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Association Between IgM Against an Aldehyde-Modified Peptide in Apolipoprotein B-100 and Progression of Carotid Disease

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Background and Purpose—Autoantibodies against antigens in oxidized low-density lipoprotein are common in people; experimental studies suggest that these immune responses have a functional role in the disease process. The aim of this study was to evaluate the relationship between the immune response against one defined oxidized low-density lipoprotein antigen, the aldehyde-modified peptide corresponding amino acids 3136 and 3155 (MDA-p210) in apolipoprotein (apo) B-100, and progression of carotid intima media thickness (IMT).

Methods—IgM and IgG against MDA-p210 were determined by enzyme-linked immunosorbent assay at baseline and after 12 months of treatment with placebo, metoprolol, fluvastatin, or metoprolol/fluvastatin in 751 individuals participating in the BCAPS. Carotid IMT was assessed by ultrasonography at baseline and after 18 and 36 months of treatment.

Results—Antibody levels did not change in response to treatment, but high baseline MDA-p210 IgM levels were associated with a more rapid progression of carotid disease both at 18 ($r=0.09$, $P<0.05$) and 36 months ($r=0.12$, $P<0.005$). At 36 months, the difference in IMT progression rate per year between those with high MDA-p210 IgM levels and those with low was 0.011 mm (95% CI=0.005 to 0.018 mm, $P<0.0001$). Treatment with fluvastatin markedly decreased the progression of IMT among subjects with high but not with low MDA-p210 IgM levels. There was no association between MDA-p210 IgG and carotid IMT progression.

Conclusions—IgM against the aldehyde-modified peptide corresponding amino acids 3136 and 3155 in apo B-100 is common in subjects with asymptomatic carotid disease, and high levels are associated with a more rapid progression of carotid IMT. The observation that the effect of fluvastatin was restricted to subjects with high MDA-p210 IgM levels may reflect the increased rate of disease progression in this group. (*Stroke*. 2007;38:1495-1500.)

Key Words: atherosclerosis ■ carotid arteries ■ echocardiography ■ immune system ■ lipoproteins

Inflammatory responses to oxidized low-density lipoprotein (LDL) particles accumulating in the arterial wall are believed to play a key role in the initiation and progression of atherosclerosis.¹⁻³ This notion is also supported by recent clinical studies showing a strong association between circulating oxidized LDL and angiographically documented coronary artery disease.⁴ The oxidative modification of LDL involves oxidation of surface phospholipids, degradation of the LDL apolipoprotein B-100 (apo B-100), and binding of malondialdehyde (MDA) and other reactive aldehydes to apo B-100 peptide fragments.⁵ These modifications activate both innate and adaptive immune responses,^{6,7} including generation of antibodies against aldehyde-modified peptide fragments of apo B-100 and phosphorylcholine-containing oxidized phospholipids.⁸⁻¹⁰ Antibodies against the latter type of antigens are exclusively IgM, whereas both IgM and IgG are generated in response to

aldehyde-modified apo B-100 peptide fragments.⁸⁻¹⁰ Autoantibodies against antigens in oxidized LDL are common in people,¹¹ but their role in the atherosclerotic disease process remains to be fully understood. Immunization of hypercholesterolemic animals with oxidized LDL inhibits the development of atherosclerosis¹²⁻¹⁸ and has generally been associated by an increase in oxidized LDL-specific IgG. We have previously characterized a number of different MDA-modified apo B-100 peptide epitopes specifically recognized by oxidized LDL autoantibodies present in human plasma⁸ and subsequently shown that immunization with some of these apo B-100 peptides significantly inhibits the development of atherosclerosis in apo E^{-/-} mice.¹⁹⁻²¹ The aim of the present study was to determine the association between autoantibodies against one of these MDA-modified apo B-100 peptides (p210, amino acids 3136 and 3155) and progression of carotid intima media thickness

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(IMT) during a 3-year period in 751 individuals participating in the Beta-blocker Cholesterol-lowering Asymptomatic Plaque study (BCAPS).²² The selection of the p210 apo B-100 sequence was based on previous studies suggesting that it is one of the major oxidized LDL antigens recognized by the immune system in people⁸ and that immunization with this antigen inhibits the development of atherosclerosis in mice.¹⁹ This sequence is also of particular interest because it is located immediately adjacent to the apo B-100 amino acid sequence that mediates binding of LDL to proteoglycans (amino acids 3148 to 3158) and which has been attributed a critical role in the atherogenicity of LDL.²³

Methods

Study Population

The background population for this study consisted of all men and women born between 1926 and 1945 and living in Malmö (n=68 905 in 1991). The population was identified by use of the Swedish National Population registries. Probands were invited by mail and by advertising to take part in the Malmö Diet and Cancer Study (MDCS).²⁴ Participation rate was 39% (n=28 098) and the participants were shown to have a lower mortality than nonparticipants.²⁵ The MDCS has a cardiovascular component randomly chosen from the participants in the MDCS²² in which the degree of atherosclerosis was determined by B-mode ultrasound.

The cohort participating in the BCAPS trial consisted of 793 men and women 49 to 70 years of age with a plaque in the right carotid artery but with no symptoms of carotid artery disease recruited from the MDCS participants.²² The present study group consisted of the 751 individuals for which both baseline and 12-month plasma samples were available. The individuals were divided into 4 treatment groups: (1) placebo (n=186), (2) metoprolol (n=190), (3) fluvastatin (n=186), and (4) metoprolol/fluvastatin (n=189). The study group did not include subjects regularly using β -blockers or statins, systolic blood pressure above 160 mm Hg, diastolic blood pressure above 95 mm Hg, total cholesterol above 8.0 mmol/L, or hyperglycemia suspected to require insulin treatment. The ethical committee of Lund University, Sweden, approved the study and the participating subjects gave informed consent.

B-mode Ultrasound

Analyses of common and bulb carotid IMT was performed using an Acuson 128 CT system with a 7-MHz transducer as described previously.²² Briefly, the right carotid bifurcation was scanned within a predefined window comprising 3 cm of the distal common carotid artery, the bifurcation, and 1 cm of the internal and external carotid arteries. Thickness of the intima media complex was measured in the far wall according to the leading edge principle with a specially designed, computer-assisted image analyzing system based on automatic detection of echo structures. Change in IMT was defined as the 18- and 36-month value, respectively, while simultaneously considering IMT at baseline. This definition was used in all analyses. Data regarding quality control and reproducibility for IMT measurements have been published previously and found satisfactory.²⁶ The investigators measuring carotid IMT were blind to the results of the autoantibody determinations.

Risk Factors for Cardiovascular Disease

Blood pressure was measured in the supine position after 5 minutes of rest with a mercury manometer attached to a rubber cuff of appropriate sizes for the arm circumference. Smoking habits were classified as current smokers, ex-smokers, or never-smokers. After overnight fasting, blood samples were drawn for the determination of baseline serum values of total cholesterol, triglycerides, high-density lipoprotein cholesterol, and whole blood glucose with the routine clinical method used at the Department of Clinical Chemistry, Malmö University. LDL cholesterol in millimoles per liter was calculated according to the Friedewald formula. Baseline plasma

C-reactive protein (CRP) was measured using a rabbit anti-human CRP (Dako A/S, Glostrup, Denmark) as capture antibody, rabbit anti-human CRP (Peroxidase-conjugated; Dako), human CRP high control (Dako) as standard, and TMB one substrate (Dako) as substrate (detection limit=0.1 μ g/L and interassay coefficient of variation 8%).

Enzyme-Linked Immunosorbent Assay Against the Malondialdehyde-Modified Apo B-100 Peptide

A polypeptide corresponding to the 3136 to 3155 amino acid sequence of human apo B-100 (KTTKQSFDSLVSVAQYKKNKH) was synthesized (KJ Ross Petersen AS, Horsholm, Denmark) and used in enzyme-linked immunosorbent assay. The peptide was modified by 0.5 mol/L MDA for 3 hours at 37°C and dialyzed against phosphate-buffered saline containing 1 mmol/L edetic acid as described.⁸ MDA content of the modified peptide was analyzed by the TBARS assay (0.56 nmol MDA per microgram peptide). To remove unbound MDA, the peptide was dialyzed against 0.15 mol/L phosphate-buffered saline containing 1 mmol/L edetic acid pH 7.4 using a dialyze tubing with 1000 Mw cutoff. MDA-modified peptides diluted in phosphate-buffered saline pH 7.4 (20 μ g/mL) were absorbed to microtiter plate wells (Nunc MaxiSorp; Nunc) in an overnight incubation at 4°C. After washing with phosphate-buffered saline containing 0.05% Tween-20, the coated plates were blocked with SuperBlock in TBS (Pierce) for 5 minutes at room temperature followed by an incubation of test plasma, diluted 1/100 in TBS-0.1% Tween-20 containing 10% Superblock (TBS-T) for 2 hours at room temperature and overnight at 4°C. After rinsing, deposition of autoantibodies directed to the peptide was detected using biotinylated rabbit anti-human IgM (ICN, Biomedicals, Inc., Aurora, Ohio) or IgG 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 detected by alkaline phosphatase conjugated streptavidin (Sigma) incubated for 2 hour 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. Data regarding the specificity and variability of the antibody enzyme-linked immunosorbent assay have been published previously.⁸ The mean intraassay coefficient of variance for the MDA-p210 IgM enzyme-linked immunosorbent assay was 8.2% and the mean inter-assay coefficient of variance was 14.7%.

Statistical Analysis

Differences regarding baseline characteristics were tested by *t* test and χ^2 test as applicable. Skewed variables (triglycerides and CRP) were log-transformed before statistical tests. The Pearson and Spearman correlation coefficients were used to examine the relationship among continuous variables. Student *t* test and analysis of covariance were used to test for significance between group means. A general linear model with progression of carotid IMT as dependent variable and age, baseline IMT, total cholesterol, high-density lipoprotein and LDL cholesterol, triglycerides, body mass index, fasting glucose, MDA-p210 IgM, systolic blood pressure, presence of diabetes, treatment group, smoking, CRP, and gender as independent variables was used to study independent correlation between progression of IMT and other variables. The selection of variables to be included in the model was based on our previous experience of factors demonstrating association with carotid IMT.

Results

Baseline Characteristics and Antibody Association

IgM and IgG against the MDA-modified 3136 to 3155 amino acid sequence in apo-B 100 (MDA-p210) was determined in baseline plasma samples and after 12 months of treatment in 751 individuals participating in BCAPS. The baseline characteristics of the study group are presented in the Table. A history of cardiovascular disease was present in 4.3% of the

Baseline Clinical Characteristics

Age, years	61.3±5.4
Male sex, %	45.5
Former smokers, %	36.9
Current smokers, %	30.8
Body mass index, kg/m ²	25.5±3.6
Systolic blood pressure, mm Hg	138.9±14.4
Diastolic blood pressure, mm Hg	84.7±7.1
Glucose, mmol/L	5.1±0.7
Total cholesterol, mmol/L	6.12±0.98
LDL cholesterol, mmol/L	4.14±0.88
High-density lipoprotein cholesterol, mmol/L	1.38±0.37
Cardiovascular disease, %	4.3
Type 2 diabetes, %	3.2

subjects and 3.2% had a history of type 2 diabetes. IgM recognizing MDA-p210 was identified in baseline samples of all individuals and with the exception of one individual, the same was true for MDA-p210 IgG. There was no relation between plasma levels of IgM and IgG. MDA-p210 IgM levels decreased with age ($r=-0.15, P<0.001$), whereas no such association was observed for MDA-p210 IgG. MDA-p210 IgM levels were weakly associated with fasting glucose ($r=-0.10, P<0.01$) and CRP ($r=0.07, P<0.05$) levels at baseline. High MDA-p210 IgG levels were associated with low CRP ($r=-0.16, P<0.001$). IgM and IgG against MDA-p210 were not related to lipoprotein lipids, blood pressure, body mass index, smoking, or gender.

Association Between Antibody Levels and Intima Media Thickness

There was a strong correlation between antibody levels at baseline and after 12 months for both IgM ($r=0.61, P<0.0001$) and IgG ($r=0.50, P<0.0001$; Figure 1) demonstrating that these immune responses are relatively stable over time. Fluvastatin and/or metoprolol had no effect on IgM or IgG antibody levels after 12 months of treatment. There was also no significant relation between antibody levels at baseline and baseline common carotid IMT. However, high baseline levels MDA-p210 IgM were weakly but significantly associated with a higher progression rate of carotid disease both at 18 ($r=0.09, P<0.05$) and 36 months ($r=0.12, P<0.005$). There was also a weak inverse association between MDA-p210 IgM levels and baseline carotid bulb IMT ($r=-0.12, P<0.005$), whereas there was no association between antibody levels and progression of IMT in the carotid bulb. Comparing individuals below or above the median MDA-p210 IgM level at baseline demonstrated marked progression of common carotid IMT in those with an antibody level above the median but no mean progression in those with antibody levels below the median value (Figure 2). The difference in IMT progression per year at 36 months between the 2 groups was 0.011 mm (95% CI=0.005 to 0.018 mm, $P<0.0001$). This difference remained significant after adjustment for baseline IMT (0.010 mm; 95% CI=0.004 to 0.017 mm, $P<0.005$). There was also a significant difference in progression of IMT per year between the 2 groups at 18 months (0.013 mm;

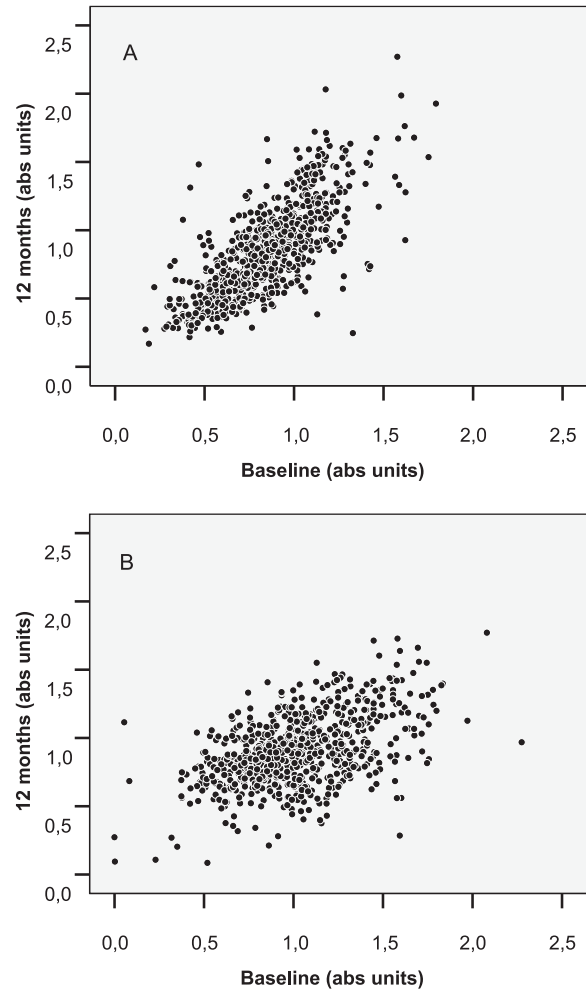


Figure 1. Correlation between (A) IgM and (B) IgG levels against the MDA-modified 3136 to 3155 amino acid sequence of apo B-100 at baseline and after 12 months (abs units, absorbance units).

95% CI=0.003 to 0.024 mm, $P<0.02$) and after adjustment for baseline IMT (0.011 mm; 95% CI=0.001 to 0.021 mm, $P<0.05$). There was no significant association between MDA-p210 IgG levels and progression of IMT (data not shown).

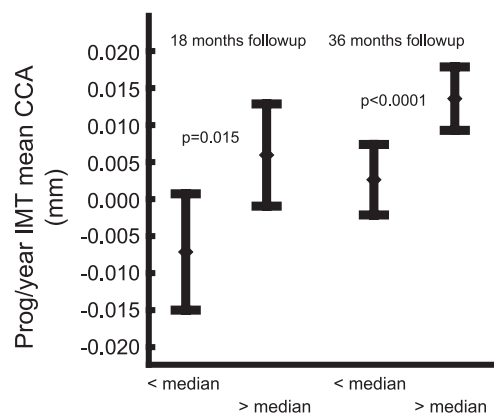


Figure 2. Progression of carotid IMT in the common carotid artery (CCA) after 18 and 36 months in subjects with IgM levels against the MDA-modified 3136 to 3155 amino acid sequence of apo B-100 below ($n=374$) or above ($n=375$) the median IgM level at baseline. Data are presented as mean and 95% CI.

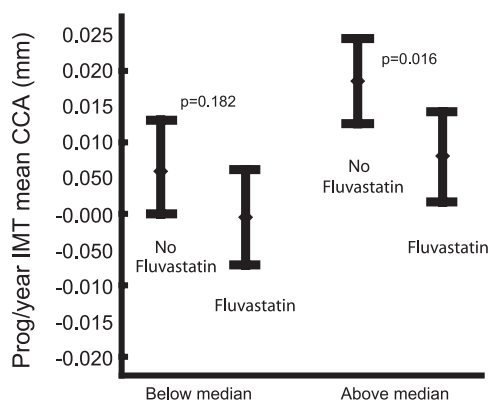


Figure 3. Progression of IMT in the common carotid artery (CCA) after 36 months in the different treatment groups (the placebo and metoprolol groups [$n=376$] versus the fluvastatin and fluvastatin/metoprolol groups [$n=375$]) in subjects with IgM levels against the MDA-modified 3136 to 3155 amino acid sequence of apo B-100 below or above the median level at baseline. Data are presented as mean and 95% CI.

In multivariate analyses, including age, baseline IMT, total cholesterol, high-density lipoprotein and LDL cholesterol, triglycerides, body mass index, fasting glucose, MDA-p210 IgM, systolic blood pressure, presence of diabetes, treatment group, smoking, CRP, and gender, independent associations with the rate of common carotid IMT progression between baseline and 36 months were found for baseline IMT (regression coefficient = -0.14 , 95% CI = -0.20 to -0.09 , $P < 0.001$), body mass index (regression coefficient = -0.32 , 95% CI = -0.61 to -0.02 , $P < 0.05$), MDA-p210 IgM (regression coefficient = 3.16 , 95% CI = 0.62 to 5.70 , $P < 0.02$), and treatment group (regression coefficient = -3.26 , 95% CI = -5.95 to -0.56 , $P < 0.02$).

Associations Between Antibodies and Intima Media Thickness in Relation to Treatment

One finding of the BCAPS was that treatment with fluvastatin reduced progression of IMT in the common carotid artery, whereas metoprolol was without effect in this respect. To determine whether antibody levels influenced the effect of treatment, we compared the rate of IMT progression at 36 months in subjects with or without fluvastatin. In subjects with MDA-p210 IgM above the median level at baseline, fluvastatin markedly reduced the progression of carotid IMT at 36 months as compared with those receiving only placebo or metoprolol (0.008 mm, 95% CI = 0.002 to 0.014 mm versus 0.019 mm, CI = 0.013 to 0.024 mm; $P = 0.016$), whereas in subjects with low MDA-p210 IgM levels, progression of common carotid IMT was minimal and there was no significant effect of fluvastatin treatment (Figure 3).

Discussion

This study demonstrates that both IgM and IgG against the aldehyde-modified peptide corresponding to amino acids 3136 and 3155 in apo B-100 are common in subjects with asymptomatic carotid disease, that the plasma levels of these autoantibodies are relatively stable over a 12-month period, and that high IgM levels are associated with a more rapid progression of the disease.

The relation between autoantibodies against this defined oxidized LDL antigen and progression of carotid IMT supports the existence of a biological association but does not clarify whether antibodies are actively involved in the disease process or only serve as markers of disease activity. However, several lines of evidence suggest that immune responses against antigens in oxidized LDL play an important functional role in atherosclerosis. Immunization of experimental animals with oxidized LDL results in inhibition of atherosclerosis.^{12–18} These effects have generally been associated with an increase in specific IgG suggesting the involvement of adaptive immunity in the protective response. Atheroprotection after immunization with apo B-100 peptides has also been associated with an increased expression of specific IgG,^{19–21} and treatment with recombinant human MDA-modified apo B-100 peptide IgG reduces atherosclerosis in apo E^{-/-} mice.²⁷ Although a significant inverse association between MDA-p210 IgG and the cardiovascular risk marker CRP was observed in the present study, the lack of an association between MDA-p210 IgG and progression of carotid IMT does not support the existence of a corresponding protective role for naturally occurring MDA-p210 IgG. This notion is also supported by a body of experimental studies suggesting that the net effect of adaptive immune responses to hypercholesterolemia is atherogenic.^{6,7,28,29}

The observation that high IgM levels against MDA-p210 are associated with a more rapid progression of carotid disease implies that these antibodies may have a proatherogenic effect. In contrast, most experimental studies have suggested that oxidized LDL-specific IgM is atheroprotective. Binder et al³⁰ demonstrated that selective induction of IgM against oxidized LDL phospholipids by immunization with *Streptococcus pneumoniae* reduced both the plasma levels of oxidized LDL and the development of atherosclerosis. Moreover, Faria-Neto et al³¹ found that passive immunization with a monoclonal antiphosphatidylcholine IgM antibody has an atheroprotective effect in mice. Accordingly, it cannot be excluded that the present observation of increased levels of MDA-p210 IgM in subjects with a more rapid progression of carotid disease reflects activation of a defense mechanism rather than suggesting a proatherogenic role of the antibodies. It is also interesting to note that the association between MDA-p210 IgM and IMT appears to differ between the common carotid artery and the carotid bulb.

The experience from other diseases involving organ-specific autoimmune responses such as type 1 diabetes, thyroiditis, celiac disease, and rheumatic diseases has convincingly demonstrated the potential of autoantibodies as diagnostic tools.³² However, the usefulness of autoantibodies as markers of atherosclerotic disease activity and as predictors of cardiovascular risk remains to be fully established. Salonen et al³³ first reported increased antibody binding to MDA-LDL in subjects with accelerated 2-year progression of carotid disease as compared with control subjects without progression. The results of several subsequent studies have been inconsistent reporting that antibodies against MDA- or oxidized LDL are increased in patients with coronary artery disease,^{34–36} predict risk for development of myocardial infarction,³⁷ have no association with coronary artery disease,^{38,39} increase with severity of

carotid disease,⁴⁰ and decrease with severity of femoral disease⁴⁰ and carotid disease.^{41–43} The approach used in the present study to analyze antibodies against one clearly defined antigen present in oxidized LDL may offer the advantage of a higher specificity and reproducibility. A limitation of this technique is that only a fraction of all antibodies against epitopes in oxidized LDL are measured. However, by studying autoantibodies against single antigens in oxidized LDL, it may be possible to identify more specific markers of disease severity and risk.

Treatment with fluvastatin significantly inhibited the progression of carotid disease in subjects with high MDA-p210 IgM levels but not in subjects with low antibody levels. It is likely that this is explained by the increased rate of disease progression in subjects with high IgM levels rather than by an interaction of the drug with the immune response because there was no difference in antibody levels between fluvastatin-treated and nontreated subjects.

In a previous prospective study of the relation between IgM autoantibodies against the MDA-p210 and carotid disease in 78 cases with coronary events and 149 matching control subjects, we found a significant association between baseline antibody levels and baseline carotid IMT.⁸ No such association was found in this study, but the circumstance that presence of carotid disease was an inclusion criteria in the BCAPS may have been a confounding factor. Another limitation of the present study is the relatively low prevalence of clinically manifest cardiovascular disease; it remains to be determined whether our observations are valid also for subjects with more advanced disease.

Summary

The findings of the present study add further support to the idea that immune responses against oxidized LDL antigens are involved in the development of atherosclerosis and suggest that measurement of antibodies against clearly defined oxidized LDL antigens may help to identify subjects with a more aggressive disease.

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Disclosures

G.N.F., B.H., G.B., R.A., J.-Å.N., A.S., and J.N. have no conflict of interests. P.K.S. has an institutional conflict of interest.

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