Short Communication

Proteomic profiling of antigens in circulating immune complexes associated with each of seven autoimmune diseases

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Keywords: autoimmune disease; immune complexome analysis; immune complex; tandem mass spectrometry

Nonstandard abbreviations: AAV, anti-neutrophil cytoplasmic antibody-associated vasculitis; CICs, circulating immune complexes; DM, dermatomyositis; ICs, immune complexes; MCTD, mixed connective tissue disease; nano-LC-MS/MS, nano-liquid chromatography-tandem mass spectrometry; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; SS, Sjögren's syndrome; SSc, systemic scleroderma; TA, Takayasu's arteritis

Abstract

Objective: Immune complexes (ICs) trigger humoral immune responses. Therefore, the identification of constituent antigens within ICs would have very different clinical significance than identification of free antigens.

Design and Methods: Here, we applied immune complexome analysis of serum to the study of seven major autoimmune diseases—anti-neutrophil cytoplasmic antibody-associated vasculitis, Takayasu's arteritis, mixed connective tissue disease, dermatomyositis, Sjögren's syndrome, systemic scleroderma, and systemic lupus erythematosus— and healthy donors to comprehensively identify antigens incorporated into circulating ICs and to find disease-specific antigens.

Results: We identified 468 distinct IC- associated antigens using this method. Importantly, 62 of those antigens were disease-specific antigens, and there were at least three disease-specific antigens for each of the seven autoimmune diseases. Of the disease-specific antigens identified, coiled-coil domain-containing protein 158 and spectrin were identified as potential autoantigens important to SSc and SS pathogenesis, respectively; notable titin and spectrin autoantibodies are reportedly found in SSc and SS patients, respectively.

Conclusion: Immune complexome analysis may be generally applicable to the study of the relationship between ICs and autoimmune diseases in animals and humans.

Introduction

For a long time, immune complexes (ICs) assembly was thought to represent a common pathogenic pathway for several diseases (infections, vasculitis, and connective tissue autoimmune disorders). Actually, concentrations of circulating ICs (CICs) in sera from patients with rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), or systemic scleroderma (SSc) were significantly higher than those in sera from healthy controls [1, 2]. Many researchers have investigated the mechanisms by which ICs could underlie pathogenicity [3]. An autoimmune response is directed against several autoantigens [4, 5]; therefore, comprehensive profiling of the autoantigens that actually assemble into ICs may provide insight into the pathophysiology of specific autoimmune diseases, and such profiling could form the basis for novel diagnosis and treatment strategies for these diseases. However, such comprehensive profiling studies for CICs are limited because tools screening of ICs are lacking.

We developed a proteomic strategy, designated immune complexome analysis, in which ICs are separated from whole serum and then subjected to direct tryptic digestion and nano-liquid chromatography-tandem mass spectrometry (nano-LC-MS/MS) to comprehensively identify and profile constituent antigens in CICs [6]. We used this method to identify CIC-associated antigens in sera from patients with RA, and found that thrombospondin-1 is a constituent of CICs and is more highly specific and sensitive for established and early RA than other conventional diagnostic markers such as rheumatoid

factor or anti-citrulline-containing protein/peptide antibody [6, 7].

In this report, we used immune complexome analysis of serum to study seven major autoimmune diseases—anti-neutrophil cytoplasmic antibody-associated vasculitis (AAV), Takayasu's arteritis (TA), mixed connective tissue disease (MCTD), dermatomyositis (DM), Sjögren's syndrome (SS), SSc, and SLE—to comprehensively identify antigens incorporated into CICs and find disease-specific antigens.

Materials and methods

Serum samples were collected from 66 patients; each patient had AAV (n=7; 35-86 years; 3 female; 4 patients are Microscopic polyantitis and 3 patients are Granulomatosis with polyantitis), TA (n=7; 28-63 years; 7 female), MCTD (n=9; 43-77 years; 9 female), DM (n=8; 30-69 years; 6 female), SS (n=14; 35-78 years; 14 female), SSc (n=7; 55-78 years; 6 female), or SLE (n=14; 16-67 years; 13 female) as diagnosed based on classification criteria from the Ministry of Health, Labour and Welfare, Japan for AAV, MCTD, and DM; classification criteria from the American College of Rheumatology for TA, SSc, and SLE; or the criteria for SS proposed by the American-European Consensus Group. All sample collection and diagnoses were performed at Nagasaki University Hospital and all the patients were newly diagnosed. Also, healthy donors (n=11; 30-65 years; 2 female) are enrolled in this study. Each serum sample was subjected to a single pretreatment process and replicate nano-LC-MS/MS analyses. All experiments were performed in accordance with the Helsinki Declaration and with approval from the institutional ethics committees of the

Graduate School of Biomedical Sciences, Nagasaki University. Each patient provided written informed consent for their participation in this study.

CICs were purified by magnetic beads with immobilized Protein G or Protein A (PureProteome®, Millipore, Darmstadt, Germany). The tryptic digestion procedure and nano-LC-MS/MS analysis were performed as previously [6].

Results and discussion

Here, we present the first comprehensive identification of the constituent antigens assembled into CICs from patients with AAV, TA, MCTD, DM, SS, SSc, or SLE (Table S1). We identified 341 and 264 human antigens via immune complexome analysis with Protein G or Protein A beads, respectively; 468 distinct antigens were identified in all, and each of these was found in independent samples from two or more patients (Table S2). Notably, only 29% (137) of these 468 distinct antigens were recovered with both Protein G and Protein A beads; 204 antigens were recovered only when using Protein G beads, and 127 antigens were recovered only when using Protein A beads. This indicates that the parallel use of Protein G and Protein A beads recovered a wider range of antigens than the use of either bead type alone. Furthermore, among 341 and 264 antigens identified with Protein G or Protein A, 127 and 92 antigens were also found in healthy donors, respectively; therefore, these antigens were thought to form common CICs in human bodies (Table S2). It is interesting that much more antigens identified with Protein G than Protein A and there is a little overlap between Protein G and Protein A immune complexome. The association constant between IgG and Protein G

was reported to be 4-times higher than that between IgG and Protein A [8]. It is known that IgG3 is captured on Protein G (but not Protein A) and IgM, IgA, IgD and IgE are captured on Protein A (but not Protein G). High association constant and binding to IgG3 of Protein G and distinct affinity of Protein A to IgM, IgA, IgD and IgE may contribute to more antigens identified with Protein G than Protein A and a little overlap between Protein G and Protein A.

Of the 468 distinct antigens identified, 62 were detected in only one disease group and not in any other disease groups by using Protein G or Protein A (Table 1). Also, these antigens were not found in healthy donors. Although there is the possibility that a protein nonspecifically binds to Protein G or Protein A beads, those disease-specific antigens are not thought to directly bind to the beads because nonspecifically binding proteins should be found in multiple diseases. Also, in this study, many disease-specific antigens are identified; therefore, it is difficult to determine if each protein nonspecifically binds to the beads by using a recombinant or natural protein as our previous study [6].

In this study, relatively many disease-specific antigens were found in SSc and SS. Namely, 9 disease-specific antigens were associated with SSc even though only seven SSc patients were enrolled in this study; moreover, SS was associated with 2-fold more disease-specific antigens than was SLE, even though the number of patients with SS was equal to that with SLE. Reportedly, concentrations of CICs are elevated in patients with SSc or SS [9, 10]; thus, these elevated CIC concentrations may contribute to the large number of distinct types of disease-specific antigens associated with each disease. Only 6 disease-specific antigens associated with AAV, TA, MCTD, or DM were detected with Protein

G beads; however, 19 of the disease-specific antigens associated with AAV, TA, MCTD, or DM were detected with Protein A beads. This finding may indicate that there were more CICs associated with IgA, IgD, IgE, or IgM than those associated with IgG. Conversely, more of the disease-specific antigens associated with SS, SSc, or SLE were recovered only with Protein G beads.

To our knowledge, most of the 62 disease-specific antigens have never been reported to be associated with the respective autoimmune disease; however, some do have a recognized association with the respective disease. Some of the autoantibodies previously reported to be associated with SS are known to recognize proteins that contain one or more coiled-coil domains. SS is known to produce autoantibodies that react with a 52-kDa protein component of the SS-A antigen. The antigenic region of this 52 kDa protein is recognized by all SS-A antibody-positive sera and contains a sequence motif reminiscent of a leucine zipper that is predicted to form a coiled-coil region [11]. Similarly, we specifically detected coiled-coil domain-containing protein 158 in SS sera. Spectrin was specifically detected with Protein A beads in serum from patients with SS. Spectrin is a protein that was first characterized in human erythrocytes; the nonerythrocyte spectrin is called fodrin [12]. Studies have suggested that 1) the α-subunit of fodrin is cleaved during apoptosis, 2) fodrin plays a role in the development of SS, and 3) fodrin is a candidate autoantigen in primary SS [13, 14]. Although some of our results were consistent with these previous reports, our method did not detect the autoantigens targeted by well-known autoantibodies (e.g. SS-A, SS-B, Jo-1, Scl-70 antibodies) that are associated with SS, DM, SSc, or some combination

thereof. Therefore, it is possible that these autoantibodies do not form ICs with their corresponding autoantigens and immune complexome analysis may specifically identify those antigens that actually assemble into ICs.

The sensitivity of each disease specific antigens was less than 33%. The sensitivity of individual disease-specific antigens may be improved by developing ELISA methods that ea ch detects a certain IC with high specificity [15]; if such ELISAs were developed, these disease-specific antigens may become promising diagnostic or pathogenic biomarkers.

Conflict of interest

Research Funding: K. Ohyama, Eisai Co., Ltd.

Acknowledgement

This work was supported by a Grant-in-aid for Scientific Research (C) and challenging Exploratory Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, a Grant-Aid for Scientific Research from Nagasaki University, the Joint Research Promotion Project of Nagasaki University Graduate School of Biomedical Sciences in 2013, The Research Foundation for Pharmaceutical Sciences and The Kato Memorial Trust For Nambyo Research.

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Table 1 Summary of disease-specific antigens in CICs isolated from patients with AAV, TA, MCTD, DM, SS, SSc, or SLE

Protein G

Accession	Description	AAV (n=7) Frequency/ peptides per hit	TA (n=7) Frequency/ peptides per hit	MCTD (n=9) Frequency/ peptides per hit	DM (n=8) Frequency/ peptides per hit	SS (n=14) Frequency/ peptides per hit	SSc (n=7) Frequency/ peptides per hit	SLE (n=14) Frequency/ peptides per hit
IPI00926948.1	Uncharacterized protein	3/1						
IPI00217511.1	Seven transmembrane helix receptor	2/1						
IPI00555948.1	Androgen-regulated short-chain dehydrogenase/reductase 1 variant (Fragment)			2/1				
IPI01012262.1	Uncharacterized protein			2/1				
IPI00910896.1	cDNA FLJ56556, highly similar to Alpha-1-syntrophin				2/1			
IPI00982295.1	Uncharacterized protein				2/1			
IPI00023711.2	Envoplakin					2/1		
IPI00103630.3	Isoform 2 of Protein phosphatase 1E					3/1		
IPI00249982.4	Isoform 1 of Death-inducer obliterator 1					2/1-9		
IPI00643437.1	Uncharacterized protein					3/1		
IPI00784980.1	Isoform 1 of Coiled-coil domain-containing protein 158					2/1-3		
IPI00796214.1	Isoform 2 of Mediator of RNA polymerase II transcription subunit 12-like protein					2/1		
IPI00796249.1	Uncharacterized protein					4/1		
IPI00796316.5	Uncharacterized protein					7/1-3		
IPI00829812.3	13 kDa protein					2/1		

IPI00844250.2	cDNA FLJ52101					2/1		
IPI00853581.1	Uncharacterized protein					3/1-2		
IPI00917789.1	Uncharacterized protein					2/2		
IPI00979799.1	Similar to VH-7 family (N54P3)D/J protein					2/2		
IPI00968154.1	Uncharacterized protein						2/1	
IPI00946590.1	26 kDa protein						2/1	
IPI00447173.1	Antigen MLAA-39						2/1	
IPI00973260.1	cDNA FLJ55558, highly similar to Regulating synaptic membrane exocytosis protein 1						2/1	
IPI01009738.1	Isoform 3 of Golgin subfamily A member 4						2/1	
IPI00436634.4	Isoform 3 of Nipped-B-like protein						2/1	
IPI00006900.1	Something about silencing protein 10						2/1	
IPI00304557.2	Short palate, lung and nasal epithelium carcinoma-associated protein 2							3/1
IPI00827548.3	cDNA FLJ44825 fis, clone BRACE3046609, highly similar to Homo sapiens inhibitor of Bruton agammaglobulinemia tyrosine kinase (IBTK), mRNA							2/1
IPI00868939.1	Similar to Sarcoma antigen NY-SAR-41							2/1
IPI00908840.1	Isoform 3 of AT-rich interactive domain-containing protein 5B							2/1
IPI00945093.1	Protein							2/1
IPI01011877.1	cDNA FLJ59435, highly similar to Homo sapiens outer dense fiber of sperm tails 2 (ODF2), transcript variant 2, mRNA							2/1

Protein A

Accession	Description	AAV (n=7) Frequency/ peptides per hit	TA (n=7) Frequency/ peptides per hit	MCTD (n=9) Frequency/ peptides per hit	DM (n=8) Frequency/ peptides per hit	SS (n=14) Frequency/ peptides per hit	SSc (n=7) Frequency/ peptides per hit	SLE (n=14) Frequency/ peptides per hit
IPI00552578.2	Serum amyloid A protein	2/1						
IPI00973428.1	Putative uncharacterized protein	2/3						
IPI01025129.1	12 kDa protein	2/1						
IPI00007138.2	Isoform 2 of Disks large-associated protein 4	2/1						
IPI00885166.2	polyadenylate-binding protein 4-like		2/1					
IPI00032931.2	Isoform 2 of Peroxisomal targeting signal 1 receptor		2/1					
IPI00965543.1	collagen alpha-1(XI) chain isoform C preproprotein		2/1					
IPI00827532.1	Anti-folate binding protein (Fragment)			3/1				
IPI00217511.1	Seven transmembrane helix receptor			2/1				
IPI00973424.1	Putative uncharacterized protein			2/3-4				
IPI00645207.2	Uncharacterized protein			2/1				
IPI00917650.2	Protein PIEZO1			2/1				
IPI00432678.1	MRSS6228			2/1				
IPI00977998.1	Uncharacterized protein			2/1				
IPI00007193.7	Isoform 2 of Ankyrin repeat domain-containing protein 26				2/1			
IPI00968154.1	Uncharacterized protein				2/1			
IPI00552943.3	V1-11 protein				2/1			
IPI00470871.6	Uncharacterized protein				2/1			
IPI00023466.2	Protein AF-17				2/1			

IPI00423464.1	Putative uncharacterized protein DKFZp686K03196					5/13-15		
IPI00896471.2	Isoform 2 of Protein SZT2					2/1		
IPI00973032.1	V1-17 protein					2/2		
IPI00022987.3	PRO0483					2/1		
IPI00736625.2	Uncharacterized protein C3orf43					2/1		
IPI00333015.7	Isoform 2 of Spectrin beta chain, brain 1					2/1		
IPI00922213.2	cDNA FLJ53292, highly similar to Homo sapiens fibronectin 1 (FN1), transcript variant 5, mRNA						2/1-9	
IPI00970895.1	Isoform 2 of PR domain-containing protein 11						2/1	
IPI01026317.1	cDNA FLJ55146, highly similar to Complement C4-B							2/1
IPI00165579.6	Isoform 2 of Cytosolic non-specific dipeptidase							2/1
IPI00924585.1	neurabin-1 isoform 5							2/1