Identification of Immune Responses Against Aldehyde-Modified Peptide Sequences in ApoB Associated With Cardiovascular Disease

Gunilla Nordin Fredrikson, Bo Hedblad, Göran Berglund, Ragnar Alm, Mikko Ares, Bojan Cercek, Kuang-Yuh Chyu, Prediman K. Shah, Jan Nilsson

Objective—Atherosclerosis is associated with an immune response against oxidized LDL, which modulates the progression of the disease process.

Methods and Results—Using a library of polypeptides covering the complete sequence of apoB-100, the only major protein of LDL, we have identified over 100 different human antibodies reacting against aldehyde-modified apoB-100 sequences. IgM antibody titer levels decreased with age and were associated with the intima-media thickness of the carotid artery in subjects younger than 60 years. There were also inverse associations between IgM levels and oxidized LDL in plasma. In prospective clinical studies, antibody levels against several aldehyde-modified apoB-100 sites were associated with cardiovascular disease in this age group. Whether this immune response is adaptive (protective) or maladaptive (causal) in atherosclerosis requires further investigation.

Conclusions—We have characterized a large number of epitopes within the apoB-100 component of oxidized LDL that provoke an immune response in humans. These findings will make it possible to study the role of immune responses against specific sites in oxidized LDL and to determine whether these immune responses influence the risk for future cardiac events. (Arterioscler Thromb Vasc Biol. 2003;23:872-878.)

Key Words: apolipoproteins ■ atherosclerosis ■ cardiovascular diseases ■ immune responses ■ peptide sequences

Recent studies suggest that the immune system modulates the atherogenic process and that epitopes generated in association with LDL oxidation are major targets for these immune responses.1–3 Oxidized LDL has been implicated in atherogenesis by its ability to induce a number of proinflammatory genes leading to endothelial adhesion molecule expression, leukocyte recruitment, and cytokine secretion4,5 as well as by its activation of both humoral and cellular immune responses. Autoantibodies binding to oxidized LDL have been demonstrated in humans, rabbits, and mice.6,7 Increased levels of oxidized LDL autoantibodies have been identified in patients with coronary artery disease, peripheral vascular disease, diabetes, and hypertension.8–10 High levels of oxidized LDL autoantibodies have also been shown to predict a more rapid progression of carotid atherosclerosis.11 However, other studies have failed to observe such associations between oxidized LDL autoantibodies and atherosclerosis.12

It was originally assumed that autoimmune responses against oxidized LDL would contribute to plaque development. However, studies in rabbits and mice immunized with oxidized LDL demonstrated that activation of these immune responses reduced atherosclerosis by 40% to 60%.13–17 These observations suggest the possibility that immunization with oxidized LDL-derived antigens may be a useful antiatherogenic strategy. Characterization of antigenic structures in oxidized LDL would provide an important first step toward this goal. This would allow the development of reliable diagnostic assays for determination of oxidized LDL antibodies as well as identification of possible epitopes for incorporation into a vaccine.

Methods

Study Population
The study subjects, born between the years 1926 and 1945, belonged to the Malmö Diet and Cancer (MDC) study cohort. A random 50% of those who entered the MDC study between November 1991 and February 1994 were invited to take part in a study on the epidemiology of carotid artery disease.18 Routines for ascertainment of information on morbidity and mortality after the health examination, as well as definition of traditional risk factors, have been reported.18 Eighty-five cases of acute coronary heart events, that is, fatal or nonfatal myocardial infarction (MI) or deaths resulting from coronary heart disease (CHD) were identified during follow-up. Participants who had a history of MI or stroke (n=6) prior to enrollment were not eligible for the present study. For each case two controls...
without a history of MI or stroke were individually matched for age, sex, smoking habits, presence of hypertension, and month of participation in the screening examination and duration of follow-up. Only 1 control was available for 7 cases and no controls for 1 case. This case was excluded from analysis. Thus, the study population consisted of 227 subjects, 78 cases and 149 controls, ages 49 to 67 (median 61 years) years at baseline.

Laboratory Analyses
After overnight fasting blood samples were drawn for the determination of serum values of total cholesterol, triglycerides, HDL cholesterol, LDL cholesterol, and whole blood glucose. LDL cholesterol in mmol/L was calculated according to the Friedewald formula. Oxidized LDL was measured using ELISA (Mercodia) in EDTA plasma supplemented with the antioxidants DTPA and BHT. The plasma samples had been stored at −80°C and not previously thawed. This oxidized LDL ELISA is a capture ELISA using the mAb4E6 antibody developed by Holvoet et al.19 The coefficient of variation for the assay is 8% and the recovery 95%.

B-mode Ultrasound Vasculography
An Acuson 128 Computed Tomography System (Acuson) with a 7-MHz transducer was used for the assessment of carotid plaques in the carotid artery as described previously.20

Development of ELISAs Against ApoB-100 Peptide Sequences
The 302 peptides, which were all 20 amino acids long and corresponded to the entire human apoB-100 amino acid sequence,21 were synthesized (Euro-Diagnostica AB and K.J. Ross Petersen AS) and used in ELISA. The peptides were produced with a 5 amino acid overlap to cover all sequences at breaking points and numbered 1 to 302 starting at the N-terminal end of the protein (a complete list of all peptide sequences used can be accessed online at http://atvb.ahajournals.org). A fraction of each synthetic peptide was modified by 0.5 mol/L malondialdehyde (MDA)22 for 3 hours at 37°C. The MDA-modified peptides were dialyzed against PBS containing 1 mmol/L EDTA with several changes for 18 hours at 4°C. The MDA modification of peptides was assessed using the thiobarbituric acid reactive substances assay.23 To determine thiobarbituric acid reactive substances, peptide samples (0.1 mL) were incubated in a boiling water bath for 15 minutes with 0.1 mL of 1 mol/L ferric chloride, 0.1 mL of 1 mol/L butylated hydroxytoluene, 1.5 mL of 0.2 mol/L glycine buffer (pH 3.6) and 1.5 mL of 3.5 mol/L thiobarbituric acid supplemented with 0.3% SDS. After cooling to room temperature, the pink chromogen was extracted with acetic acid (1 mL) and chloroform (2 mL). The optical density of the upper layer was measured at 532 nm against water blank. 1,1,3,3-Tetraethoxyxpropane was used as a standard. No aldehydes were detected in preparations of native peptides. After the MDA-modification procedure, all peptides were found to contain aldehydes. The mean aldehydes content was 243.2±212.6 nmol per mg peptide. The lowest aldehyde content was found in MDA peptide 12 (16 nmol per mg peptide) and the highest in peptide 75 (1427 nmol per mg peptide).

Native or modified synthetic peptides diluted in PBS pH 7.4 (20 µg/mL) were absorbed to microtiter plate wells (Nunc MaxiSorp) in an overnight incubation at 4°C. After washing with PBS containing 0.05% Tween-20 (PBS-T) the coated plates were blocked with SuperBlock in Tris-buffered saline (TBS, Pierce) for 5 minutes at room temperature followed by an incubation of pooled human plasma, diluted 1/100 in TBS-0.05% Tween-20 (TBS-T) for 2 hours at RT and overnight at 4°C. Plasma from 50 healthy blood donors (25 men and 25 females) was pooled and used for the first screening of the peptide ELISA library. In the second screening pooled plasma from subjects with clinical signs of CHD (acute MI, and unstable angina, n=10) was tested. All plasma from CHD patients was obtained within 5 days of admittance to the coronary care unit. Accordingly, an effect of the acute event on antibody levels against epitopes in apoB-100 cannot be entirely excluded. After rinsing, deposition of autoantibodies directed to the peptides was detected by using biotinylated rabbit anti-human IgG or IgM antibodies (Dako A/S) appropriately diluted in TBS-T. After another incubation for 2 hours at room temperature, the plates were washed and the bound biotinylated antibodies were detected by alkaline phosphatase conjugated streptavidin (Sigma) and incubated for 2 hours at room temperature. The color reaction was developed by using phosphatase substrate kit (Pierce), and the absorbance at 405 nm was measured after 1 hour of incubation at room temperature. In screening of the complete polypeptide library peptide 298 (which in the initial studies had an absorbance equal to that of the background control) was used as an internal standard on each ELISA plate and the absorbance values for each peptide expressed as the ratio against peptide 298. In the prospective clinical study ELISAs based on 7 MDA-modified peptide sequences were used (#32: GNMGQTMEQLTPELKSSILK; 45: IEIGLEGKGFEPTLEALFGK; 102: SLTSTSDLQS GIKN-TASLK; 129: GSTSHHLVSRKSISALEHK; 216: IREVTRQ-LNGEQIALPQPQK; 210: KTTKQPSDFLSVKAAQYYKNNKH; 240: FPDLQGEVLANTKNNQJKIR). The mean plating efficiency was 33% for both native and MDA peptides as assessed by measuring the peptide content in the coating solution before and after the coating procedure. The mean intra-assay coefficient of variance for the 7 ELISAs used in the clinical study was 8.2% and the mean inter-assay coefficient of variance was 14.7%. Binding of IgM to ELISA plates was inhibited by about 40% after addition of relevant MDA peptides, by 30% after addition of MDA LDL, by 20% to 25% after addition of copper-oxidized LDL, and by 10% to 15% after addition of relevant native peptides, nonrelevant MDA-peptide, nonrelevant native peptide, and native LDL, respectively. Detailed data regarding analysis of peptide plating efficiency and ELISA specificity and reproducibility are presented online.

Statistics
SPSS was used for the statistical analyses. The results are presented as median and range and as proportions when appropriate. In cases and controls, separately, partial correlation coefficients, adjusted for age and sex, were computed among selected peptides and blood lipid levels and common carotid intima-media thickness (IMT). Age- and sex-adjusted partial correlation coefficients were also computed between common carotid IMT and selected peptides in cases and controls below and over the median age. An independent sample t test was used to assess normally distributed continuous variables and a χ² test for proportions between cases and controls. Nonparametric test (Mann-Whitney) was used to assess non-normally distributed continuous variables between cases and controls. All probability values are two-tailed.

Results
Identification of Peptide Sequences in apoB-100 Recognized by Antibodies in Human Plasma
During oxidation of LDL, polyunsaturated fatty acids in phospholipids and cholesteryl esters undergo peroxidation, leading to formation of highly reactive breakdown products, such as MDA.24 MDA may then form covalent adducts with lysine and histidine residues in apoB-100, making them highly immunogenic.2,25 In these experiments peptides, were used in their native state and after MDA modification.

We performed a screening of the complete peptide library using pooled plasma derived from healthy control subjects and native and MDA-modified peptides as antigens. Using twice the absorbance of the background control as positive titer cut off, antibodies were detected against 102 of the 302 peptides constituting the complete apoB-100 sequence (Figure 1). IgM binding was substantially more abundant than that of IgG. Generally, binding was higher to MDA-modified peptide sequences than to the corresponding native sequence,
Figure 1. A library of 302 polypeptides covering the complete amino acid sequence of apoB-100 was used for detection of antibodies in pooled healthy control plasma against native and MDA-modified peptide sequences. Peptide 298 was used as an internal standard on each ELISA plate and the absorbance values for each peptide expressed as the ratio against peptide 298. Arrows indicate location of specified peptides.

but there was a striking correlation between the two (Figure 1). Binding to MDA-modified sequences and to some extent to native peptides was competitively inhibited by addition of the respective MDA peptide, MDA-modified LDL, and copper-oxidized LDL but not by the relevant native peptide, nonrelevant MDA peptide, nonrelevant native peptide, and native LDL. The inability of native LDL to compete antibody binding to native apoB-100 peptide sequences is intriguing but may indicate that these sequences only become exposed after the degradation of apoB-100 that occurs as a result of LDL oxidation. Comparison with published data on hydrophobic and hydrophilic sequences of apoB demonstrated that there was no preferential binding as a result of the degree of hydrophobicity/hydrophilicity. Moreover, there were no significant relations between binding of IgG and IgM to MDA-modified peptides and the number of lysine, arginine, and histidine residues in the respective peptide.

Antibodies in pooled CHD plasma bound to the same sequences and with the same overall distribution as for antibodies in plasma from healthy control. However, antibody titers to several peptides (#1, 30 to 34, 100, 107, 129, 148, 149, 162, 169, 236, 252, and 301; Figure 1) were at least twice as high in control plasma compared with plasma from CHD subjects, whereas titers against a few peptides (#10, 45, 111, 154, 199, 222, and 240) were higher in plasma from CHD patients compared with controls.

Associations Between Antibodies to Peptide Sequences in ApoB-100 and Cardiovascular Disease

We then performed a prospective clinical study to investigate whether antibody levels against MDA-modified peptide sequences in apoB-100 predict risk for development of CHD. Using a nested case-control design we selected 78 subjects with coronary events (acute MI or death from CHD) and 149 controls from the MDC Study. Neither cases nor control individuals had a history of previous MI or stroke before their enrollment in the study. The baseline characteristics of the study groups are shown in Table 1. The median time from inclusion to the acute coronary event was 2.8 years (range 0.1 to 5.9 years) among cases. Using the carotid IMT as assessed by ultrasonography at baseline, we also analyzed associations between antibody levels and degree of existing vascular disease. We studied 7 MDA-modified peptide sequences that in the initial screening studies were associated with high plasma antibody levels (#102 and 210) and/or marked differences between control and CHD plasma pools (#32, 45, 129, 162, and 240). There were no differences in antibody levels between cases and controls. However, associations between IMT and IgM against MDA peptides #102, 129, and 162 (r=0.233, 0.232, and 0.234, respectively, P<0.05) were observed in cases and between IMT and MDA peptide 45 (r=0.18, P<0.05) in controls. Weak correlations were observed between antibodies to MDA peptide 129 and both total and LDL cholesterol (r=0.19 and r=0.19, P<0.01, respectively), otherwise peptide antibody levels showed no associations with total plasma cholesterol, LDL cholesterol, HDL cholesterol, or plasma triglycerides, systolic, or diastolic blood pressure. There were strong covariations between antibody levels to the different peptides (r values ranging from 0.6 to 0.9).

Antibodies against all sequences were inversely associated with age among cases (r values ranging from −0.38 to −0.58, P<0.01 to 0.001), but not in controls (Figure 2a-d). Plasma levels of oxidized LDL, in contrast, increased with age. Again this association was stronger in cases than in controls (r=0.44 versus 0.29, P<0.001; Figure 2e and f). To investigate whether the associations between immune responses against MDA-modified peptide sequences and cardiovascular disease were different in different age groups, a subgroup analysis was performed on cases and controls under and above the median age (61 years). In the younger age group, cases had increased antibody levels against peptides 32 and 45, whereas no differences were seen in the older age group (Figure 3). Antibodies against all MDA peptide sequences were significantly associated with IMT in the younger age group but not in the older (Table 2, Figure 4). In addition to this, strong inverse correlations between antibody levels and plasma oxidized LDL were detected in the younger age group. Similar, but weaker, associations were observed among the controls (Table 3).

Discussion

These studies identify a number of MDA-modified sequences in apoB-100 that are recognized by human antibodies. MDA modification of apoB-100 occurs as a result of LDL oxidation, indicating that these antibodies belong to the family of previously described oxidized LDL autoantibodies. This notion is also supported by the observation that antibody binding to MDA-modified apoB-100 peptides is inhibited by addition of oxidized LDL. Antibodies were also identified against a large number of native apoB-100 sequences with a striking covariation between antibodies to native and MDA-
modified sequences. Preincubation of plasma with the respective MDA peptides inhibited IgM binding in the ELISA, whereas incubation with native peptides was without effect. This suggests that the covariation in binding between antibodies to native and MDA-modified sequences is not explained by crossreactivity of the same antibody, but by binding of different antibodies to native and MDA-modified sequences. If antibodies against native apoB-100 sequences

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Scattergrams demonstrating the relations between age and IgM against MDA-modified apoB-100 peptide 32 in patients (a) and controls (b), IgM against MDA-modified peptide 45 in patients (c) and controls (d), and plasma levels of oxidized LDL in patients (e) and controls (f).
bind also to native LDL particles, this is likely to have a major influence on LDL metabolism. However, the finding that native LDL does not compete antibody binding to native apoB-100 sequences, as well as the lack of correlation between antibodies against native apoB-100 sequences and LDL cholesterol levels, argue against the existence of such a phenomena. Unexpectedly, there was no significant association between antibody binding to MDA-modified peptides and the content of epsilon amino groups (ie, lysine, histidine, and arginine). This suggests that aldehyde binding to the free amino group at the amino-terminal end of peptide fragments is of importance for activation of immune responses.

Antibodies against MDA-modified peptide sequences decreased progressively with age in the cases, but not in the controls. IgM antibodies against MDA peptides were significantly associated with carotid IMT in the younger age group (less than 62 years of age), but not in the older age group. These findings suggest that significant change in the interactions between the immune system and the atherosclerotic vascular wall takes place between ages 50 and 70 years. One possibility is that in younger individuals the atherosclerotic disease process is at a more active stage with a more prominent involvement of immune cells. Another possibility is that the decreased levels of antibodies against MDA-modified peptide sequences in older subjects reflect a senescence of the immune cells involved in atherosclerosis. An impaired function of immune cells caused by immunosenescence has been proposed to contribute to an increased susceptibility to infections and cancer in the older population.26 Interestingly, immunosenescence is inhibited by antioxidants indicating involvement of oxidative stress.27 Immune cells that interact with epitopes in oxidized LDL are likely to be particularly exposed to oxidative stress. Because oxidized LDL is present in arteries already at a very early age28 these immune response are being continuously challenged for several decades, which may further contribute to a development of immunosenescence.

Increased antibodies against two sites in apoB-100 were observed in CHD patients less than 62 years of age. Antibodies against MDA-modified apoB-100 peptide 102 in patients (a) and controls (b).

**TABLE 2.** Age- and Sex-Adjusted Correlation Coefficient for Different Baseline MDA Peptides and Common Carotid Artery Intima-Media Thickness Among Younger (49–61 Years) and Older (62–67 years) Cases With Myocardial Infarction and Their Corresponding Controls Matched for Age, Sex, Smoking and Hypertension

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>IgM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDA 32</td>
<td>0.235*</td>
<td>-0.101</td>
</tr>
<tr>
<td>MDA 45</td>
<td>0.366†</td>
<td>-0.030</td>
</tr>
<tr>
<td>MDA 102</td>
<td>0.255†</td>
<td>-0.039</td>
</tr>
<tr>
<td>MDA 129</td>
<td>0.330†</td>
<td>-0.009</td>
</tr>
<tr>
<td>MDA 162</td>
<td>0.245*</td>
<td>0.001</td>
</tr>
<tr>
<td>MDA 210</td>
<td>0.233†</td>
<td>0.013</td>
</tr>
<tr>
<td>MDA 240</td>
<td>0.284†</td>
<td>0.006</td>
</tr>
<tr>
<td>IgG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDA 210</td>
<td>0.119</td>
<td>-0.059</td>
</tr>
</tbody>
</table>

*P<0.05. †P<0.01.

**TABLE 3.** Age- and Sex-Adjusted Correlation Coefficient for Different Baseline MDA Peptides and Plasma Oxidized LDL Among Younger (49–61 Years) Cases With MI and Their Corresponding Controls Matched for Age, Sex, Smoking and Hypertension

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Cases, Aged 49–61 y, n=36</th>
<th>Controls, Aged 49–61 y, n=71</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDA 32</td>
<td>-0.350*</td>
<td>-0.169</td>
</tr>
<tr>
<td>MDA 45</td>
<td>-0.501†</td>
<td>-0.374†</td>
</tr>
<tr>
<td>MDA 102</td>
<td>-0.147</td>
<td>0.146</td>
</tr>
<tr>
<td>MDA 129</td>
<td>-0.344*</td>
<td>-0.221</td>
</tr>
<tr>
<td>MDA 162</td>
<td>0.004</td>
<td>-0.066</td>
</tr>
<tr>
<td>MDA 210</td>
<td>-0.429†</td>
<td>-0.186</td>
</tr>
<tr>
<td>MDA 240</td>
<td>-0.443†</td>
<td>-0.175</td>
</tr>
</tbody>
</table>

*P<0.05. †P<0.01.
ies against these sites showed a high level of covariation, suggesting that they were produced in response to the same underlying pathophysiological processes. The fact that the median time from blood sampling to coronary event was only 2.8 years makes these antibodies particularly interesting as potential markers for increased CHD risk. However, considerably larger prospective studies with multivariate analysis are required before the clinical value of determining antibodies against apoB-100 MDA-modified peptide sequences can be fully established. Another limitation of the present clinical study is that we only analyzed antibodies against a small number of the antigenic sites in apoB-100 and that antibody titers against other sites may be even better markers of cardiovascular risk and will need further evaluation.

In subjects less than 60 years of age, IgM antibodies against MDA-modified sites in apoB-100 were correlated with the extent of existing vascular disease as assessed by carotid IMT (Table 2). Although carotid IMT has obvious limitations as a measure of general atherosclerotic burden, these observations still suggest that determination of IgM against MDA-modified sequences in apoB-100 may be one method to assess the severity of existing atherosclerosis. These observations are also in line with several previous studies that have reported associations between coronary and carotid artery disease and IgM antibodies against oxidized LDL.11,29

An important question is why these associations occur. They clearly demonstrate that immune responses against MDA-modified apoB-100 sites somehow are involved in the atherosclerotic disease process. Because high antibody levels are associated with more severe atherosclerosis and increased risk for development of acute coronary events, one obvious possibility is that these immune responses promote atherogenesis. Studies demonstrating that immune responses against heat shock proteins, such as heat shock protein 65, are atherogenic provide some support for this notion.30 However, experimental animal studies have shown an atheroprotective effect of oxidized LDL immunization.13–17,31 Reduced atherosclerosis has also been observed in apoE-null mice given repeated injections of immunoglobulin.32 The present observations do not necessarily argue against an atheroprotective role of immune responses against oxidized LDL. Proatherogenic processes, such as LDL oxidation, activate these immune responses. Accordingly, they are also likely to be in proportion to the severity of the disease process and could serve as markers of disease severity and CHD risk without contributing to disease progression. The finding that immunization of apoE-null mice with apoB-100 peptide sequences inhibits development of atherosclerosis as reported in the accompanying article demonstrates that an atheroprotective role is likely to be the case. The observation that the decrease in antibodies against MDA-modified peptide sequences in apoB-100 that occurs with age is accompanied by an increase in plasma levels of oxidized LDL suggest that an increased clearance of minimally oxidized LDL from the circulation may be one mechanism by which these antibodies could protect against atherosclerosis.

IgM levels against MDA-modified peptide sequences in apoB-100 were substantially more abundant than IgG. IgM levels were also significantly associated with clinical outcome, carotid IMT, and plasma oxidized LDL levels, suggesting that measuring IgM may be of clinical significance. Although less extensive, in studies performed on IgG against MDA-modified peptide sequences in apoB-100, the association was generally weaker. This may be the result of involvement of specific IgG subclasses not reflected in total IgG measurements and should be further studied.

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