Elevation of Autoantibody in Patients with Ischemic Stroke

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Abstract

Recent clinical research has revealed a significant correlation between atherosclerosis, one of the primary etiologies of ischemic stroke, and the immune system. Assuming that "disease-specific autoantibodies are produced in the sera of patients with ischemic stroke," we investigated multiple arteriosclerosis-related antibodies using the serological identification of antigens by recombinant cDNA expression cloning (SEREX), an established method for identifying antigenic proteins. We either screened a human aortic endothelial cell cDNA library or conducted protein array screening using the sera from patients with ischemic stroke, such as carotid artery stenosis or transient ischemic attack (TIA). Next, we measured serum antibody levels using amplified luminescent proximity homogeneous assay-linked immunosorbent assay (AlphaLISA) in patient/healthy donor (HD) cohorts and identified several antigens, the antibody levels of which were significantly higher in patients with ischemic stroke than in HDs. This review introduced the method of identifying antigens by the SEREX and protein microarray and summarized antigenic proteins. In particular, it focused on anti-replication protein A2 antibody and anti-programmed cell death 11 antibody, which are significantly related to atherosclerotic plaque and ischemic brain tissue, respectively, and proposed the mechanism of elevated autoantibody levels against them. Furthermore, this review suggests a possibility of clinical application as an atherosclerotic disease diagnostic marker for TIA or cerebral infarction.

Key words: autoantibody, TIA, cerebral infarction, biomarker, atherosclerosis

Introduction

Ischemic stroke is one of the leading causes of morbidity and mortality worldwide, with arteriosclerosis as one of its primary etiologies, the suppression of which is imperative for the prevention of onset.[1–3] To date, several factors have been recognized as underlying the progress of arteriosclerosis, such as age, high blood pressure, hyperlipidemia, diabetes, smoking, and obesity.[4] However, in recent years,

some studies have highlighted that the immune system is profoundly involved in the progression of arteriosclerosis.[5–9] Assuming that "disease-specific autoantibodies are produced in the sera of patients with ischemic stroke," we investigated multiple arteriosclerosis-related antibodies using the serological identification of antigens by recombinant cDNA expression cloning (SEREX), an established method for identifying antigenic proteins.[10,11]

Of these antibodies, we focused on anti-replication protein A2 (RPA 2) antibody and anti-programmed cell death 11 (PDCD 11) antibody, which were significantly related to atherosclerotic plaque and ischemic brain tissue, respectively. RPAs are composed of three subunit proteins of 70, 32, and 14 kDa (RPA1,

RPA2, and RPA3, respectively). In the rat brain, DNA double-strand breaks (DSBs) were induced by ischemia,[12] and BRCA1 which is involved in the repair of DNA DSBs has been shown to be involved in heart function and survival following myocardial infarction.[13] It can be speculated that the RPA complex works in the upstream pathway of BRCA1 in the DSB repair pathway.[14,15] In atherosclerotic plaque, cells are exposed to reactive oxygen species, which can provoke extensive oxidative DNA damage,[16,17] then; the RPA complex which is required for DSB repair might be overexpressed in atherosclerotic lesion. On the other hand, PDCD11 is a NF-κB-binding protein that colocalizes with U3 RNA in the nucleolus and is required for rRNA maturation and generation of 18S rRNA. PDCD11 is necessary for Fas ligand (FasL) expression, and PDCD11 overexpression is known to induce transcription of FasL, leading to the induction of apoptosis through Fas/FasL/caspase death pathway.[18–21] It is also reported that post-stroke inflammatory response of FasL is an important contributing mechanism in ischemic brain lesion.[22] Therefore, it can be speculated that Fas/FasL/caspase death pathway may be activated in the ischemic brain tissue and that PDCD11 may be overexpressed.

This review introduces the method of identifying antigens by the SEREX and protein microarray and summarizes antigenic proteins. In addition, it discusses a proposed mechanism of the elevation of autoantibody and a possibility of clinical application as a disease diagnostic marker.

Autoantibody against Atherosclerotic Lesion

Immune system associated with atherosclerosis

In the arteriosclerotic lesions, several histopathological studies have observed macrophage and lymphocyte infiltration simultaneously as the deposition of lipid in the subintimal membrane,[23] suggesting that the immune system is intensely involved in the establishment of arteriosclerosis.

Apparently, macrophages release several substances, such as free radicals and various proteases, to induce tissue damage and secrete fibrogenic cytokines and play a central role in the formation of advanced arteriosclerosis, such as fibrous thickening of the intima and remodeling of vascular architecture.[24] In atherosclerotic lesions, T-lymphocytes and vascular dendritic cells are reportedly involved in macrophages activation,[23,25] suggesting the presence of an immune mechanism strongly through antigen presentation for arteriosclerosis.

Several studies have been conducted on antigens related to the occurrence of arteriosclerosis,[26] and oxidized low-density lipoproteins (oxLDL) constitute a typical example for candidate antigens. Autoantibodies against oxLDL are detected in the circulating blood, as well as atherosclerotic lesions locally; research has reported a correlation between their autoantibody levels and the degree of arteriosclerosis. In addition, many studies have reported that autoantibodies against heat shock protein (Hsp) family, such as Hsp 27, 60, or 70, are elevated in patients with arteriosclerotic diseases. Furthermore, it is suggested that cross-immunization with bacteria-derived Hsp is its pathogen.

SEREX

The SEREX, a combination of molecular cloning using phage expression libraries with serological typing, is an established method for comprehensively recognizing cancer-specific antigenic proteins and was first reported by Sahin et al.[10] First, mRNA was extracted from a cancer tissue to prepare a cDNA library, which was incorporated into the DNA of the phage vector for protein expression. Next, *Escherichia coli* were infected with the phage, and the expression of resident cDNA clones was induced after blotting inflectional bacteria onto the nitrocellulose membrane. Membranes were then incubated with the sera of patients. Finally, cancer antigens were recognized in patients' immune system by collecting clones that exhibited a positive reaction and analyzing the base sequences.

Protein microarray screening

In addition to the SEREX, we used the ProtoArray v-4.0 human protein microarray system as another screening method,[27] wherein we extracted antigen proteins identified by IgG autoantibodies in the sera of patients. This method identified more candidate antigenic proteins.

Second screening by AlphaLISA

Although the SEREX screening comprehensively recognized positive clones accountable for specific diseases, it could not quantify autoantibody levels in the sera of patients. Next, we compared the serum antibody levels against the candidate antigenic protein using the sera of patients and healthy donors (HDs) to ascertain a relationship between candidate antigens and diseases. We used amplified luminescent proximity homogeneous assay-linked immunosorbent assay (AlphaLISA) for evaluating the serum antibody level. AlphaLISA is an immunoassay using anti-human IgG-conjugated acceptor

beads and glutathione-conjugated donor beads, in which the antigen–antibody reaction in the serum and antigenic protein substitute with luminescence because of a chemical reaction between these beads and is detected with a dedicated measuring instrument; this luminescence reflects the antibody level. Finally, antigens with antibody levels by AlphaLISA significantly higher in patients than in HDs were determined as disease-related antigens.

Identified antigens associated with ischemic stroke

The SEREX is one of the most effective methods for identifying antigenic targets on a genomic scale and has been used to identify more than 1,000 novel cancer antigens.[28–30] Despite initially being developed to screen cancer-associated antigens, this method has been applied for vascular disorders, such as transplant-associated coronary disease,[31] Kawasaki disease,[32] and Moyamoya disease.[33] Thus, we conducted the SEREX screening using a commercially available human aortic endothelial cell cDNA library and the sera of patients, followed by the identification of several antigens accountable for atherosclerotic diseases, such as cerebral infarction, cardiovascular disease (CVD), and diabetes mellitus (DM).[34–40] Of these antigens, we introduced antigens screened using the sera of patients with carotid artery stenosis and transient ischemic attack (TIA).

Identified associated antigens responsible for ischemic stroke

First, we screened a human aortic endothelial cell cDNA library using the sera of patients with ischemic stroke due to severe (>90%) carotid stenosis. Among isolated candidates in the SEREX screening, RPA2,[34] TUBB2C,[35] ATP2B4, and BMP-1[36] were considered to be associated with ischemic stroke, as proven in patient/HD cohorts by AlphaLISA. Subsequently, we performed immunological screening using the sera of patients with TIA and considered PDCD11,[37] MMP1, CBX1, and CBX5[38] to be associated with cerebral infarction or TIA. In addition, we performed protein microarray screening and recognized DHPS[39] and SH3BP5[40] to be associated with ischemic stroke, as demonstrated in patient/HD cohorts.

Compared to HDs, serum antibody levels against RPA2 (RPA2-Ab levels) were significantly higher in patients with ischemic stroke. Thus, we further investigated RPA2-Ab levels using synthetic peptides by enzyme-linked immunosorbent assay to identify the antigenic epitopes of RPA2.[34] In this study, the sensitivity and specificity with autoantibody levels against peptide bRPA2-132 (biotin-LRSFQNKKSLVAFKI; amino acids between positions 132 and 146) were higher than those with RPA2Ab. Next, we assessed

antibody levels against peptide antigen of RPA2-132 (bRPA2-Ab levels) in the following study. Similarly, serum autoantibody levels against TUBB2C were significantly higher in patients with cerebral infarction than in HDs. Furthermore, autoantibody levels were elevated in patients with CVD and DM.[35] Similar results were obtained with anti-ATP2B4 antibody, anti-BMP-1 antibody, anti-DHPS antibody, and anti-SH3BP5 antibody.[36,39,40] Incidentally, these autoantibody levels were elevated not only in patients with cerebral infarction but also in patients with TIA. Conversely, serum autoantibody levels against PDCD11 (PDCD11-Ab levels) and CBX1, which were screened using the sera of patients with TIA, were significantly higher in patients with cerebral infarction or TIA than in HDs. However, autoantibody levels were not elevated in patients with acute myocardial infarction (AMI) and DM.[37,38] Table 1 summarizes the above results.

Therefore, various antigenic proteins considered responsible for ischemic stroke differed in results, resulting in speculation that the mechanism of autoantibody production correlated with this background of difference. An immunohistological examination on RPA 2 and PDCD 11 revealed that RPA 2 was

Table 1 Association with autoantibody biomarkers and atherosclerotic diseases

	CI	DM	CVD
Replication protein A2 (RPA2)[23]	↑	↑	↑
Tublin beta 2C (TUBB2C)[24]	↑	↑	↑
ATPase, Ca^{++} transporting, plasma membrane 4 (ATP2B4)[25]	↑	↑	↑
Bone morphogenetic protein 1 (BMP-1)[25]	↑	↑	↑
Matrix metalloproteinase 1 (MMP1)[27]	↑	↑	↑
Deoxyhypusine synthase (DHPS)[28]	↑	↑	↑
SH3 domain-binding protein 5 (SH3BP5)[29]	↑	↑	↑
Programed cell death 11 (PDCD11)[26]	↑	—	—
Chromobox homolog 1 (CBX1)[27]	↑	—	—

We examined autoantibody levels against above antigens in patients with atherosclerotic diseases, such as cerebral infarction (CI), diabetes mellitus (DM), and cardiovascular disease (CVD). "↑" showed that serum antibody levels were significantly higher in patients compared to those in healthy donors (HDs). On the other hand, "—" showed that serum antibody levels were not elevated in patients. We added the number of reference.
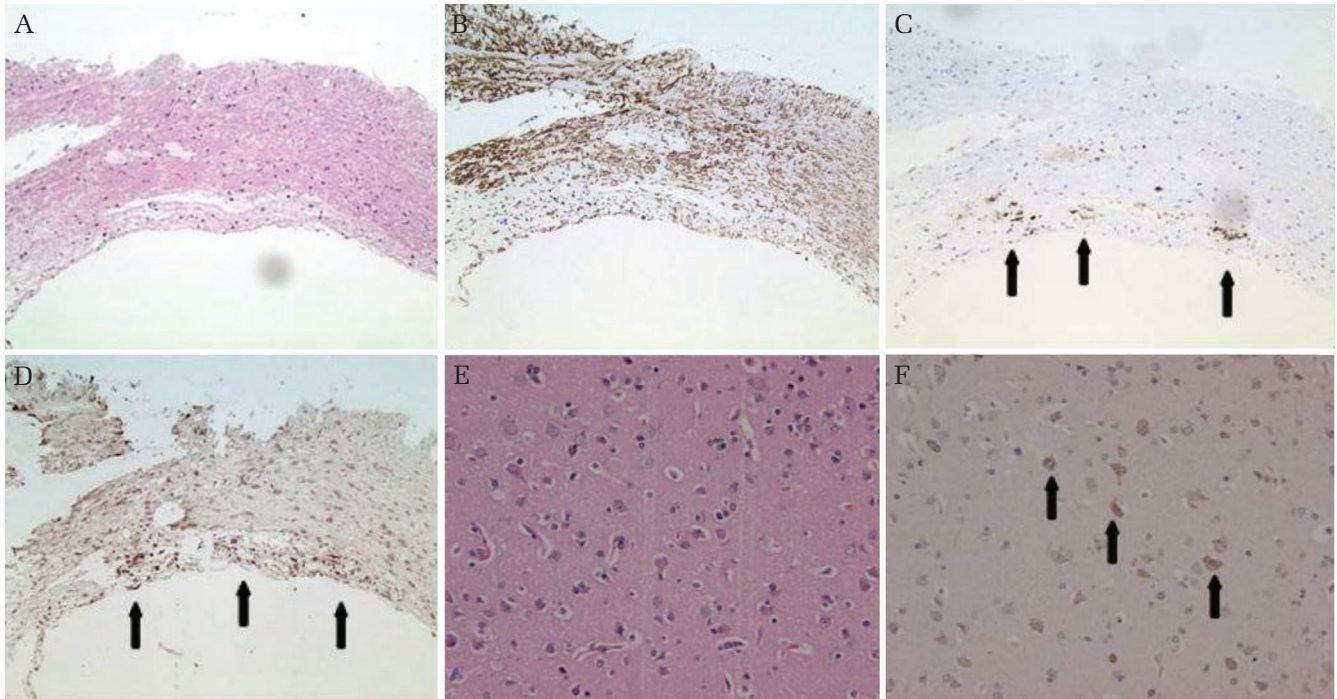
Fig. 1 Immunohistochemistry. Surgically excised carotid plaque (A–D) was stained with hematoxylin only (A), anti-SMC; which identified vascular smooth muscle cells (B), anti-CD68; which identified macrophages (C, *arrow*), or anti-RPA2 antibody (D, *arrow*). Surgically resected ischemic brain tissue (E and F) was stained with hematoxylin only (E) and anti-PDCD11 antibody (F, *arrow*). Photos reprinted with the permission of BioMed Central journals (A–D) and Oncotarget (E and F).

significantly expressed in the intima of arteriosclerotic plaque (Figs. 1A–1D),[34] resulting in the autoantibody production. In contrast, it was confirmed that the PDCD11 expression was closely co-localized with ischemic brain cells (Figs. 1E and 1F),[37] and this expression was considered to have led to the production of PDCD11 autoantibody through an autoimmune response.

As mentioned above, it could be considered that while the elevation of serum RPA2-Ab levels reflects the presence of arteriosclerotic lesions, the elevation of serum PDCD11-Ab levels reflects the presence of a cerebral ischemic lesion, thereby both having different autoantibody production mechanisms. In addition, this finding verified the reason why serum PDCD11-Ab levels were not elevated in patients with AMI and DM. Instead, it was considered that the elevation of serum RPA2-Ab levels reflecting the arteriosclerotic lesion was also observed in other arteriosclerotic diseases. In fact, we investigated correlations between bRPA2-Ab levels and AMI and DM in another validation cohort using the sera of each 128 age-matched patients and HDs. AlphaLISA revealed statistically significantly higher bRPA2-Ab levels in patients with AMI ($P < 0.001$) or DM ($P < 0.001$) compared to those in HDs (Fig. 2, Table 1).
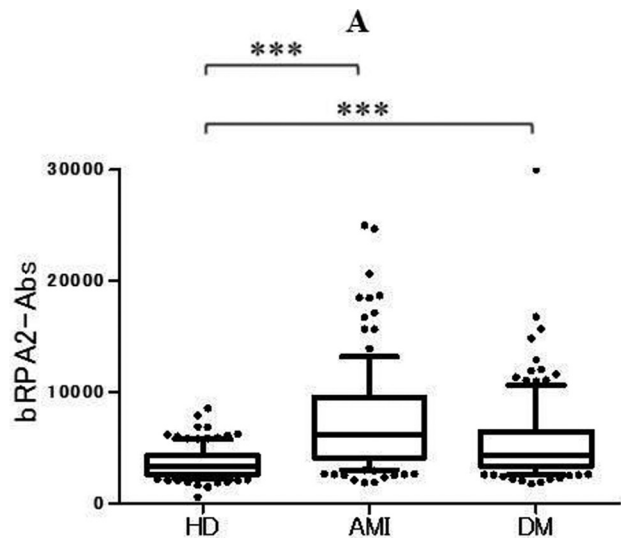

Fig. 2 The correlation between autoantibody levels and other atherosclerotic diseases, including AMI and DM. The levels of bRPA2-Ab (A) were examined by AlphaLISA. The mean age ± SDs of HDs and patients with AMI or DM were 58.29 ± 5.63, 58.28 ± 8.5, and 58.37 ± 9.11 years, respectively. ***$P < 0.001$ was calculated by the Mann–Whitney *U*-test with type I error adjustment using the Bonferroni procedure. bRPA2-Ab, antibody against peptide antigen of RPA2-132; AMI, acute myocardial infarction; DM, diabetes mellitus; HD, healthy donors.

Utility of identified autoantibody as a diagnostic marker

The determination of a disease-specific elevation in the autoantibody level suggests the involvement of some form of immune system in the pathology of the disease and elucidating its mechanism might illustrate the pathology of the disease. Conversely, the elevation in serum disease-specific antibody levels could be a potential disease diagnostic marker.

Furthermore, it is likely that antigen proteins leak out from vascular lesion by recurrent microenvironmental artery destruction, which frequently occurs before the onset of ischemic stroke. Although the antigen proteins disappeared rapidly, antibodies can be amplified after repeated exposure to antigen proteins. Based on this perspective, antibody markers might be more sensitive than antigen markers because repeated exposure of a small amount of an antigen to immune cells leads to the production of a large number of antibodies. Thus, increasing the number of antibody markers, such as those against oxidized LDL for atherosclerosis,[41] p53 antibodies for cancer,[42] and glutamic acid decarboxylase antibodies for type-1 DM,[43] has been recommended for practical use.

Transient ischemic attack was one of the significant risk factors for ischemic stroke, and approximately 15–20% of patients with ischemic stroke are estimated to experience TIA before the onset of a stroke.[44,45] Recent clinical studies have investigated early intervention benefits in patients with TIA to prevent the subsequent development of cerebral infarction.[46] However, the diagnosis of TIA is occasionally challenging because physicians have to diagnose TIA only by considering history and they have few objective pieces of evidence detected by various medical examinations, such as magnetic resonance imaging, echocardiogram, and Holter electrocardiogram. Hence, the diagnosis of TIA simply by blood examination will significantly contribute to the clinical practice in preventing stroke development, as well as facilitating medical economy. Despite highlighting that the elevated PDCD11-Ab level was an independent predictor of TIA, unfortunately, the diagnostic value of PDCD11 alone was weak (sensitivity and specificity were 73.6% and 55.8%, respectively).[37]

We believe that the diagnostic value will increase by a combination of the measurement of multiple autoantibodies with the variable mechanism of production. Next, we examined serum bRPA2-Ab and PDCD11-Ab levels using the sera of 92 patients with TIA and 285 HDs; either bRPA2-Ab or PDCD11-Ab levels were elevated in 79.3% of patients with TIA,

Table 2A Positive predictive values for TIA (*n* = 92)

		bRPA2 (>1731)*		Total
		Positive	Negative	
PDCD11 (>13,921)**	Positive	35	33	68
	Negative	5	19	24
	Total	40	52	92

Table 2B Negative predictive values for HDs (*n* = 285)

Positive		bRPA2 (>1731)*		Total
		Positive	Negative	
PDCD11 (>13,921)**	Positive	63	63	126
	Negative	27	132	159
	Total	90	195	285

Either bRPA2-Ab or PDCD11-Ab levels were elevated in 73 patients with TIA (*n* = 92). Both of antibody levels were not elevated in 19 patients with TIA and 132 HDs (total = 151). *RPA2, elevated RPA2-Ab levels. RPA2-Ab cut-off was 1731 based on ROC curve analysis. **PDCD11, elevated PDCD11-Ab levels. PDCD11-Ab cut-off was 13921 based on ROC curve analysis.

and 87.4% of those who were both of antibody levels were not elevated was HDs (Tables 2A and 2B). Assumedly, this diagnostic value was superior to diagnosis by a single marker. In addition, we are now attempting to produce a peptide antigen of PDCD11 with a localized epitope, which might enhance the sensitivity and specificity. Moreover, the finding that the diagnostic value can increase by a combination of the measurement of multiple autoantibodies with difference production mechanism led us to anticipate the possibility that the diagnostic value will be further enhanced by novel combining markers in the future. Furthermore, it was documented that positive predictive values for TIA with the combination of PDCD11-Ab and clinical risk factors were higher than those with clinical risk factors alone, and all individuals with these four factors developed TIA, including age, hypertension, diabetes, and elevated PDCD11-Ab levels.[37] Thus, the evaluation of antibody markers along with clinical factors might be useful for diagnosis.

Conclusion

This review introduced the ischemic stroke-related antigens determined using the SEREX, particularly, the antibody against RPA2 and PDCD11 implying the arteriosclerotic lesion and the cerebral ischemic tissues, respectively. In addition, it revealed that the elevation of the serum PDCD11-Ab level was an independent predictor of TIA. Thus, combining autoantibody markers with various mechanisms of

production might increase the diagnostic value and be valuable for the estimation of the risk of developing atherosclerotic cerebral infarction.

Ethical Approval

Author Contributions

Acknowledgments

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Conflicts of Interest Disclosure

The author declares no conflict of interest.

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