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Detection of circulating IgG antibodies to apolipoprotein B_{100} in acute myocardial infarction

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A B S T R A C T

A number of studies have reported an association between increased levels of antibodies against oxidized low-density lipoprotein (oxLDL) and cardiovascular disease, but the anti-oxLDL antibody has not been confirmed to serve as an effective biomarker for prediction of acute myocardial infarction (AMI). Apolipoprotein B_{100} ($ApoB_{100}$)-derived peptide fragments generated by proteolytic degradation and aldehyde modification are the major antigens in oxLDL, and so the present work was undertaken to detect circulating IgG for Apo-B_{100}-derived peptide antigens. An in-house enzyme-linked immunosorbent assay (ELISA) was developed with eight $ApoB_{100}$-derived peptide antigens (Ag1–Ag8) to detect circulating anti-$ApoB_{100}$ IgG levels in 267 patients with AMI and 201 control subjects. Binary logistic regression analysis revealed that circulating IgG for Ag1 was significantly higher in the patient group than the control group ($P < 0.001$) after adjustment for age, gender, smoking, hypertension, diabetes and circulating levels of cholesterol, HDL, LDL, ApoA and $ApoB_{100}$. None of the other seven antigens detected an increase in IgG levels in AMI patients compared with control subjects. Spearman correlation analysis showed no correlation between IgG antibody for Ag1 and clinical characteristics. In conclusion, the linear peptide antigens derived from $ApoB_{100}$ may be suitable for the development of an ELISA antibody test for prediction of AMI, although further confirmation is still needed in large-scale clinical studies.

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1. Introduction

Acute myocardial infarction (AMI) is defined as myocardial cell death due to prolonged ischemia [14], characterized mainly by rupture or erosion of coronary atherosclerotic plaque and subsequent complete or incomplete occlusive thrombosis. Patients can be diagnosed as having AMI when they have increased blood concentrations of sensitive and specific biomarkers, such as circulating troponin or the membrane-bound fraction of creatinine kinase [30]. AMI is the leading cause of death in coronary heart disease, but there are still no effective biomarkers available for prediction of AMI or identification of unstable coronary plaques. Atherosclerosis of coronary arteries is the main cause of AMI. Sub-endothelial retention of low-density lipoprotein (LDL) on account of accumulation of the oxidized form of LDL (oxLDL) in macrophages and formation of foam cells as a critical early event in atherogenesis [10,17]. Many immunocytochemical studies have identified both oxLDL epitopes and anti-oxLDL immunoglobulins within atherosclerotic lesions [36]. Normal levels of oxLDL are as low as 0.1 ng/μg LDL protein in healthy people [13], making measurement difficult. In addition, spot oxLDL measurements only provide a snap-shot of concentration at a particular moment and are likely to be susceptible to extraneous factors, including diet, smoking and exercise. However, the oxidative modification of LDL leads to the formation of immunogenic epitopes that can induce the secretion of specific antibodies against oxLDL [29,1]. Circulating antibodies against oxLDL represent a durable measure of lipid peroxidation [21].

Abbreviations: AMI, acute myocardial infarction; $ApoB_{100}$, apolipoprotein B_{100}; cAg, control antigen; ELISA, enzyme-linked immunosorbent assay; hAgs, human antigens; HLA, human leukocyte antigen; IgG, immunoglobulin G; LDL, low-density lipoprotein; oxLDL, oxidized low-density lipoprotein

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Circulating anti-oxLDL antibodies are natural antibodies that can be detected almost in every individual, and the concentrations of anti-oxLDL antibodies often peak just before the onset of clinical disease [32]. There are numerous studies demonstrating the association between circulating anti-oxLDL antibodies and cardiovascular disease, but the results reported to date have been inconsistent. Some studies have determined a positive correlation between the levels of circulating anti-oxLDL antibodies and atherosclerotic diseases, whereas others have shown an opposite result [3,12,11,33,16,2,34]. The reasons for inconsistent findings may be due to the difficulties in standardizing the LDL antigens used for testing of antibodies to oxLDL, which can also be altered on account of oxidation ex vivo [11,20]. An approach to overcome the problem of standardization is to detect antibodies against specific native or MDA-modified $ApoB_{100}$ peptide sequences [5,4,19].

$ApoB_{100}$ is the only component permanently associated with formation of LDL in the body. Its peptide fragments generated by the proteolytic degradation and aldehyde-modification are the major antigens in oxLDL [25]. These antigens have the advantage of being specific to oxLDL for the unique amino acid sequence. The mature $ApoB_{100}$ protein is composed of 4563 amino acids in a single polypeptide chain that may carry many human leukocyte antigen (HLA)-restricted epitopes. The present work was thus undertaken to develop an in-house enzyme-linked immunosorbent assay (ELISA) with $ApoB_{100}$-derived linear peptide antigens to detect circulating IgG antibodies against these linear peptides in AMI.

2. Methods

2.1. Subjects

A total of 267 patients who were diagnosed with AMI between January 2012 and January 2014 were recruited by the Department of Cardiovascular Medicine, Fourth Affiliated Hospital of China Medical University, Shenyang, China. Of these 267 patients aged 60.89 ± 11.01 years, 200 were male and 67 were female. They were all diagnosed with spontaneous AMI (namely type 1) based on the Third Universal Definition of Myocardial Infarction published by the European Society of Cardiology [31], including both ST elevation myocardial infarction and non-ST-elevation myocardial infarction, one of whom had atrial septal defect without any symptom and treatment. All patients who had coronary imaging data underwent assessment for the severity of coronary heart disease by calculating the Gensini score of the coronary lesions [7]. Blood samples were taken from each patient within 24 h of the onset of AMI. Two hundred and one healthy subjects aged 59.44 ± 5.43 years were also recruited as controls from local communities, of whom 136 were male and 65 were female. Clinical interview and biomarker detection were applied to rule out those subjects who had history of AMI and other atherosclerotic cardiovascular diseases. The subjects who had malignant tumor(s), rheumatic and connective tissue diseases, organ transplantation or were long-term recipients of immunosuppressive drugs were excluded from this study. The main clinical characteristics of these subjects are listed in Table 1. All subjects were of Chinese Han origin and all gave written consent to participate in this study as approved by the Ethics Committee of the Fourth Affiliated Hospital of China Medical University and conformed to the requirements of the Declaration of Helsinki.

Table 1
Clinical characteristics of patients with AMI and control subjects.

Characteristics	AMI (n = 267)	Control (n = 201)	P value
Sex (Male)	200 (74.9%)	136 (67.7%)	0.085
Age (years)	60.9 ± 11.0	59.4 ± 5.4	0.086
Smoker (%)	160 (59.9%)	67 (37.4%)	<0.001
Drinker (%)	77 (28.8%)	42 (21.1%)	0.072
Hypertension (%)	138 (51.7)	46 (22.9%)	<0.001
Diabetes (%)	71 (26.6%)	30 (15.1%)	0.004
Obesity (%)	39 (17.3%)	38 (19.0%)	0.666
SBP (mmHg)	129.2 ± 22.9	130.9 ± 18.1	0.400
DBP (mmHg)	77.5 ± 14.5	78.4 ± 10.8	0.484
Heart rate (ppm)	73.2 ± 15.8	76.2 ± 11.6	0.021
BMI	24.7 ± 3.7	25.3 ± 3.1	0.083
Cholesterol (mM)	4.50 ± 1.09	5.27 ± 0.97	<0.001
Triglyceride (mM)	1.69 ± 1.28	1.91 ± 1.92	0.153
HDL (mM)	1.01 ± 0.25	1.29 ± 0.33	<0.001
LDL (mM)	3.02 ± 0.96	3.13 ± 0.81	0.242
ApoA (mM)	0.92 ± 0.43	1.34 ± 0.22	<0.001
ApoB (mM)	0.73 ± 0.38	0.91 ± 0.18	<0.001
Gensini score	55.2 ± 26.7	–	

Abbreviation: SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; HDL, high density lipoprotein; LDL, low density lipoprotein; ApoA, apolipoprotein A; ApoB, apolipoprotein B.

2.2. Autoantibody testing

Eight human $ApoB_{100}$-derived peptide antigens (hAgs) were designed, based on the computational prediction of HLA-II restricted epitopes, 6 of which have been used in our previous study [37]. A control antigen (cAg) was also designed based on a maize protein (NCBI accession 1BFA_A). Information of these 9 peptide antigens is detailed in Table 2. An in-house ELISA test for the detection of circulating IgG antibodies to $ApoB_{100}$ was performed as described in our previous study [37]. In brief, Corning 96-Well Microtiter EIA Plates (ImmunoChemistry Technologies, USA) were half-coated in 0.1 ml/well of each hAg and half-coated in 0.1 ml/well cAg. After overnight incubation at 4 °C, antigen-coated plates were washed three times with wash buffer (PBS containing 0.1% Tween-20), 100 μl serum sample diluted 1:150 in assay buffer (DS98200, Life Technologies) was added to each sample well and 100 μl assay buffer was also added to the negative control (NC) wells. Following 1.5 h incubation at room temperature, the plate was washed three times and 100 μl peroxidase-conjugated goat antibody to human IgG (A8667, Sigma–Aldrich) diluted 1:30,000 in wash buffer was added to each well. After incubation at room temperature for an hour, color development was initiated by adding 100 μl Stabilized Chromogen (SB02, Life Technologies) and terminated 20 min later by adding 50 μl of stop solution (SS04, Life Technologies). The measurement of optical density (OD) was performed on a microplate reader within 10 min at 450 nm with a reference wavelength of 620 nm. Each sample was tested in duplicate. To reduce the interference from a nonspecific signal produced by passive absorption of various IgG antibodies in serum to the surface of 96-well microplate, a specific binding index (SBI) was used to express the levels of circulating antibodies to these 8 hAgs. SBI was calculated as follows: $SBI = (OD_{hAg} - OD_{NC})/(OD_{cAg} - OD_{NC})$.

Table 2
Apo-B derived peptide antigens used.

Antigen	Sequence of antigen	Position of amino acid
Ag1	H-DRFKPIRTGISPLALIKGMTRPLSTLIS-OH	213–240
Ag2	H-LQWLKRVHANPLLIDVVTYLVALIPEPS-OH	395–422
Ag3	H-TFLDDASPGDKRLAAYLMLMRSPSQA-OH	547–572
Ag4	H-TVMDFRKFSRNYQLYKSVSLPSLDP-OH	626–650
Ag5	H-KIKGVISIPRLQAEARSEILAHWSPAKLL-OH	1119–1147
Ag6	H-DMTFRHVGSKLIVAMSSWLQKASGSLPY-OH	1220–1247
Ag7	H-SCKLDFREIQIYKKLRTSSFALNLPT-OH	3760–3785
Ag8	H-FLIYITELLKKLQSTTVMNPYMKLAPGELT-OH	4531–4560
cAg	H-HAQLEGRLHDLPGCPREVQRGFAATLVTN-OH	

Abbreviation: Ag, antigen; cAg, control antigen from a maize protein.

2.3. Data analysis

All antibody testing data were expressed as mean ± standard deviation (SD). Student's *t*-test was initially applied to detect the differences in SBI between the patient group and the control group; binary logistic regression was then used to analyze the differences in antibody levels for an antigen of interest between the patient group and the control group, with adjustment for multiple potential confounders listed in Table 1. Spearman correlation analysis was used to test the correlation between the anti-$ApoB_{100}$ IgG levels and clinical characteristics. Because eight antigens were used to develop the in-house antibody testing, the significance level was set at $P = 0.006$. To minimize intra-assay deviation, the ratio of the difference between duplicate sample OD values to their sum was used to assess the assay accuracy. If the ratio was found to be >15% for a given sample, the test for that sample was considered to be invalid and the test was repeated. The inter-assay deviation that represents the reproducibility of the in-house ELISA test was estimated using SBI tested with pooled serum samples, namely quality control (QC) samples that were randomly collected from 100 healthy subjects and tested on every 96-well plate. The coefficient of variation (CV) from the test of QC samples was then worked out based on their mean SBI and SD. The in-house ELISA test was considered as being repeatable when CV <20%.

3. Results

As shown in Table 1, there was no significant difference in gender, age, alcohol consumption, obesity, triglyceride levels or LDL between patients with AMI and control subjects. However, AMI patients were more likely to have high rates of smoking, hypertension and diabetes than controls, but lower heart rate, serum cholesterol, high density lipoprotein (HDL), ApoA and ApoB (Table 1).

Of these eight antigens used in this study, circulating IgG for Ag1 was significantly higher in AMI patients than control subjects ($P < 0.001$, adjusted for age, gender, heart rates, smoking, hypertension, diabetes and circulating levels of cholesterol, HDL, LDL, ApoA and ApoB). Increased IgG levels were not detected for any of the other seven antigens in patients with AMI as compared with control subjects (Table 3).

Analysis of QC samples showed that the inter-assay deviation was less than 20% for all eight individual antibody tests (Table 4). Spearman correlation analysis showed no correlation between IgG antibody for Ag1 and clinical characteristics (Table 5).

4. Discussion

There are differences in many clinical characteristics between AMI patients and control subjects. For example, the incidence of smoking, hypertension and diabetes are significantly higher in the patient group than the control group, all of which have been recognized as traditional risk factors of AMI for several decades [38]. Heart rate, serum cholesterol, HDL, ApoA and ApoB are significantly lower in AMI patients than control subjects (Table 1), which may be caused by the characteristics of the disease and the administration of β-blockers and statins. However, no correlation between anti-$ApoB_{100}$ IgG levels and clinical characteristics was found (Table 5), suggesting that the IgG antibody levels are independent of the clinical states.

Circulating antibodies to oxLDL and $ApoB_{100}$ have been studied for many years in coronary heart disease and other atherosclerotic diseases [28,24,23,15,22,8]. The increased levels of circulating antibodies to oxLDL promote the formation of immune complexes with oxLDL, which could adhere to the artery intima and cause damages to the endothelium [35]. It has been shown that increased levels of circulating antibodies to oxLDL may be regarded as predictors of atherosclerosis and ACS [18], while the highest anti-oxLDL IgG levels were observed in patients with multi-vessel CAD [9]. In fact, increased anti-oxLDL antibody levels appear to be pathological and may damage the walls of blood vessels, leading to atherosclerotic changes [9] and instability of atherosclerotic plaques [16]. The association between anti-oxLDL antibodies and cardiovascular diseases has been found to be inconsistent across studies, most of which have used the whole molecule of oxLDL as antigens so that the outcome may not be accurate. Recently, some studies have primarily used the p45 (amino acids 661–680) and p210 (amino acids 3136–3155) $ApoB_{100}$ peptides to explore the mechanism of immune response [28,24,23,15,22,8] and the development of immune-modulatory therapies [26]. An inverse association has been found between the levels of antibodies against these $ApoB_{100}$ peptides and the severity of atherosclerosis. It has been reported that AMI patients had lower anti-ApoB antibody levels than control subjects matched for age and sex [6,27,4], but these studies were all for immune-modulatory therapies but not for diagnosis.

In this study, we found that only IgG antibodies to the Ag1 sequence derived from $ApoB_{100}$ were significantly increased in patients with AMI (Table 3). Failure to show an increase in the levels of IgG antibodies to the other $ApoB_{100}$-derived antigens sug-

Table 3
The levels of anti-Apo-B IgG antibodies in the circulation.

Antigen	Patients (SBI)	Control (SBI)	P
Ag1	0.800 ± 0.148	0.744 ± 0.140	<0.001[a]
Ag2	2.164 ± 0.661	2.087 ± 0.691	0.231
Ag3	0.944 ± 0.147	0.958 ± 0.218	0.526
Ag4	0.923 ± 0.142	0.889 ± 0.133	0.034
Ag5	0.889 ± 0.177	0.926 ± 0.215	0.064
Ag6	0.702 ± 0.151	0.686 ± 0.166	0.345
Ag7	0.913 ± 0.163	0.921 ± 0.174	0.639
Ag8	0.702 ± 0.176	0.696 ± 0.124	0.768

Values are presented as mean ± SD in SBI.
[a] $P < 0.001$ after adjustment for age, gender, smoking, hypertension, diabetes and circulating levels of cholesterol, HDL, LDL, ApoA and ApoB.

Table 4
Inter-assay deviation between plates tested.

Antigen	Number of plates	Mean ± SD	CV (%)
Ag1	38	0.738 ± 0.074	10.03
Ag2	36	2.101 ± 0.279	13.28
Ag3	38	0.879 ± 0.057	6.48
Ag4	37	0.905 ± 0.082	9.06
Ag5	37	0.884 ± 0.122	13.80
Ag6	35	0.708 ± 0.115	16.24
Ag7	38	0.889 ± 0.101	11.36
Ag8	34	0.723 ± 0.052	7.19

Table 5
Spearman correlation between levels of IgG for Ag1 and clinical characteristics.

	Patients			Control		
	N	r	P	N	r	P
Age	261	−0.065	0.295	189	0.141	0.053
SBP	261	−0.072	0.246	171	−0.104	0.177
DBP	261	−0.009	0.891	171	−0.058	0.450
Heart rate	261	0.031	0.617	171	0.061	0.431
BMI	221	−0.118	0.081	171	−0.068	0.377
CHOL	249	−0.067	0.294	171	−0.064	0.409
TG	249	−0.102	0.107	171	−0.048	0.53
HDL	249	−0.076	0.23	171	−0.007	0.93
LDL	249	−0.029	0.645	170	−0.096	0.212
Apo-A	234	−0.087	0.187	171	0.016	0.837
Apo-B	234	−0.075	0.251	170	−0.107	0.165
Gensini score	226	0.032	0.629			

gests that HLA-restriction may be involved in influencing the humoral immune response in AMI. Interestingly, the increased levels of anti-Ag1 IgG observed in AMI patients were not correlated with their clinical characteristics and the severity of coronary plaques characterized by the Gensini score (Table 5). Possibly, the linear peptide antigen derived from $ApoB_{100}$ is more suitable for the development of a diagnostic tool for AMI than the whole oxLDL molecule, although further screening is needed to identify more such peptide antigens present in the $ApoB_{100}$ protein. Testing of circulating antibodies to linear antigens derived from $ApoB_{100}$ may have a potential benefit for diagnosis and prognosis of AMI.

5. Conclusion

The linear peptide antigens derived from $ApoB_{100}$ may be suitable for the development of an ELISA antibody test for prediction of AMI, although further confirmation is still needed in large-scale clinical studies.

Conflict of interest

All the authors declared that they had no conflict of interest.

Author Contributions

Xueying Zhang, Xiaohong Zhang, Mingming Lei and Yingzi Lin mainly carried out the laboratory work, identification of patients and collections of blood samples. Bo Yu, Yuanzhe Jin and Jun Wei conceived of this study and were partially involved in supervision of laboratory work and drafting manuscript. Ian L. Megson was involved in drafting the manuscript.

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