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Serum Anti-Apo B Antibody Level as Residual CVD Marker in DM Patients under Statin Treatment

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Aim: In the pathogenesis of atherosclerosis, autoantibodies have two-facedness of progression and protection. Previous reports have indicated that low autoantibody levels against apolipoprotein B-100 (apo B-100) could increase the risk of atherosclerotic cardiovascular diseases (CVD) in healthy subjects. In this study, we investigated the relationship between circulating anti-apo B-100 autoantibodies and the clinical parameters in Japanese diabetic patients with or without CVD.

Methods: We measured the serum levels of anti-apo B-100 autoantibodies against native and malondialdehyde (MDA)-modified p45 or p210 epitopes, as well as anti-apo E autoantibodies, using enzyme-linked immunosorbent assay.

Results: In patients with CVD, the circulating levels of IgG against native p45, MDA-modified p45, and MDA-modified p210 ($IgG_{N\text{-}45}$, $IgG_{MDA\text{-}45}$, and $IgG_{MDA\text{-}210}$) were significantly lower than those in patients without CVD, whereas no difference was observed in anti-apo E autoantibody levels. In addition, $IgM_{N\text{-}45}$, $IgM_{MDA\text{-}45}$, and $IgG_{MDA\text{-}45}$ were negatively correlated with LDL-C levels, whereas $IgG_{N\text{-}45}$ and $IgG_{N\text{-}210}$ were positively correlated with HbA1c levels. No correlation was observed between autoantibody levels and diabetic microangiopathy. In the statin-treated subgroup, $IgG_{MDA\text{-}45}$ and $IgG_{MDA\text{-}210}$ were significantly lower in patients with CVD than in those without CVD.

Conclusion: Measurement of serum anti-apo B-100 autoantibodies can be useful for the evaluation of CVD risk in patients with diabetes receiving statin treatment.

Key words: Apolipoprotein B-100, Autoantibody, Cardiovascular disease, Diabetes

Abbreviations: apo, apolipoprotein; CVD, cardiovascular diseases; DM: diabetes mellitus; HDL, high-density lipoprotein; Ig, immunoglobulin; LDL, low-density lipoprotein; MDA, malondialdehyde

Introduction

The prevalence of diabetes mellitus is steadily increasing, with more than 400 million people being reported to have type 2 diabetes globally in 2014 [1]. Cardiovascular disease (CVD) comprises a major cause of death in patients with diabetes[2]. Although multiple clinical trials have demonstrated that statin treatment reduces primary and secondary CVD events and mortality[3], a considerable residual CVD risk has been noted. Therefore, biomarkers of CVD risk need to be identified for targeted diabetic therapies.

CVD events are developed based on the progression of atherosclerotic plaques, recognized as inflammatory lesions occurring in large- and medium-sized arteries[4]. Atheromatous plaques contain different

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types of inflammatory cells, such as macrophages and lymphocytes[5-7], that are involved in the innate recognition of disease-specific antigens, followed by adaptive immunity[8, 9]. At the initiation of atherosclerosis, monocytes attach to the vascular endothelial surface and mature into macrophages. The activated macrophages uptake modified lipoproteins in the arterial intima, subsequently stimulating B cells to generate antibodies against modified lipoproteins.

Accordingly, the roles of B cells on the development of atherosclerosis have been studied in several experimental models. Caligiuri *et al.* demonstrated that B cell-associated protective immunity reduced atherosclerotic progression, observed as aggravated atherosclerosis in the apolipoprotein (apo) E-deficient, B cell-deprived mice[10]. Similarly, Major *et al.* showed that B cell depletion increased atherosclerosis in LDL receptor knockout mice[11], and Doran *et al.* demonstrated that adoptive B cell-transfer reduced diet-induced atherosclerosis in mice deficient in B cells[12]. In addition, some immunization studies have supported athero-protective roles of B cell-derived humoral immunity[13].

Of the several antigens involved in these adaptive responses of atherosclerosis, apo B-100, oxidized LDL (oxLDL), heat shock protein 60 (HSP60), and HSP65 are the promising candidates for T cell activation[14-16]. However, clinical studies have showed conflicting data on the relationship between the serum levels of these antibodies and CVD. Some studies have reported that the plasma antibody titers against HSP60 and oxLDL were elevated in patients with CVD[17, 18], whereas some have reported an inverse correlation between anti-oxLDL antibody levels and carotid artery atherosclerosis[19]. Apo B-100 is the primary apolipoprotein on LDL, and elevated serum apo B levels are supposed to drive plaque formation[20]. The relationship between anti-apo B autoantibodies and atherosclerosis has been mostly studied in patients with CVD, but the role of these autoantibodies on CVD has not been well clarified in patients with diabetes.

We proposed to investigate the association of serum autoantibody titers against apo B-100 peptides with the macro- and microangiopathies in Japanese patients with diabetes.

Methods

Patients

We enrolled outpatients with diabetes presenting to the NTT West Osaka Hospital between September and November 2014. In total, 90 patients with records on diabetic complications who provided informed consent were consecutively enrolled. Those aged >85 years or having renal dysfunction (serum creatinine [s-Crn] >2.0 mg/dL) were excluded from this study. This study was approved by the ethics committees of both NTT West Osaka Hospital and the Osaka University Hospital.

Diabetic retinopathy was diagnosed by the treating ophthalmologist, based on the presence of characteristic microvascular changes in the retina observed by ophthalmoscopy through dilated pupils. Severity of diabetic retinopathy was determined according to the Davis classification[21], and staged according to "a new classification of diabetic nephropathy 2014" of Japan Diabetes Society[22]. Diabetic neuropathy was diagnosed based on the presence of at least two positive findings among abnormal sensation, vibration abnormality on both sides of the ankle, and ankle tendon reflex abnormality on both sides.

Measurement of Serum Parameters

Morning blood samples were obtained after overnight fast, and the biochemical markers were measured in the hospital laboratory.

Measurement of Serum Levels of Antibodies against Apolipoprotein B by Enzyme-Linked Immunosorbent Assay

The apo B peptides, p45 (amino acids 661–680; IEIGLEGKGFEPTLEALFGK) and p210 (amino acids 3136–3155; KTTKQSFDLSVKAQYKKNKH), were synthesized (Sigma-Aldrich; Saint-Louis, MO), and their MDA-modified peptides were produced according to the previously mentioned method[23]. Apo E peptide (amino acids 158–178; HLRKLRKRLLRDADDLQKRLA) containing the LDL receptor-binding domain was also generated (Sigma-Aldrich; Saint-Louis, MO). These peptides were diluted at 4 μg/mL in dimethyl sulfoxide, and 50 μL was dropped into each microtiter plate well for 2 hours using peptide coating kit (TAKARA; Shiga, Japan), according to the manufacturer's protocol. After washing with distilled water three times, the peptide-coated plates were incubated with blocking solution (TAKARA) for 1 h at room temperature (RT).

The test serum was then diluted at 1:100 with TBS-containing 0.01% Tween-20 (Santa Cruz Biotechnology; Dallas, TX), and 100 μL of the diluted serum was added into each well of the ELISA plate for 2-h incubation at RT. After rinsing with 0.1% Tween 20-containing PBS (pH 7.5) three times, deposition of autoantibodies directed to the peptide was detected using HRP-conjugated rabbit polyclonal secondary antibodies against human IgG, IgM, and IgA (Agilent Technologies; Santa Clara, CA) with an appropriate dilution with TBS-T. After washing the wells using

Table 1. Characteristics of the patients

	Mean ± SD (range)
Age (years)	67 ± 9 (42–83)
Diabetes history (years)	16 ± 10 (1–43)
Sex (M/F)	73/17
BMI (kg/m^2)	24.7 ± 3.7 (17.4–38.0)
Systolic blood pressure (mmHg)	130 ± 13 (84–161)
Smoking (+/−)	51/39
TC (mg/dL)	174 ± 26 (122–255)
TG (mg/dL)	126 ± 75 (31–530)
HDL-C (mg/dL)	54 ± 13 (33–95)
LDL-C (mg/dL)	96 ± 21 (38–160)
RemL-C (mg/dL)	8.0 ± 5.5 (1.7–38.4)
Fasting plasma glucose (mg/dL)	129 ± 26 (78–223)
HbA1c (%)	7.0 ± 0.7 (5.5–9.2)

TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; RemL-C, remnant cholesterol; HbA1c, hemoglobin A1c.

TBS-T three times, 100 µL TMB substrate (Abcam; Cambridge, UK) was added into each well. The ELISA plates were incubated for 15 min at RT with agitation and protection from light. The reaction was stopped by adding 100 µL stop solution to each well (Abcam). The absorbance at 450 nm was measured using Multiskan FC (Thermo Fisher Scientific; Waltham, MA).

Statistical Analysis

JMP version 11.2.2 (SAS Institute Inc., Cary, North Carolina) was used for statistical analysis. Data are presented as mean ± standard deviation (SD) values. Spearman correlation coefficient was used to evaluate the association between two variables. In the stepwise multiple regression analysis, the F-value was set at 2.0 for the inclusion of variables. Mann–Whitney test was used for statistical analyses of independent samples, and a p value of <0.05 was considered significant.

Results

Patients

The demographic and clinical characteristics of the enrolled patients are detailed in **Table 1**. The study population was predominantly men, with an average age of 67 ± 9 years. Most patients were not obese, with a mean body mass index of 24.7 ± 3.7 kg/m^2. Moreover, their blood glucose levels were well controlled with oral hypoglycemic agents and/or insulin (**Supplemental Table 1**), as HbA1c and FPG were 7.0 ± 0.7% and 129 ± 26 mg/dL, respectively. Serum lipid levels were in good control with the use of statins and fibrates (**Supplemental Table 1**); the mean serum triglyceride was 126 ± 75 mg/dL and the mean LDL-C level was 96 ± 21 mg/dL.

The diabetic micro- and macroangiopathy complications in patients are listed in **Table 2**. The number of patients with ischemic heart disease, stroke, and arteriosclerosis obliterans was 23, 8 and 6, respectively. Patients who had at least one of these three diseases were defined as "atherosclerotic patients" ($n=29$). Missing data numbers were 4, 4, and 5 in retinopathy, nephropathy, and neuropathy, respectively. There were no missing data for macroangiopathy.

Measurement of Anti-Apo B-100 Autoantibodies by ELISA

The serum levels of autoantibodies against native and MDA-modified apo B peptides, p45 and p210, were measured using a homemade ELISA (**Supplemental Table 2**). The titers of the autoantibodies against native p45 and MDA-p45 in all immunoglobulin subclasses were significantly and positively correlated (**Supplemental Fig. 1**). Similarly, positive correlations were observed between the antibody titers to native p210 and MDA-p210 in each subclass (**Supplemental Fig. 1**).

The serum levels of IgG class antibodies against native p45 ($IgG_{N\text{-}45}$) and $IgG_{N\text{-}210}$ were both positively correlated with HbA1c levels ($\rho=0.230$, $p<0.05$ and $\rho=0.300$, $p<0.05$, respectively; **Supplemental Table 3**), whereas no such correlation was observed for MDA-modified antibodies. The $IgM_{N\text{-}45}$ and $IgM_{MDA\text{-}45}$ levels were negatively correlated with serum LDL-C

Table 2. Complications in the patients with diabetes

Microangiopathy		
Nephropathy	Stage (1/2/3/4/5)	47/27/11/1/0
Retinopathy	(NDR/SDR/PrePDR/PDR)	64/13/4/5
Neuropathy	(−/+)	52/33
Macroangiopathy		
Atherosclerotic changes		29
Ischemic heart disease		23
Stroke		8
Arteriosclerosis obliterans		6

NDR, no diabetic retinopathy; SDR, simple diabetic retinopathy; PrePDR, preproliferative diabetic retinopathy; PDR, proliferative diabetic retinopathy.
Atherosclerotic changes were defined as having at least one previous cardiovascular disease, stroke, or arteriosclerosis obliterans.

levels ($\rho = -0.262$, $p < 0.05$ and $\rho = -0.259$, $p < 0.05$, respectively); HDL-C levels ($\rho = -0.267$, $p < 0.05$ and $\rho = -0.228$, $p < 0.05$, respectively); and total cholesterol ($\rho = -0.299$, $p < 0.005$ and $\rho = -0.262$, $p < 0.05$, respectively), but not with triglycerides (data not shown). LDL-C levels had tendencies for negative correlation with $IgM_{N\text{-}210}$ and $IgM_{MDA\text{-}210}$ levels ($\rho = -0.206$, $p = 0.053$ and $\rho = -0.201$, $p = 0.059$, respectively; **Supplemental Table 3**). The IgG levels of antibodies were not correlated with LDL-C, except for $IgG_{MDA\text{-}45}$ ($\rho = -0.219$, $p < 0.05$; **Supplemental Table 3**).

Relationship between Autoantibodies against Apo B-100 or Anti-Apo E and Diabetic Complications

We next investigated the association between anti-apo B-100 autoantibodies and diabetic complications (**Table 3** and **Fig. 1**). Compared with the non-atherosclerotic group, the atherosclerotic group had significantly lower serum $IgG_{N\text{-}45}$, $IgG_{MDA\text{-}45}$, and $IgG_{MDA\text{-}210}$ levels. The serum $IgG_{N\text{-}210}$ levels tended to be low in the atherosclerotic group. No differences were observed in the titers of the IgM class autoantibodies against apo B-100 between these two groups. There was no association between anti-apo E autoantibodies and diabetic complications (**Supplemental Fig. 2**). In addition, the antibody titers against both apo B-100 and apo E were similar, regardless of the severity of diabetic microangiopathy (**Table 3** and data not shown).

Relationship between Anti-Apo B Autoantibodies and Atherosclerotic Diseases According to Statin Treatment

Interestingly, compared with the atherosclerotic group without statin treatment, the statin-treated atherosclerotic group had significantly lower IgG autoantibody levels, including $IgG_{N\text{-}45}$ (0.223 ± 0.178 vs. 0.306 ± 0.281, $p = 0.056$); $IgG_{MDA\text{-}45}$ (0.360 ± 0.284 vs. 0.510 ± 0.345, $p < 0.05$); $IgG_{N\text{-}210}$ (0.282 ± 0.236 vs. 0.357 ± 0.287, $p = 0.092$); and $IgG_{MDA\text{-}210}$ (0.446 ± 0.285 vs. 1.067 ± 1.257, $p < 0.05$) (**Table 4**). No significant difference was observed in the group without statin treatment.

Because LDL-C levels are recognized as a risk factor for atherosclerosis and can be reduced by statin treatment, we examined the effects of statin treatment on the relationship between the autoantibody titers and the clinical parameters. Overall, LDL-C level was negatively correlated with $IgM_{N\text{-}45}$ ($\rho = -0.262$, $p < 0.05$; **Supplemental Table 3**) and $IgM_{MDA\text{-}45}$ ($\rho = -0.259$, $p < 0.05$); in the group without statin treatment, these correlations were stronger ($\rho = -0.358$, $p < 0.05$ and $\rho = -0.410$, $p < 0.05$, respectively; **Supplemental Table 4**). Moreover, in the group without statin treatment, LDL-C level was significantly and negatively correlated with $IgM_{N\text{-}210}$ ($\rho = -0.473$, $p < 0.01$) and $IgM_{MDA\text{-}210}$ ($\rho = -0.403$, $p < 0.05$) levels. No associations were observed in the statin-treated group. The HbA1c level was positively correlated with the serum levels of $IgG_{N\text{-}45}$ and $IgG_{N\text{-}210}$, but not with the MDA-modified antibodies.

Finally, we conducted stepwise regression analyses to identify independently associated clinical parameters for atherosclerosis in the statin-treated group. These analyses revealed that $IgG_{N\text{-}210}$ and $IgG_{MDA\text{-}210}$ had stronger association with atherosclerosis, compared with other clinical parameters. $IgG_{MDA\text{-}210}$ was found to be the strongest explanatory variable for atherosclerosis (data not shown).

Discussion

In this study, we investigated the relationship

Table 3. Relationship between the autoantibody titers and diabetes complications

		Atherosclerosis			Nephropathy		
	Class	(−)	(+)	*p*	Stage < 3	Stage ≥ 3	*p*
N-45	IgG	0.264 ± 0.233	0.196 ± 0.150	< 0.05	0.232 ± 0.156	0.331 ± 0.434	n.s.
	IgM	0.732 ± 0.447	0.723 ± 0.659	n.s.	0.708 ± 0.547	0.813 ± 0.389	n.s.
MDA-45	IgG	0.443 ± 0.329	0.313 ± 0.243	< 0.05	0.391 ± 0.269	0.492 ± 0.524	n.s.
	IgM	0.978 ± 0.606	0.818 ± 0.455	n.s.	0.915 ± 0.584	0.987 ± 0.538	n.s.
N-210	IgG	0.313 ± 0.242	0.248 ± 0.198	0.065	0.279 ± 0.184	0.392 ± 0.438	n.s.
	IgM	0.754 ± 0.509	0.708 ± 0.580	n.s.	0.731 ± 0.559	0.788 ± 0.413	n.s.
MDA-210	IgG	0.826 ± 1.051	0.384 ± 0.255	< 0.05	0.681 ± 0.919	0.761 ± 0.891	n.s.
	IgM	1.025 ± 0.656	0.844 ± 0.690	n.s.	0.967 ± 0.691	0.954 ± 0.644	n.s.

		Retinopathy			Neuropathy		
	Class	(−)	(+)	*p*	(−)	(+)	*p*
N-45	IgG	0.251 ± 0.229	0.228 ± 0.172	n.s.	0.231 ± 0.150	0.266 ± 0.294	n.s.
	IgM	0.725 ± 0.521	0.673 ± 0.496	n.s.	0.692 ± 0.547	0.786 ± 0.499	n.s.
MDA-45	IgG	0.418 ± 0.324	0.368 ± 0.283	n.s.	0.371 ± 0.229	0.450 ± 0.415	n.s.
	IgM	0.925 ± 0.498	0.889 ± 0.746	n.s.	0.879 ± 0.501	1.016 ± 0.678	n.s.
N-210	IgG	0.297 ± 0.250	0.292 ± 0.189	n.s.	0.271 ± 0.184	0.331 ± 0.302	n.s.
	IgM	0.712 ± 0.487	0.795 ± 0.665	n.s.	0.689 ± 0.491	0.832 ± 0.608	n.s.
MDA-210	IgG	0.700 ± 0.961	0.533 ± 0.515	n.s.	0.526 ± 0.505	0.959 ± 1.295	n.s.
	IgM	0.984 ± 0.670	0.847 ± 0.642	n.s.	0.972 ± 0.717	0.971 ± 0.634	n.s.

Values are presented as mean ± SD.
n.s = not significant.

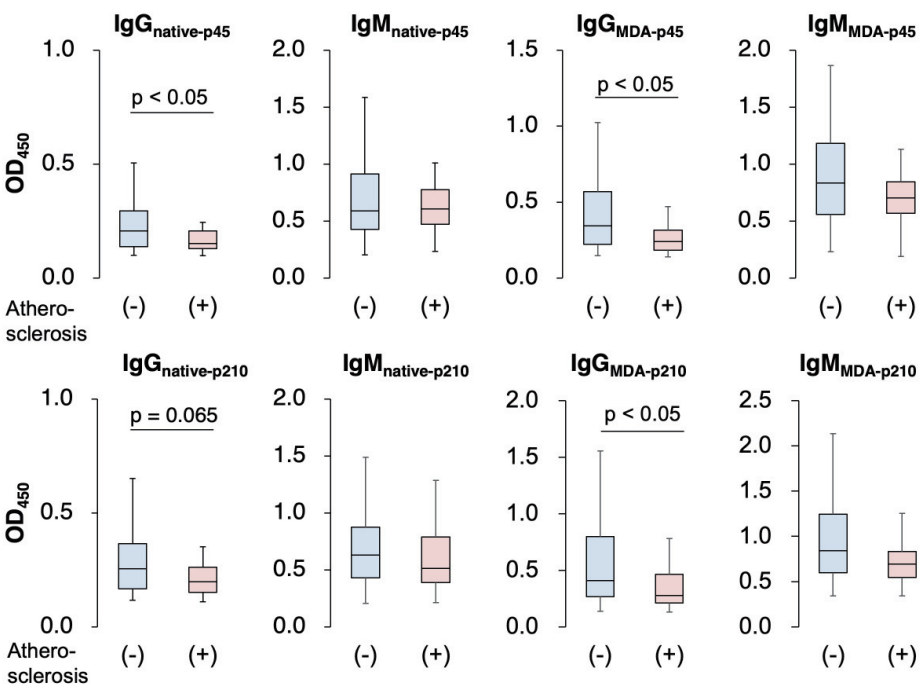

Fig. 1. Association between serum levels of anti-apolipoprotein B autoantibodies and atherosclerosis in patients with diabetes

The serum levels of anti-apolipoprotein B autoantibodies in the IgG or IgM class were compared between the atherosclerotic (+) or non-atherosclerotic (−) group (n = 29 and 60, respectively).

Table 4. Relationship of the autoantibodies with atherosclerosis according to the intake of statins

Statin (+)	Class	Atherosclerosis (−)	Atherosclerosis (+)	*p*
N-45	IgG	0.306 ± 0.281	0.223 ± 0.178	0.056
	IgM	0.727 ± 0.397	0.769 ± 0.804	n.s.
MDA-45	IgG	0.510 ± 0.345	0.360 ± 0.284	< 0.05
	IgM	1.009 ± 0.541	0.848 ± 0.522	n.s.
N-210	IgG	0.357 ± 0.287	0.282 ± 0.236	0.092
	IgM	0.745 ± 0.444	0.736 ± 0.684	n.s.
MDA-210	IgG	1.067 ± 1.257	0.446 ± 0.285	< 0.05
	IgM	1.077 ± 0.684	0.950 ± 0.823	n.s.

Statin (−)	Class	Atherosclerosis (−)	Atherosclerosis (+)	*p*
N-45	IgG	0.217 ± 0.152	0.145 ± 0.050	n.s.
	IgM	0.737 ± 0.504	0.641 ± 0.221	n.s.
MDA-45	IgG	0.368 ± 0.298	0.231 ± 0.098	n.s.
	IgM	0.943 ± 0.681	0.768 ± 0.323	n.s.
N-210	IgG	0.262 ± 0.170	0.189 ± 0.069	n.s.
	IgM	0.764 ± 0.584	0.655 ± 0.341	n.s.
MDA-210	IgG	0.551 ± 0.675	0.281 ± 0.130	n.s.
	IgM	0.966 ± 0.630	0.636 ± 0.253	n.s.

Values are presented as mean ± SD.
n.s. = not significant.

between circulating anti-apo B-100 autoantibodies and the clinical parameters in 90 Japanese diabetic patients with or without CVD. The serum levels of IgG class anti-apo B-100 antibodies were significantly lower in patients with CVD than in those without CVD, but there was no correlation between autoantibody levels and any diabetic microangiopathy. Even in statin-treated patients with diabetes (n=52), serum levels of IgG class anti-apo B-100 autoantibodies were significantly lower in those with CVD complications.

Numerous large-scale trials have demonstrated that statin therapy reduces the rates of primary and secondary cardiovascular events. However, several statin-treated patients continue to experience life-threatening vascular events, usually described as "residual risk." An important effect of statin treatment is the reduction of LDL-C, which is clearly associated with decreased cardiovascular events and plaque regression. However, statin-mediated risk reduction was only about 30%, indicating the presence of unidentified residual risks other than LDL-C[3, 24-26]. Analysis of patients whose cardiac plaque progressed despite considerably low LDL-C levels identified that diabetes mellitus, hypertension, low HDL-C levels, and high apo B levels were independent risk factors[27]. It is conceivable that there are unknown risk factors in addition to these risk factors.

Recently, the role of immune response by autoantibodies against self-antigens in the pathogenesis of atherosclerosis has been the focus of many studies. Several immunohistochemical studies have revealed that oxidized LDL epitopes, anti-oxidized LDL autoantibodies, and T cells recognizing oxidized LDL were found in atherosclerotic plaques[28]. These Th1 immune responses to self-antigens could accelerate the process of atherosclerosis. On the contrary, anti-atherosclerotic immune responses induced by autoantibodies have been reported by some studies; for example, reduced atherosclerosis was found in immunized animals with anti-LDL or anti-apo B-100 antibodies[29]. Circulating autoantibodies against oxidized LDL are commonly detected in almost all individuals[30]. These immunogenic targets are generated from lipids and apolipoproteins contained in the oxidized

LDL; the oxidized LDL-associated apo B-100 are fragmented during LDL oxidation, both in proteolytical and aldehyde-modified manners[31]. However, the association between the autoantibody titer against oxidized LDL and CVD had been inconsistent in early reports, probably because of the lack of established ELISA systems to detect autoantibodies against mixed anti-oxidized LDL antigens[32-35]. The oxidized LDL used in experiments is generated in several different ways. Such inconsistent results were overcome in subsequent studies by using ELISA systems with specific anti-apo B-100 fragment as an antigen[36-43]. Based on the initial screening of apo B-100 peptide epitopes that are specifically recognized by oxidized LDL autoantibodies in human plasma[44], p45 and p210 peptides have been identified as the most important targets for immune response against LDL in patients with CVD[39, 45].

The correlation between atherosclerosis and serum autoantibody levels against p45 or p210 has been investigated in previous reports. Serum $IgG_{MDA\text{-}45}$ and $IgG_{N\text{-}210}$ levels were significantly lower in patients with coronary artery disease[42, 45]. $IgG_{MDA\text{-}210}$ levels were also negatively correlated with coronary plaque size[40]. $IgM_{MDA\text{-}210}$ levels in patients with carotid stenosis, or $IgM_{MDA\text{-}210}$ levels in patients with coronary artery disease, were reported to be increased[39, 42]. Although the mechanism of anti-atherogenic effects remains to be completely elucidated, Zeng *et al.* recently reported that anti-apo B peptide 210 antibodies ameliorate atherosclerosis by altering the phagocyte of macrophages in apolipoprotein E knockout mice[46].

In our study, serum IgG levels against p45 and p210 were significantly lower in patients with CVD events than those without CVD events. This result was similar to previously reported results that serum $IgG_{N\text{-}45}$ and $IgG_{N\text{-}210}$ levels were relatively low in type 2 diabetic patients with advanced coronary artery disease[38]. Interestingly, these associations were observed even in the statin-treated subgroup. Because the recommendation for patients with diabetes is the strict reduction of LDL-C levels, novel methods of evaluating the residual CVD risk are required for diabetic patients whose LDL-C levels are relatively well controlled. In the statin-free group, the tendency of autoantibody levels was similar, although no significant difference was observed probably because of the small sample size. Based on our results, we propose that serum IgG levels against p45 and p210 could be useful markers for residual CVD risk.

In addition to anti-apo B-100 autoantibodies, we studied anti-apo E autoantibodies, because both apo B and apo E are reported in human atherosclerotic tissues[47]. Apo E is essential for the catabolism of remnant lipoproteins, and apo E deficiency is known to result in the development of type III hyperlipemia. In contrast to anti-apo B autoantibodies, however, no association was observed between CVD and anti-apo E autoantibodies. These results suggest that the roles of autoantibodies on atherosclerosis might be antigen-specific, although further investigations are warranted to clarify the detailed mechanism.

In this study, serum LDL-C levels were negatively correlated with anti-apo B autoantibody levels, especially with the IgM class autoantibody against p45 and p210, but not with the IgG-class. The negative correlation between IgM autoantibodies and LDL-C levels was observed more strongly in the subgroup without statin treatment. Previous reports suggested that statin treatment itself might lead to various alterations in the serum levels of anti-oxLDL autoantibody by modulating adaptive immunity[48-52]. We speculate that catabolism of immunoglobulin-conjugated LDL might be accelerated through the reticuloendothelial system, although further investigation is necessary. In addition, a positive correlation was observed between IgG autoantibodies and HbA1c levels. In patients with type 1 diabetes, an inverse correlation between the levels of the anti-oxLDL antibody and HbA1c was reported, but their mechanism of action remains to be elucidated[33, 53].

We observed that IgM class antibodies (N-45, MDA-45, N-210, and MDA-210) were correlated with LDL-C and TC, as well as HDL-C levels (except MDA-210). Because apo B-100 is a major apolipoprotein on LDL particle, the correlation between LDL-C and anti-apo B autoantibodies can be derived from direct effects of the autoantibodies on LDL metabolism. On the contrary, some previous reports have shown the mutual correlation between LDL and HDL metabolism, suggesting that an indirect effect of anti-apo B-100 autoantibodies on HDL metabolism. The decreased production and increased catabolism of apo A-1 were reported in familial hypercholesterolemia (FH)[54]. Similarly, defective apo B-100 affected both catabolism and production rate of apo A-1 in patients with familial defective apo B-100 (FDB)[55]. Although the cross-talk mechanism between LDL and HDL metabolism are still unclear, anti-apo B-100 autoantibodies could influence not only LDL metabolism but also HDL metabolism.

This study has some limitations. We conducted a cross-sectional, single-center study and the sample size was markedly small. We did not measure hydroxynonenal- and methylglyoxal-modified forms of anti-apo B-100 antibodies. Further larger case-control studies

are necessary to determine whether the anti-apo B-100 autoantibodies IgG$_{MDA-45}$ and IgG$_{MDA-210}$ are risk factor of CVD in patients with diabetes.

In conclusion, low serum levels of the anti-apo B-100 autoantibodies IgG$_{MDA-45}$ and IgG$_{MDA-210}$ could be a CVD risk factor in Japanese patients with diabetes. The measurement of these autoantibodies might be useful to identify patients with high residual CVD risk under statin treatment.

Contribution Statement

H.Y., M.K., I.K., M.I. and Y.M. performed research and clinical data analysis; T.S., Y.C., and K.H. performed clinical work and analysis; H.Y., K.H., and S.K. designed research, and H.Y. and S.K. wrote the manuscript.

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Disclosure Summary

There are no financial conflicts of interest to disclose.

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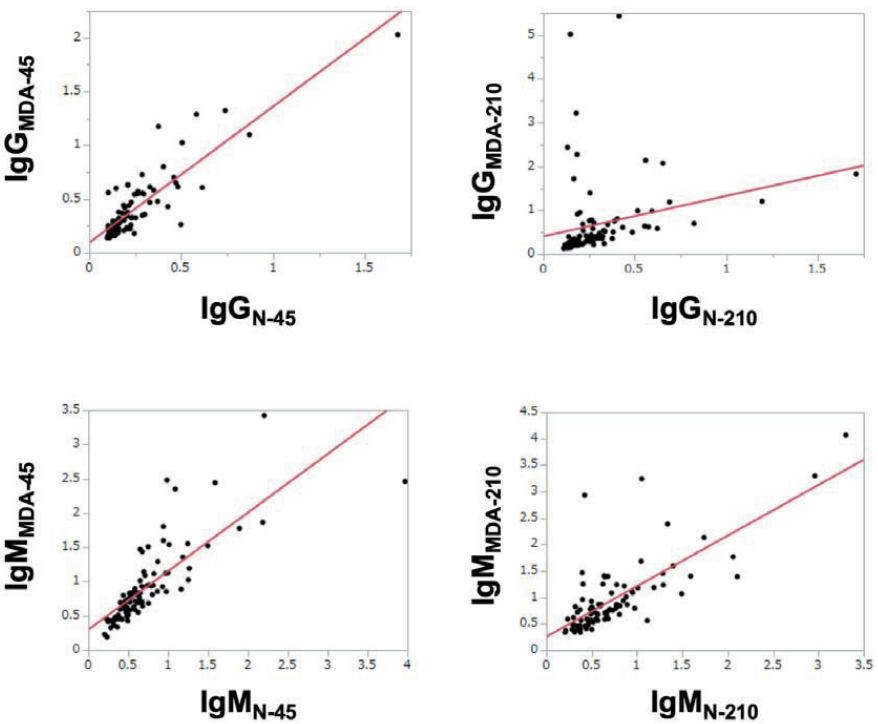
Supplemental Table 1. Medical treatment of the patients

	n (%)
Statins	52 (58%)
Fibrates	8 (9%)
Anti-diabetic agents	
OHA only	51 (57%)
Insulin only	13 (14%)
OHA + Insulin	12 (13%)
Anti-hypertensive agents	57 (63%)
Anti-platelet agents	28 (31%)

Supplemental Table 2. Titres of antibodies against apo B-100

		OD_{450}
N-45	IgG	0.244 ± 0.210
	IgM	0.726 ± 0.516
MDA-45	IgG	0.404 ± 0.306
	IgM	0.920 ± 0.560
N-210	IgG	0.295 ± 0.229
	IgM	0.734 ± 0.526
MDA-210	IgG	0.682 ± 0.887
	IgM	0.961 ± 0.664



Supplemental Fig. 1. Correlation of the titres of native and MDA-modified apo B-100 autoantibodies

The serum levels of anti-apo B-100 autoantibodies against native and MDA-modified apo B-100 autoantibodies in the IgG or IgM class were compared.

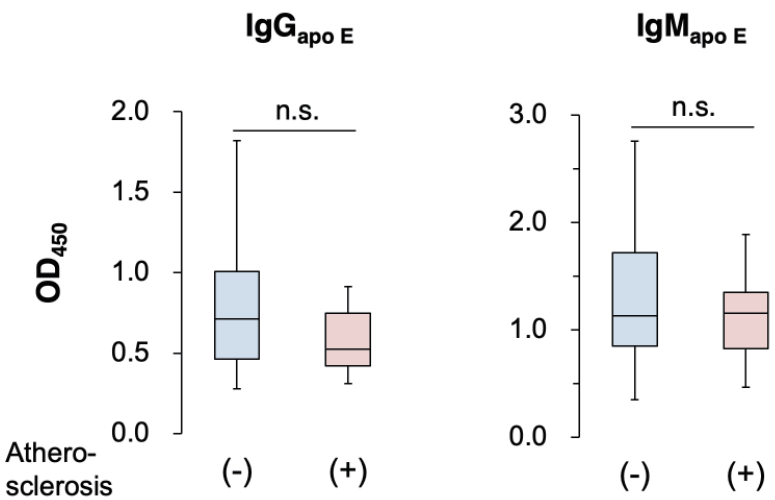
Supplemental Table 3. Correlation between autoantibodies against apolipoprotein B-100 and the clinical parameters

	Class	Age	BMI	FBS	HbA1c	LDL-C	HDL-C	TC
N-45	IgG	n.s.	n.s.	n.s.	0.230***	n.s.	n.s.	n.s.
	IgM	n.s.	n.s.	n.s.	n.s.	−0.262*	−0.267*	−0.299***
MDA-45	IgG	n.s.	n.s.	n.s.	0.185	−0.219*	n.s.	n.s.
	IgM	n.s.	n.s.	n.s.	n.s.	−0.259*	−0.228*	−0.262*
N-210	IgG	n.s.	n.s.	n.s.	0.300***	n.s.	n.s.	n.s.
	IgM	n.s.	n.s.	n.s.	n.s.	−0.206	−0.214*	−0.250*
MDA-210	IgG	n.s.	n.s.	0.301*	n.s.	n.s.	−0.182	n.s.
	IgM	n.s.	n.s.	n.s.	n.s.	−0.201	n.s.	−0.200

Values are presented as Spearman's correlation coefficient (ρ)

n.s., not significant

*$p<0.05$, **$p<0.01$, ***$p<0.005$



Supplemental Fig. 2. Association between serum levels of anti-apolipoprotein E autoantibodies and atherosclerosis in the diabetic patients

The serum levels of anti-apolipoprotein E autoantibodies in the IgG or IgM class were compared between the atherosclerotic (+) or non-atherosclerotic (−) group ($n=29$ and 60, respectively).

Supplemental Table 4. Relationship of the autoantibodies with HbA1c or LDL-C according to the intake of statins

		HbA1c		LDL-C	
	Class	statin (−)	statin (+)	statin (−)	statin (+)
N-45	IgG	n.s.	0.231	n.s.	n.s.
	IgM	n.s.	n.s.	−0.358*	n.s.
MDA-45	IgG	0.319	n.s.	n.s.	n.s.
	IgM	n.s.	n.s.	−0.410*	n.s.
N-210	IgG	n.s.	0.337*	n.s.	n.s.
	IgM	n.s.	n.s.	−0.473**	n.s.
MDA-210	IgG	n.s.	n.s.	n.s.	n.s.
	IgM	n.s.	n.s.	−0.403*	n.s.

Values are presented as Spearman's correlation coefficient (ρ)
Statin (−), $n=37$; Statin (+), $n=52$
n.s. = not significant, $^{*}p<0.05$, $^{**}p<0.01$