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ORIGINAL ARTICLE

A study of IgG antibodies to the ApoB protein in non-ST segment elevation acute coronary syndrome

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Abstract

Objectives. It has long been noted that there is an association of antibodies against oxidized low-density lipoprotein (oxLDL) with cardiovascular disease, but the anti-oxLDL antibody has not been confirmed as a biomarker for prediction of acute coronary syndrome (ACS). Apolipoprotein B (ApoB) may carry the epitopes for the immune response to oxLDL. The present work was thus undertaken to detect circulating antibodies to ApoB in non-ST segment elevation ACS (NSTE-ACS). *Design*. A total of 130 patients with NSTE-ACS and 201 control subjects were recruited. Six ApoB-derived peptipe antigens (Ag1–Ag6) were used to develop an in-house enzyme-linked immunosorbent assay to examine circulating anti-ApoB IgG levels. *Results*. The anti-Ag1 IgG level was significantly higher in the patient group than the control group ($P < 0.001$) and the non-ST segment elevation myocardial infarction appeared to be the main form of NSTE-ACS contributing to the increased levels of anti-Ag1 IgG ($P < 0.001$); there was no significant alteration in the levels of IgG to the other 5 antigens in NSTE-ACS. *Conclusions*. Circulating anti-ApoB IgG test may be useful for prediction of NSTE-ACS although further confirmation is needed in large-scale clinical studies.

Key words: *antibody, ApoB, atherosclerosis, Non-ST segment elevation acute coronary syndrome*

Introduction

Acute coronary syndrome (ACS) refers to a spectrum of conditions compatible with acute myocardial ischemia and/or infarction due to an abrupt reduction in coronary blood flow. The absence of persistent ST elevation is suggestive of non-ST segment elevation ACS (NSTE-ACS), which can be further subdivided into non-ST segment elevation myocardial infarction (NSTEMI) and unstable angina (UA) on the basis of cardiac biomarkers of necrosis (e.g. cardiac troponin) (1). Thus, UA and NSTEMI are closely related conditions whose pathogenesis and clinical presentation are similar but vary in severity. Current epidemiological data indicate more frequent occurrence of NSTE-ACS than ST segment elevation myocardial infarction (STEMI) (2). It is estimated that approximately 70% of patients who have suffered ACS each year are NSTE-ACS in the USA (3). Patients with NSTE-ACS typically have more comorbidities, both cardiac and noncardiac, than those with STEMI (4). However, there are still no effective biomarkers available for prediction of unstable coronary plaques.

Oxidized low-density lipoprotein (oxLDL) is pivotal in the development of atherosclerosis (5), which is the main cause of NSTE-ACS. Normal levels of oxLDL are as low as ~0.1 ng/μg LDL protein in healthy people (6), making it difficult to detect. However, the oxidative modification of LDL leads to the formation of immunogenic epitopes that can induce the secretion of specific antibodies against oxLDL (7). It is possible that circulating antibodies

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against oxLDL can serve as a biomarker indicating lipid peroxidation (8). The fact that antibodies to oxLDL exist in healthy people is well-established, but their levels significantly increase in patients who have cardiovascular disease (9) and often peak just before the onset of clinical disease (10). The increased levels of antibodies to oxLDL promote the formation of immune complexes with oxLDL, which then bind to the intima and cause additional damage to the endothelium (11). Several studies have shown that elevated levels of circulating antibodies to oxLDL may be regarded as predictors of atherosclerosis and ACS (9). It has been suggested that the levels of IgG antibodies to oxLDL change with the severity of coronary artery disease (CAD), and that the highest antibody levels are observed in patients with multivessel CAD (12). Increased anti-oxLDL antibody levels might be pathological, leading to damage of the vessel wall and eventually to atherosclerotic changes (12). The association between anti-oxLDL IgG and ACS has recently been established and such antibodies may be associated with atherosclerotic plaque instability (13). The detection of circulating anti-oxLDL antibody levels is likely to have diagnostic values for prediction of acute coronary event. Unfortunately, the most recent studies have failed to draw a firm conclusion (14,15). The reasons for inconsistent findings may be because of the difficulties in standardizing the LDL antigens used to measure antibodies to oxLDL, which can also be altered on account of oxidation ex vivo (16). An approach to overcome the problem of standardization is to detect antibodies against specific native or malondialdehyde-modified $apoB_{100}$ (Apo-B) peptide sequences (17) instead of measuring oxLDL itself.

Apolipoprotein B (ApoB) is the only component permanently associated with formation of LDL in the body. Its proteolytic degradation and aldehyde-modification can generate the major antigens, inducing immune responses to oxLDL. The mature ApoB protein is composed of 4563 amino acids in a single polypeptide chain that carries many of overlapped human leukocyte antigen (HLA)-restricted epitopes. The present work was thus undertaken to develop an enzyme-linked immunosorbent assay (ELISA) in-house with ApoB-derived linear peptide antigens to detect circulating antibodies against these linear peptides in patients with NSTE-ACS.

Materials and methods

Subjects

This study recruited a total of 130 patients, who were diagnosed with NSTE-ACS between January 2012 and January 2014 at the Department of Cardiovascular Medicine, the Fourth Affiliated Hospital of China Medical University, Shenyang, China, including 83 with UA and 47 with NSTE-AMI. Of these 130 patients aged 60.4 ± 9.1 years, 88 were male and 42 were female. They were all diagnosed as having NSTE-ACS based on 2014 AHA/ACC guideline for the management of NSTE-ACS for both NSTE-AMI and UA (4); diagnosis was confirmed by coronary angiography. In addition, all patients underwent assessment for the severity of coronary heart disease by calculating the Gensini Score of the coronary lesions (18). Blood samples were taken from each patient within 24 h after the onset of NSTE-ACS. A total of 201 healthy subjects aged 59.4 ± 5.4 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 who had a history of coronary heart disease. The subjects who had malignant tumor(s), rheumatic and connective tissue diseases, organ transplantation, and those receiving long-term immunosuppressive drugs were excluded from this study. The clinical characteristics of these subjects are given in Table I. All subjects were of Chinese Han origin and they all gave written informed consent to participate in this study as approved by the Ethics

Table I. Clinical characteristics of patients with NSTE-ACS and control subjects.

Characteristics	NSTE-ACS (n = 130)	Control (n = 201)	P
Male, n (%)	88 (68)	136 (68)	0.873
Age (years)	60.4 ± 9.1	59.4 ± 5.4	0.208
Smoking*, n (%)	55 (42)	67 (33)	<0.001
Alcohol*, n (%)	31 (24)	42 (21)	0.512
HTN#, n (%)	87 (67)	46 (23)	<0.001
DM#, n (%)	42 (32)	30 (15)	0.004
Obesity#, n (%)	25 (19)	38 (19)	0.999
SBP (mmHg)	136.0 ± 19.1	130.9 ± 18.1	0.020
DBP (mmHg)	80.8 ± 11.3	78.4 ± 10.8	0.055
Heart rate (ppm)	71.6 ± 13.1	76.2 ± 11.6	0.001
BMI	25.05 ± 3.67	25.27 ± 3.11	0.606
Cholesterol (mM)	4.54 ± 1.30	5.27 ± 0.97	<0.001
Triglyceride (mM)	2.12 ± 2.52	1.91 ± 1.92	0.415
HDL (mM)	1.02 ± 0.30	1.29 ± 0.33	<0.001
LDL (mM)	2.88 ± 0.96	3.13 ± 0.81	0.019
ApoA (mM)	0.99 ± 0.40	1.34 ± 0.22	<0.001
ApoB (mM)	0.76 ± 0.34	0.91 ± 0.18	<0.001
Gensini score	52.4 ± 28.9	–	

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; HTN, Hypertension; DM, Diabetes mellitus.

*Current or history of smoking or alcohol consumption.

#Based on WHO diagnostic criteria, HTN was defined as SBP ≥ 140 mmHg or DBP ≥ 90 mmHg or HTN history, DM as fasting blood-glucose ≥ 7.0 mmol/l or DM history, and obesity as BMI ≥ 30.

Committee of the Fourth Affiliated Hospital of China Medical University and conformed to the requirements of the Declaration of Helsinki.

Autoantibody testing

Six human ApoB-derived peptide antigens (hAgs) were designed based on the computational prediction of HLA-II restricted epitopes (19). These 6 hAgs were applied to develop a relative ELISA test for detection of circulating IgG antibodies to ApoB. Each hAg carried more than 10 overlapping HLA-II restricted epitopes that could be recognized by antigen-presenting cells in more than 90% of the Chinese Han population. A control antigen (cAg) was also designed based on a maize protein (NCBI accession 1BFA_A). Detailed information of these seven peptide antigens is given in Table II. These peptide antigens were synthesized by solid-phase chemistry with a purity of $>95\%$; the synthetic peptides were dissolved in 67% acetic acid to obtain a concentration of 5 mg/ml (stock solution kept at $-20°C$), and diluted with phosphate-buffered saline (PBS)-based coating buffer (P4417, Sigma-Aldrich, Beijing, China) to obtain a working solution that was optimized by testing a series of concentrations ranging from 0 to 20 μg/ml. Corning 96-Well Microtiter EIA Plates (ImmunoChemistry Technologies, Bloomington, MN, USA) were half-coated in 0.1 ml/well of each hAg and half-coated in 0.1 ml/well of the cAg. After overnight incubation at 4°C, antigen-coated plates were washed three times with PBS containing 0.1% Tween-20 (PBS-T), 100 μl serum sample diluted 1:150 in assay buffer (DS98200, Life Technologies, Carlsbad, CA, USA) was added to each sample well and 100 μl assay buffer was also added to the negative control (NC) wells. Following a 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 PBS-T 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, Carlsbad, CA, USA) and terminated 20 min later by adding 50 μl of stop solution (SS04, Life Technologies, Carlsbad, CA, USA). The measurement of optical density (OD) was completed 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 6 hAgs. SBI was calculated as follows: $SBI = (OD_{hAg} - OD_{NC})/(OD_{cAg} - OD_{NC})$.

Data analysis

All statistical analyses were performed using IBM SPSS Statistics 19.0 software. Antibody testing data were expressed as mean ± standard deviation (SD) in SBI. 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 used to analyze the differences in circulating levels of IgG to an antigen of interest between the patient group and the control group, with adjustment for multiple potential confounders listed in Table I. One-way analysis of variance (ANOVA) with Bonferroni post-hoc test was applied to compare the differences in anti-ApoB antibody levels between subgroups of NSTE-ACS. Spearman correlation analysis was used to test the correlation between the anti-ApoB IgG level and clinical characteristics. Because 6 antigens were used to develop the in-house antibody testing, the significance level was set at $P \leq 0.008$. The sensitivity of the ELISA antibody test was determined against a specificity of $\geq 90\%$.

To minimize intra-assay deviation, the ratio of the difference between duplicate sample OD values to their sum was used to assess the assay precision and accuracy. If the ratio was found to be $>15\%$, the test of this sample was treated as being invalid and this sample was repeated. The inter-assay deviation that represents the reproducibility of the in-house ELISA was estimated using SBI tested with pooled serum

Table II. Apo-B derived peptide antigens used.

Antigen	Sequence of antigen	Position
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-SCKLDFREIQIYKKLRTSSFALNLPT-OH	3760–3785
Ag6	H-FLIYITELLKKLQSTTVMNPYMKLAPGELT-OH	4531–4560
cAg	H-HAQLEGRLHDLPGCPREVQRGFAATLVTN-OH	–

Ag, antigen; cAg, control antigen derived from maize protein.

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, and the CV of $< 20\%$ was considered as being acceptable.

Results

There was no significant difference in gender, age, alcohol consumption, body weight, or triglyceride levels between the patient group and the control group. However, patients with NSTE-ACS had higher rates of smoking, hypertension, and diabetes than control subjects, but lower heart rates, serum cholesterol, LDL, high-density lipoprotein (HDL), ApoA, and ApoB (Table I).

Circulating IgG against Ag1 was significantly higher in patients with NSTE-ACS than control subjects ($P = 0.003$ adjusted for age, gender, heart rates, smoking, hypertension, diabetes, cholesterol, HDL, LDL, ApoA, and ApoB). All the other 5 antigens failed to detect an increase in corresponding IgG levels in patients with NSTE-ACS (Table III). As shown in Table IV, one-way ANOVA showed a significant difference in anti-Ag1 IgG levels between patients with NSTEMI, those with UA and control subjects ($F = 8.89$, $df = 2,328$, $P < 0.001$), and the Bonferroni post-hoc test revealed that NSTEMI appeared to be the main form of NSTE-ACS contributing to the increased levels of anti-Ag1 IgG ($P < 0.001$), while there was no significant difference in anti-Ag1 IgG levels between patients with UA and those with NSTEMI ($P = 0.112$).

Analysis of QC samples showed an inter-assay deviation of $<20\%$ for all 6 individual antibody tests (Table V). Spearman correlation analysis showed no correlation between IgG antibody to Ag1 and clinical characteristics (Table VI). The sensitivity of IgG antibody to Ag1 was 21.2% against a specificity of 90.5%.

Table III. The levels of circulating anti-Apo-B IgG antibodies in NSTE-ACS circulation.

Antigen	Patients (SBI)	Control (SBI)	*P*
Ag1	0.810 ± 0.155	0.744 ± 0.140	<0.001[a]
Ag2	2.259 ± 0.721	2.087 ± 0.691	0.034
Ag3	0.974 ± 0.314	0.958 ± 0.218	0.575
Ag4	0.933 ± 0.253	0.889 ± 0.133	0.041
Ag5	0.919 ± 0.133	0.921 ± 0.174	0.928
Ag6	0.669 ± 0.110	0.696 ± 0.124	0.189

Values are presented as mean ± SD in SBI.
[a]$P = 0.003$ after adjustment for age, gender, smoking, hypertension, diabetes, cholesterol, HDL, LDL, ApoA, and ApoB.

Discussion

In the present study, we detected anti-ApoB IgG antibodies in plasma taken from patients who had onset of NSTE-ACS within 24 h. Because total IgG antibodies have a half-life of 4–5 weeks in humans, circulating IgG levels should reflect the status before the MI event. Our work revealed that circulating IgG for Ag1 derived from the ApoB protein was significantly higher in patients with NSTE-ACS than in control subjects (Table III). The results suggest that the anti-ApoB IgG antibody might be a useful biomarker for prediction of ACS. To our knowledge, this is the first report of a significant increase in anti-ApoB IgG antibodies in NSTE-ACS. Our study is novel.

The mechanism behind that underpins ACS is the erosion and/or rupture of atherosclerotic plaques, which results in myocardial hypoperfusion (20). The oxLDL particles can activate endothelial cells by inducing the expression of several cell surface adhesion molecules; after adhesion to the endothelium, leukocytes migrate into the intima in response to chemokines. Monocytes then differentiate into macrophages and upregulate expression of scavenger receptors that engulf oxLDL, resulting in lipid accumulation and foam cell formation (21). Macrophage activation leads to the release of pro-

Table IV. The levels of anti-Apo-B IgG antibodies in subgroups of NSTE-ACS.

Antigen	UA ($n = 83$) (SBI)	NSTE-AMI ($n = 47$) (SBI)	Control ($n = 201$) (SBI)	F (df)	*P*
Ag1	0.793 ± 0.152[a,b]	0.836 ± 0.156[c]	0.744 ± 0.139	8.89 (2,328)	<0.001
Ag2	2.293 ± 0.701	2.212 ± 0.754	2.087 ± 0.691	2.60 (2,328)	0.076
Ag3	0.985 ± 0.375	0.952 ± 0.137	0.958 ± 0.218	0.37 (2,328)	0.688
Ag4	0.933 ± 0.300	0.932 ± 0.119	0.889 ± 0.133	2.10 (2,328)	0.124
Ag5	0.921 ± 0.137	0.921 ± 0.132	0.921 ± 0.174	<0.001 (2,328)	1.000
Ag6	0.663 ± 0.128	0.678 ± 0.068	0.696 ± 0.124	1.01 (2,328)	0.367

Values are presented as mean ± SD in SBI.
[a]$P = 0.011$, compared with the control group; [b]$P = 0.112$, compared with the NSTE-AMI group; [c]$P < 0.001$, compared with the control group.

Table V. Inter-assay deviation between plates tested.

Antigen	Number of plates	Mean ± SD	CV (%)
Ag1	30	0.729 ± 0.084	11.52
Ag2	28	2.055 ± 0.298	14.50
Ag3	26	0.900 ± 0.066	7.33
Ag4	27	0.876 ± 0.065	7.42
Ag5	28	0.929 ± 0.168	18.08
Ag6	30	0.709 ± 0.043	7.52

inflammatory cytokines, reactive oxygen species, proteolytic enzymes involved in matrix degradation and then in atherosclerotic plaque destabilization. An association of circulating antibodies to oxLDL and ApoB with ACS or other atherosclerotic diseases has been suggested, but the findings reported to date have been inconsistent across studies (22–24). Most of these studies have used antibodies raised against whole oxLDL particles; the difference in HLA background between ethnic groups may be responsible for variability amongst results. Recently, some studies have primarily used the p45 (amino acids 661–680) and p210 (amino acids 3136–3155) $ApoB_{100}$ peptides to investigate the mechanism of immune response (22,24) and to develop immune-modulatory therapies. An inverse association was found between the levels of antibodies against these $ApoB_{100}$ peptides and the severity of atherosclerosis; patients with AMI were found to have lower anti-ApoB antibody levels than control subjects matched for age and sex (25), but these studies were all designed for immune-modulatory therapies rather than diagnosis.

While there are differences in some clinical characteristics between NSTE-ACS patients and control subjects, a correlation between anti-ApoB IgG levels and clinical characteristics was not found in this study (Table VI). Instead, we found that there was a significantly increased level of IgG antibody to the Ag1 sequence in NSTE-ACS (Table III). Failure to show an increase in the levels of IgG antibody to other ApoB-derived antigens suggests that HLA-restriction may be involved in influencing the humoral immune response in NSTE-ACS. Interestingly, the increased levels of anti-Ag1 IgG observed in patients with NSTE-ACS were not correlated with their clinical characteristics and the severity of coronary plaques characterized by the Gensini Score (Table VI). It is possible that linear peptide Ag1 is more suitable than the whole molecule antigen for the development of a diagnostic tool for ACS, although further screening is needed to identify a panel of such peptide antigens for the development of a highly sensitive test that can be used for prediction and prognosis of NSTE-ACS.

Table VI. Spearman correlation between anti-Ag1 IgG levels and clinical characteristics.

	Patients			Control		
	N	r	*P*	N	r	*P*
Age	130	0.100	0.261	189	0.141	0.053
SBP	127	−0.043	0.629	171	−0.104	0.177
DBP	127	0.025	0.780	171	−0.058	0.450
Heart rate	127	0.083	0.356	171	0.061	0.431
BMI	127	−0.069	0.492	171	−0.068	0.377
CHOL	130	−0.029	0.752	171	−0.064	0.409
TG	130	−0.179	0.050	171	−0.048	0.530
HDL	130	0.033	0.723	171	−0.007	0.930
LDL	130	0.079	0.389	170	−0.096	0.212
Apo-A	130	−0.019	0.842	171	0.016	0.837
Apo-B	130	−0.068	0.466	170	−0.107	0.165
Gensini score	130	0.098	0.289			

There is a limitation of this study. Patients with ACS often receive medication at presentation. This may be a possible confounder for analysis of circulating antibody levels. Although there is no theoretical reason to suspect that the drugs used (e.g. antiplatelet agents, nitrates, anticoagulants, analgesics, and β-blockers) would affect circulating levels of IgG antibodies to the ApoB protein, it remains a possibility. Drug treatment was not recorded in the present study, but such data would be useful in future research in order to exclude the confounding effect of drug treatment.

In conclusion, the present study suggests that circulating anti-ApoB IgG may be a useful biomarker for prediction of NSTE-ACS, although further confirmation is needed in large-scale clinical studies.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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