (22.0 %)
Previous or current	Intravenous cyclophosphamide pulse	9/41 (22.0 %)
Immunosuppressant (in total)	Oral cyclophosphamide	6/41 (14.6 %)
	Tacrolimus	6/41 (14.6 %)
	Cyclosporine	6/41 (14.6 %)
	Mizonbine	5/41 (12.2 %)
Current prednisolone dose (mean ± SD, mg/day)		9.3 ± 9.5
Quantification of HBV DNA		41/41 (100 %)
Positive PCR results		1/41 (2.4 %)

current PSL dose was 9.3 mg/day. Quantification of serum HBV DNA was performed in all resolved carriers, and positive results were obtained in 1 (2.4 %) patient, a 61-year-old woman requiring hemodialysis because of lupus nephritis type IV (WHO classification) and who was treated with PSL 5 mg/day. Although serum HBV DNA was detected (2.4 log copies/ml) in this patient, her liver function tests were within normal range (aspartate amino transferase [AST] level, 20 IU/l; alanine amino transferase [ALT] level, 12 IU/l). Her condition was diagnosed as occult HBV infection and was treated with entecavir. Administration of entecavir resulted in negative serum HBV DNA, but after 2 months, the patient suddenly died of unknown reason.

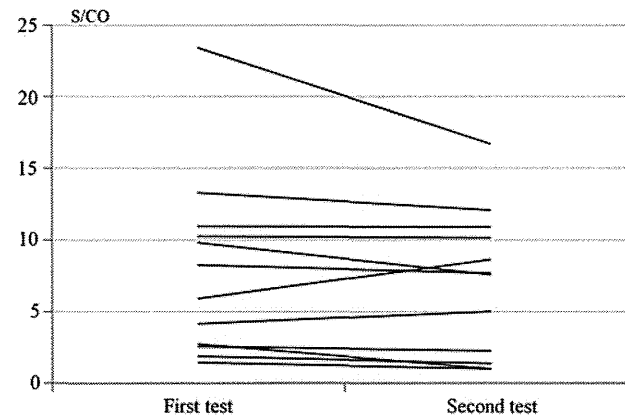

Fig. 3 Repeated examination of the titer of anti-HBc in 12 patients after a mean follow-up of 6 months

Decrease in anti-HBs and anti-HBc titer

Positive markers of HBV infection (anti-HBs or anti-HBc) in resolved HBV carriers were repeatedly examined in 18 (43.9 %) patients. Anti-HBc titer of 12 patients after a mean follow-up of 6.0 months was shown in Fig. 3. The average anti-HBc titer did not differ significantly between the first and the second test (7.9 ± 6.3 vs. 7.0 ± 5.0 S/CO, $p = 0.71$); however, surprisingly anti-HBc titer decreased below the cut-off point in 2 (2/12, 16.7 %) patients. Similar to the anti-HBc titer, anti-HBs titer decreased below the threshold in 2 (2/10, 20 %) patients. Clinical characteristics, events, and treatment modifications during the follow-up of 4 patients whose anti-HBs or anti-HBc disappeared are summarized in Table 5. Immunosuppressive therapy of 2 patients was intensified because of disease onset or flare of lupus nephritis, but that of the others was not changed.

Table 5 Clinical characteristics, events, and treatment modifications during the follow-up of 4 patients whose anti-HBs or anti-HBc disappeared

	Patient 1	Patient 2	Patient 3	Patient 4
Age (years)	23	46	23	20
Disease duration (years)	0	6	8	7
Anti-HBs	Positive	Positive	Negative	Negative
Anti-HBc (titer, S/CO)	Negative	Negative	Positive (146)	Positive (2.72)
HBVDNA	Negative	Negative	Negative	Negative
Disappearance of anti-HBs or anti-HBc	After 1 month	After 16 months	After 8 months	After 15 months
Main clinical manifestations	Arthritis, pleuritis	Arthritis	Psychosis	Nephropathy
Events during the follow-up	Disease onset	Particularly not	Particularly not	Flare of lupus nephritis
Prednisolone dose	Commencement of 60 mg/day	No change (2 mg/day)	No change (5 mg/day)	Increase to 30 mg/day
Immuno suppressant	None	None	None	Addition of tacrolimus 3 mg/day

Discussion

Occult HBV infection is defined as the presence of HBV DNA in the sera or liver, despite HBsAg negativity [8]. Occult HBV infection can result from viral mutants that are not detected by commercial assays or by very low levels of viral replication [8]. In this study, among 41 resolved HBV carriers, only 1 61-year-old female patient (2.4 %) had occult HBV infection. Her age at the onset of disease was 27 years, and she was treated with PSL (maximum dose, 40 mg/day; current dose, 5 mg/day) and intravenous cyclophosphamide pulse therapy (300 mg each, total 3 times, 20 years ago), but initiation of hemodialysis was required. Serological examination showed that she was HBsAg-negative, anti-HBs-positive, and anti-HBc-positive. She had a history of blood transfusion at the time of diagnosis. She suddenly died of unknown reason after administration of entecavir that resulted in negative serum HBV DNA after 2 months. It remained unclear from what point occult HBV infection existed and how immunosuppressive therapy affected it. However, at least one of the important insights obtained from this patient was that occult HBV infection was a possible complication in lupus patients treated with low-dose PSL therapy.

Occult HBV infection has been reported in various settings. Minuk et al. reported that among 239 HBsAg-negative hemodialysis patients, 9 (3.8 %) patients showed serum HBV DNA [9]. Of these 9 patients, 7 had G145R mutant HBV. In addition, Demir et al. reported that 11 % of the 100 HBsAg-negative patients with type 2 diabetes mellitus and 3 % of 100 HBsAg-negative healthy donors were positive for serum HBV DNA [10]. A study of a blood donor population in Hong Kong indicated that the prevalence of occult HBV infection was 0.13 % (4/3044, cohort 1) and 0.11 % (11/9967, cohort 2) [11]. In cohort 2, 10 out of 11 patients were positive for anti-HBc; however, 1 patient was negative for HBsAg, anti-HBs, and anti-HBc. This report gave us the important finding that even if all HBV markers were negative, serum HBV DNA could be detected. In our study, although liver function in anti-HBs- and anti-HBc-negative patients was regularly tested, serum HBV DNA was not measured because of the issue of cost-effectiveness. A new criteria for measurement of serum HBV DNA in patients with autoimmune diseases is required in the future.

In Japan, the prevalence of HBsAg is estimated to be 0.6–0.8 %, and about 20 % of individuals are positive for at least 1 HBV marker [12]. In our study, the rate of resolved HBV carriers was 16.5 %. In the case of other rheumatic diseases such as RA, the prevalence of resolved HBV carriers is 31.5 % [5] and 25 % [13] in Japan. These reports suggest that the rate of resolved HBV carriers in our study was slightly lower than the estimated prevalence and that in other rheumatic diseases. This might be related to the small number of patients examined, previous immunosuppressive therapy, lupus-specific manifestation, or younger age profile of lupus patients in our study.

We have summarized results from previous studies about the prevalence of HBV infection in lupus patients (Table 6) [14–19]. Previous studies indicated that anti-HBs and anti-HBc were measured using a radioimmunoassay. In our study, these antibodies were measured using CLIA, which is more sensitive than radioimmunoassay and is now used worldwide. Therefore, our study has 3 advantages over the previous studies. First, to our knowledge, this study is the first detailed analysis about the prevalence of HBV infection using CLIA. Second, the number of lupus patients who underwent serological examination for HBV was the largest. Third, this is the first study comparing the clinical characteristics of resolved HBV carriers with those of anti-HBs- and anti-HBc-negative patients. It remains to be clarified why the positive rate of anti-dsDNA was significantly higher in resolved HBV carriers. Ram et al. [19] reported that the percentage of anti-HBc in lupus patients was lower (2.5 %) than that in normal controls (10.7 %). They suggested a protective role of HBV in lupus pathogenesis [19]. In our study, nephropathy, which is directly induced by anti-dsDNA, did not differ significantly between 2 groups. Although HBV may contribute to the pathogenesis of SLE, we cannot conclude the significance of HBV in SLE.

Table 6 Summary of previous reports about the prevalence of HBV infection in lupus patients

		Denmark	South Africa	Singapore	Israel	Taiwan	Israel	Japan
Reported year		1982	1986	1993	1997	1997	2008	2010
Healthy donors	Number tested (*n*)	ND	ND	100	ND	692	140	ND
	HBsAg-positive			Total 19 % (19/100)	2 %	14.7 % (102/692)	ND	
	Anti-HBs-positive				ND	ND	ND	
	Anti-HBc-positive				ND	ND	10.7 % (25/140)	
Lupus patients	Number tested (*n*)	32	100	76	95	173	117	248
	HBsAg-positive	0 % (0/32)	1 % (1/100)	Total 19.7 % (15/76)	0 % (0/95)	3.5 % (6/173)	ND	0.8 % (2/248)
	Anti-HBs-positive	25 % (8/32)	25 % (25/100)		ND	ND	ND	Total 16.5 % (41/248)
	Anti-HBc-positive	ND	ND		ND	ND	2.5 % (3/117)	
Reference		18	17	14	16	15	19	

ND no data

During follow-up, the levels of anti-HBs and anti-HBc decreased below the threshold in 4 patients. Although we cannot exclude non-specific reaction and testing failures because anti-HBc disappeared only in the patients whose titer was low at first test, findings similar to ours were observed when RA patients were treated using anti-TNF [20]. The incidence of de novo hepatitis B in RA patients treated with anti-TNF is estimated to be 0–5 % per year [21]; however, decrease in anti-HBs titer has been shown to precede HBV reactivation in hematological disorders [22], which suggests the strong need for close follow-up of these patients. In our study, both anti-HBs and anti-HBc disappeared in nearly 20 % of resolved HBV carriers. Therefore, if patients have already taken immunosuppressive therapy, we must recognize the risk of reactivation even if all HBV markers are negative. Further, because even low-dose PSL therapy induced disappearance of these HBV-related serum markers in lupus patients, measurement of anti-HBs and anti-HBc before immunosuppressive drug administration is strongly suggested in all lupus patients.

In conclusion, the prevalence of HBV infection in Japanese lupus patients was 16.5 % in our study. Administration of immunosuppressive therapy to lupus patients is relatively safe, because reactivation of HBV was not observed; however, occult HBV infection and decrease in anti-HBs and anti-HBc titer were observed, which suggest that all lupus patients should undergo serological examination for HBV before treatment, and that serum HBV DNA should be measured at least once in resolved HBV carriers. In addition, if patients have already taken immunosuppressive therapy, we must closely monitor their liver function even if all HBV markers are negative.

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Conflict of interest None.

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RESEARCH ARTICLE Open Access

A novel autoantibody against fibronectin leucine-rich transmembrane protein 2 expressed on the endothelial cell surface identified by retroviral vector system in systemic lupus erythematosus

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Abstract

Introduction: Anti-endothelial cell antibodies (AECAs) are thought to be critical for vasculitides in collagen diseases, but most were directed against molecules localized within the cell and not expressed on the cell surface. To clarify the pathogenic roles of AECAs, we constructed a retroviral vector system for identification of autoantigens expressed on the endothelial cell surface.

Methods: AECA activity in sera from patients with collagen diseases was measured with flow cytometry by using human umbilical vein endothelial cells (HUVECs). A cDNA library of HUVECs was retrovirally transfected into a rat myeloma cell line, from which AECA-positive clones were sorted with flow cytometry. cDNA of the cells was analyzed to identify an autoantigen, and then the clinical characteristics and the functional significance of the autoantibody were evaluated.

Results: Two distinct AECA-positive clones were isolated by using serum immunoglobulin G (IgG) from a patient with systemic lupus erythematosus (SLE). Both clones were identical to cDNA of fibronectin leucine-rich transmembrane protein 2 (*FLRT2*). HUVECs expressed FLRT2 and the prototype AECA IgG bound specifically to *FLRT2*-transfected cells. Anti-FLRT2 antibody activity accounted for 21.4% of AECAs in SLE. Furthermore, anti-FLRT2 antibody induced complement-dependent cytotoxicity against FLRT2-expressing cells.

Conclusions: We identified the membrane protein FLRT2 as a novel autoantigen of AECAs in SLE patients by using the retroviral vector system. Anti-FLRT2 antibody has the potential to induce direct endothelial cell cytotoxicity in about 10% of SLE patients and could be a novel molecular target for intervention. Identification of such a cell-surface target for AECAs may reveal a comprehensive mechanism of vascular injury in collagen diseases.

Introduction

Vascular endothelial cells (ECs) represent the boundary between blood and tissue, and contribute to the process of inflammation. Anti-endothelial cell antibodies (AECAs) were first described in 1971 and defined as autoantibodies that target antigens present on the EC membrane [1,2]. AECAs have been detected in a number of patients with collagen diseases, including systemic lupus erythematosus (SLE), and were shown to be correlated to disease activity [3,4]. SLE is one of the diseases in which AECAs are frequently detected, and they are considered to play a role in the pathogenesis, especially in lupus nephritis [3,4]. In addition, SLE patients have an increased risk of cardiovascular disease originating from SLE itself, and it has been reported that AECAs play roles in atherosclerotic events [5].

AECAs have the potential to induce vascular lesions directly because their targets are expressed on ECs, which are always in contact with these circulating antibodies. AECAs are considered to play roles in the

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