Identification of autoantibodies in human plasma recognizing an apoB-100 LDL receptor binding site peptide

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Abstract The aim of this study was to test the hypothesis that autoantibodies recognize amino acid sequences in the LDL receptor binding region of apolipoprotein B-100 (apoB-100). Autoantibodies against an unmodified or malondialdehyde (MDA)-modified LDL receptor binding site peptide were determined by ELISA in baseline plasma samples of 78 cases with coronary events and 149 matched controls recruited from the prospective Malmö Diet Cancer Study. IgG and IgM recognizing this peptide were detected in all subjects but did not differ between cases and controls. Inverse associations were observed between IgG against the native binding site and plasma oxidized LDL ($r = -0.21$, $P < 0.005$), but there were no significant associations with total or LDL cholesterol levels. In univariate analyses, inverse associations were found between baseline carotid intima-media thickness and IgG against the MDA-modified binding site ($r = -0.14$, $P < 0.05$), but this association was lost when controlling for other major cardiovascular risk factors. Specificity studies demonstrated that the binding of autoantibodies to these sequences could be inhibited by oxidized but not by native LDL. Autoantibodies recognizing the LDL receptor binding site in apoB-100 are frequently expressed. Their association with plasma oxidized LDL suggests that they have been generated in response to breakdown products of LDL oxidation, but their influence on cholesterol metabolism and the development of atherosclerosis appears limited.— Fredrikson, G. N., G. Berglund, R. Alm, J-Å. Nilsson, P. K. Shah, and J. Nilsson. **Identification of autoantibodies in human plasma recognizing an apoB-100 LDL receptor binding site peptide. *J. Lipid Res.* 2006.** 47: **2049–2054.**

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The importance of innate and adaptive immune responses in the development of atherosclerosis is being increasingly recognized (1, 2). Particular interest has focused on the role of immune responses against oxidatively modified LDL (3). A substantial fraction of T-cells present in atherosclerotic plaques are reactive to epitopes in oxidized LDL (4). Both IgM and IgG against oxidized LDL are present in human plasma and have been associated with disease severity and the risk for development of acute cardiovascular events (5–12). These antibodies primarily bind to oxidized phospholipids and aldehyde-modified peptide fragments of degraded apolipoprotein B-100 (apoB-100) in oxidized LDL (3). Recent detailed characterization of the epitopes present in oxidized LDL identified >100 different aldehyde-modified amino acid sequences recognized by antibodies present in human plasma (13). Interestingly, several autoantibodies recognizing non-aldehyde-modified apoB-100 sequences were also identified.

The functional role of oxidized LDL autoantibodies remains to be fully understood. IgM against oxidized phospholipids blocks lipid uptake in macrophages through the scavenger receptor pathway (14). Recombinant human IgG against specific aldehyde-modified peptide sequences in apoB-100 inhibits the development of atherosclerosis in apoE-deficient mice (15). Atheroprotective immunization with oxidized LDL as well as aldehyde-modified apoB-100 peptides is also associated with a selective upregulation of IgG (16, 17).

It is likely that the fragmentation and aldehyde modification of apoB-100 that occurs as a result of LDL oxidation may also affect the peptides that mediate binding to the LDL receptor. Immune responses resulting in the expression of antibodies against the native and/or aldehyde-modified LDL receptor binding site in apoB-100 could potentially have important effects on both cholesterol homeostasis and atherosclerosis. The aim of this study was to investigate whether antibodies against unmodified and

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aldehyde-modified peptides corresponding to the LDL receptor binding site are expressed in humans. Moreover, the associations of these antibodies with other cardiovascular risk factors, severity of atherosclerosis as assessed by the carotid intima-media thickness (IMT), and risk for the development of acute myocardial infarction and cardiac death were investigated.

MATERIALS AND METHODS

Study population

The study subjects, born between 1926 and 1945, belong to the Malmö Diet and Cancer Study cohort. A random 50% of those who entered the Malmö Diet and Cancer Study between November 1991 and February 1994 were invited to take part in a study of the epidemiology of carotid artery disease (18). Routines for the ascertainment of information on morbidity and mortality after the health examination, as well as definitions of traditional risk factors, have been reported (19). The ethical committee of Lund University approved this study.

Eighty-five cases of acute coronary heart events (i.e., fatal or nonfatal myocardial infarction or death due to coronary heart disease) were identified during follow-up. Participants who had a history of myocardial infarction or stroke (n = 6) before enrollment were not eligible for the study. For each case, two controls without a history of myocardial infarction or stroke were individually matched for age, sex, smoking habits, presence of hypertension, month of participation in the screening examination, and duration of follow-up. Only one control was available for seven cases, and no control was available for one case. This case was excluded from analysis.

Blood pressure was measured in the supine position after 5 min of rest with a mercury manometer attached to a rubber cuff of appropriate size for the arm circumference.

Laboratory analyses

After overnight fasting, blood samples were drawn between 8 and 10 AM 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 (Mercordia, Uppsala, Sweden) in EDTA plasma supplemented with the antioxidants diethylenetriamine-penta-acetic acid and butylhydroxytoluene (BHT). The plasma samples had been stored at −80°C and not previously thawed. This oxidized LDL ELISA is based on the direct sandwich technique, in which two monoclonal antibodies are directed against separate antigenic determinants on the oxidized apoB molecule, using the mAb4E6 antibody developed by Holvoet et al. (20) as the capture antibody. The coefficient of variation for the assay is 8%, and the recovery is 95%.

B-mode ultrasound vasculography

An Acuson 128 Computed Tomography System (Acuson, Mountain View, CA) with a 7 MHz transducer was used for the assessment of carotid plaques in the carotid artery as described previously (21).

ELISA using the apoB-100 binding site peptide as coating

Polypeptide corresponding to the 3,356–3,375 amino acid sequence of human apoB-100 (GTTRLTRKRGLKLATALSLS; named PLDLr) was synthesized (KJ Ross Petersen AS, Horsholm, Denmark) and used in ELISA. The peptide was modified by 0.5 M malondialdehyde (MDA) for 3 h at 37°C as described (13). MDA content of the modified peptide was analyzed by the thiobarbituric acid-reactive substances assay (9.5 pmol MDA/μg peptide). To remove unbound MDA, the peptide was dialyzed against 0.15 M PBS containing 1 mM EDTA, pH 7.4, using dialysis tubing with a molecular weight cutoff of 1,000. Unmodified (PLDLr) and MDA-modified (MDA-PLDLr) peptides diluted in PBS, pH 7.4 (20 μg/ml), were absorbed to microtiter plate wells (Nunc MaxiSorp; Nunc, Roskilde, Denmark) in an overnight incubation at 4°C. After washing with PBS containing 0.05% Tween-20, the coated plates were blocked with SuperBlock in TBS (Pierce, Rockford, IL) for 5 min at room temperature, followed by an incubation of test plasma, diluted 1:100 in TBS and 0.1% Tween-20 containing 10% SuperBlock (TBS-T) for 2 h 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 antibodies (ICN Biomedicals, Inc., Aurora, OH) or biotinylated rabbit anti-human IgG antibodies (Dako A/S, Glostrup, Denmark) appropriately diluted in TBS-T. After another incubation for 2 h at room temperature, the plates were washed and the bound biotinylated antibodies were detected by alkaline phosphatase-conjugated streptavidin (Dako) incubated for 2 h at room temperature. The color reaction was developed using the phosphatase substrate kit (Pierce), and the absorbance at 405 nm was measured after 1 h of incubation at room temperature.

Specificity tests were performed on PLDLr or MDA-PLDLr ELISAs by preincubating 0.5 ml of test plasma diluted 1:5 in 0.15 M PBS, pH 7.4, containing 1% BSA, 2.5 mM EDTA, 50 μM BHT, with 500 μg of native LDL, MDA-LDL, or copper-oxidized LDL for 1 h at room temperature and overnight at 4°C. Modified lipoproteins were prepared as described by Palinski et al. (22). After centrifugation (1,000 *g* for 10 min) at 4°C, the supernatant was diluted (final dilution, 1:100) and used in ELISAs. As a control, preincubation was performed with buffer alone.

A solid-phase specificity test was also performed. Microtiter plates were coated with apoB-100 peptides: PLDLr, MDA-PLDLr, P2 (ATRFKHLRKYTYNYEAESSS; amino acids 16–35, used as a nonspecific peptide), or MDA-P2. The plates were coated with 0, 10, 20, 40, 80, and 160 μg/ml of each peptide in an overnight incubation. After washing, test plasma diluted 1:100 and purified human IgG (0.135 mg/ml; No. I4506; Sigma) in 0.15 M PBS, pH 7.4, containing 1% BSA, 2.5 mM EDTA, and 50 μM BHT were added to the wells and incubated for 1 h at room temperature and overnight at 4°C. As a control, preincubation was performed with the buffer alone (0 μg/ml). The supernatants were thereafter used in ELISAs.

Statistical analysis

Differences regarding baseline characteristics were tested by *t*-test and Chi-square test, as applicable. Skewed variables were log-transformed before statistical tests. The Pearson and Spearman correlation coefficients were used to examine the relationship among continuous variables. Student's *t*-test and analysis of covariance were used to test for significance between group means. Multiple regression analysis was used to study independent correlations between the progression of IMT and other variables. $P < 0.05$ was considered significant.

RESULTS

Antibodies recognizing the LDL receptor binding site peptide

The baseline clinical characteristics of this study group have been reported previously (13). IgG and IgM recog-

nizing both the unmodified and MDA-modified LDL receptor binding site peptides were detected in all subjects (**Fig. 1**). Both IgG and IgM levels were higher against the MDA-modified than against the unmodified binding site peptide. There was no difference in antibody levels between cases and controls (Fig. 1).

IgG against the unmodified LDL receptor binding site peptide increased with age ($r = 0.19$, $P = 0.005$). After controlling for age and gender, a significant inverse association was found between IgG against the native binding site and the plasma level of oxidized LDL ($r = -0.21$, $P < 0.005$), whereas no significant associations were found between LDL cholesterol levels and antibodies against the unmodified or the MDA-modified binding site (**Table 1**). There were also no significant associations between antibody levels and total cholesterol, HDL cholesterol, triglycerides, fasting glucose, or systolic and diastolic blood pressure.

Using the carotid IMT assessed by ultrasonography, we also analyzed associations between antibody levels and degree of existing vascular disease. In univariate analyses, inverse associations were found between baseline carotid IMT and IgG against the MDA-modified binding site peptide ($r = -0.14$, $P < 0.05$). However, this association did not remain significant when controlling for age, gender, blood lipids, blood pressure, glucose, body mass index, and smoking habits.

Specificity of LDL receptor binding site autoantibodies

To evaluate the specificity of autoantibodies binding to the unmodified (PLDLr) and MDA-modified (MDA-PLDLr) LDL receptor binding site peptides, pooled healthy control plasma and purified human IgG were preincubated in wells coated with different concentrations of PLDLr, MDA-PLDLr, or the control peptide (P2 and MDA-P2), respectively. The preincubation of pooled healthy control plasma and purified human IgG in peptide-coated wells resulted in a dose-dependent inhibition of antibody binding in the subsequent ELISA (**Fig. 2** and data not shown). When a peptide concentration of 20 μg/ml was used to coat the preincubation plates (i.e., the same peptide concentration used to produce the ELISA plates), the following results were obtained: PLDLr removed >40% of the IgG in human plasma recognizing the unmodified LDL receptor binding site, whereas MDA-PLDLr, P2, and MDA-P2 de-

TABLE 1. Partial correlation coefficients between autoantibodies to the unmodified and MDA-modified LDL receptor binding site sequence in apolipoprotein B-100 and lipoprotein lipids, oxidized LDL, and fasting glucose

Lipid	Unmodified Sequence IgG	Unmodified Sequence IgM	MDA-Modified Sequence IgG	MDA-Modified Sequence IgM
Total cholesterol	0.08	−0.06	0.07	−0.08
LDL cholesterol	0.06	−0.05	0.03	−0.11
Oxidized LDL	−0.21[a]	−0.05	0.00	−0.07
HDL cholesterol	0.10	0.05	−0.01	0.04
Triglycerides	0.08	−0.05	0.01	−0.07
Glucose	0.06	−0.06	0.07	−0.04

MDA, malondialdehyde.
[a] $P < 0.005$.

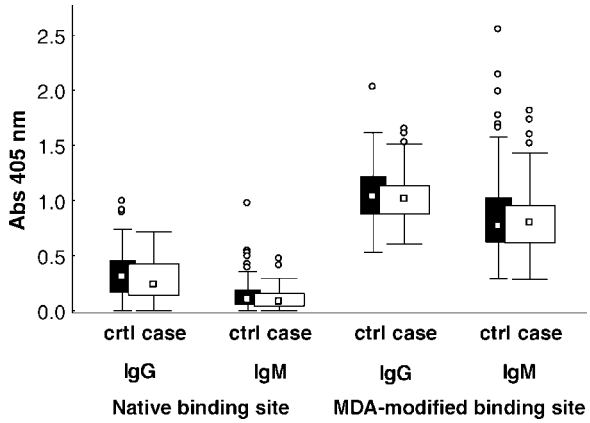

Fig. 1. IgG and IgM recognizing the LDL receptor binding site. Both IgG and IgM levels were higher against the malondialdehyde (MDA)-modified binding site peptide than against the unmodified peptide. No differences between controls (ctrl; black blocks) and cases (white blocks) were detected. Box plots demonstrate median, first, and third quartiles, with error bars showing the highest and lowest values within one and a half quartile of the median value. Small open circles depict extreme values. Abs, absorbance.

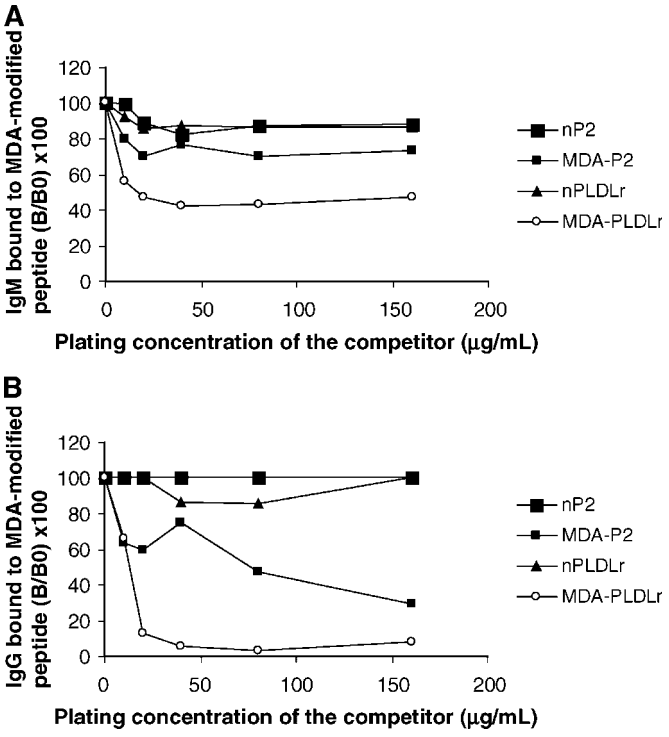

Fig. 2. Specificity tests with unmodified and MDA-modified apolipoprotein B-100 (apoB-100) peptides. Competition dilution curves showing levels of IgM in a healthy human plasma pool (A) and levels of IgG in purified human IgG recognizing the MDA-modified LDL receptor binding peptide (B). The plasma pool and the purified IgG were preincubated in wells coated with different concentrations of unmodified (nPLDLr) or MDA-modified (MDA-PLDLr) binding site peptides as well as unmodified (P2) or MDA-modified control (MDA-P2) peptides, respectively. As a control, buffer was used for the preincubation (0 μg/ml).

creased binding by <15% (data not shown). IgG in the plasma pool recognizing the MDA-PLDLr was decreased ~40% after preincubation with MDA-PLDLr, whereas no effect was detected with PLDLr, P2, and MDA-P2 (data not shown). In addition, preincubation of the plasma pool with MDA-PLDLr removed ~55% and MDA-P2 removed ~30% of the IgM recognizing the MDA-modified LDL receptor binding site, whereas PLDLr and P2 did not affect the binding (Fig. 2A). IgM in human plasma recognizing the PLDLr was decreased >40% after preincubation with PLDLr, ~25% with P2 and MDA-P2, and only 10% with MDA-PLDLr (data not shown). Competition of antibody binding could also be obtained after liquid-phase preincubation. However, the peptide concentrations needed to achieve competition were higher than those required for competition using the solid-phase technique (data not shown). To study whether plasma proteins do not interfere with the binding to peptides, purified human IgG also was used for the preincubation. This preincubation of human IgG with MDA-PLDLr removed >90% and MDA-P2 removed ~40% of the IgG recognizing the MDA-modified LDL receptor binding site, whereas unmodified peptides did not affect the binding (Fig. 2B). IgG in purified human IgG recognizing the PLDLr was decreased by ~95% after preincubation with MDA-PLDLr, by >60% with PLDLr, and by ~35% with the unmodified control peptide, and MDA-P2 had no effect (data not shown). In summary, these results demonstrate the existence of two specific LDL receptor binding site autoantibodies, one recognizing unmodified sequence and the other recognizing MDA-modified sequence. These autoantibodies seem to be sequence-specific, because no or only minor competition was seen after preincubation with a nonbinding site sequence in apoB-100.

In addition, pooled healthy control plasma was preincubated with native LDL, MDA-LDL, and copper-oxidized LDL. The addition of copper-oxidized LDL inhibited ~20% of the binding of IgG to both unmodified and MDA-modified LDL receptor binding site peptide (**Fig. 3B, D**), whereas preincubation with MDA-LDL only resulted in a 10% inhibition of IgG binding to the aldehyde-modified LDL receptor binding site and did not significantly affect the binding of IgG to the unmodified LDL receptor binding site (Fig. 3B, D). No inhibition of IgG binding to the unmodified and aldehyde-modified LDL receptor binding site peptide was observed after preincubation with native LDL (Fig. 3B, D). IgM antibodies recognizing the MDA-modified peptide were significantly inhibited by copper-oxidized LDL but not by MDA-modified or native LDL (Fig. 3A, C). These results suggest that the autoantibodies against the LDL receptor binding site recognize the amino acid sequence as expressed in oxidized but not in native LDL.

DISCUSSION

This study demonstrates for the first time the existence of autoantibodies recognizing amino acid sequences in the LDL receptor binding region of apoB-100 and that they are commonly expressed in humans. These autoantibodies have no or only very poor affinity for the LDL receptor binding site as expressed in intact LDL, but oxidation appears to change the conformation of this peptide such that it becomes targeted by autoantibodies. The question then arises of how these autoantibodies have been generated and what is their in vivo target. The fact that some of these autoantibodies are of IgG type demonstrates the involvement of specific, T-cell-dependent adaptive immunity. Because the autoantibody levels were significantly associated with the levels of oxidized LDL in the circulation, one possibility is that they were generated in response to exposure of breakdown fragments of apoB-100. Such fragments could potentially undergo sufficient conformational changes to escape self-tolerance and generate antibodies that would only react with oxidized LDL particles. This notion is in agreement with previous studies demonstrating that copper oxidation, but not MDA modification, of LDL is associated with the fragmentation of apoB (23). MDA modification of these altered peptide fragments may then further enhance their immunogenicity, resulting in the generation of autoantibodies that specifically recognize the MDA-modified and structurally altered peptide sequence.

The pathophysiological role of these autoantibodies remains to be elucidated. They did not predict the risk for the development of acute cardiovascular events in this study. In univariate analyses, low levels of IgG against the MDA-modified binding site sequence were significantly associated with a thicker carotid IMT, but this relation was weakened after adjustment for conventional cardiovascular risk factors. Together, these observations do not support a major role for LDL receptor binding site autoantibodies in the development of disease. However, the inverse association between IgG autoantibodies against the LDL receptor binding site and oxidized LDL in plasma suggests that they may play a role in the clearance of oxidized LDL. Inverse associations between circulating oxidized LDL and IgM against MDA-modified peptides outside of the LDL receptor binding site have been reported previously (13). The observation by Binder et al. (24) that immunization of LDL receptor-deficient mice with pneumococcal antigens that through molecular mimicry induce the expression of IgM against oxidized LDL phospholipids results in decreased levels of oxidized LDL in plasma further supports the notion that antibodies against oxidized LDL antigens may promote plasma clearance.

In this study, we found no associations between autoantibodies against the LDL receptor binding site peptide and total or LDL cholesterol levels, suggesting that they do not have any significant influence on the metabolism of apoB-100. This may not be surprising in view of the poor affinity of these antibodies for the binding site, as expressed in intact LDL. However, the population investigated in this study was relatively small and selected on the basis of risk for the development of acute cardiovascular events rather than for lipoprotein phenotype. Accord-

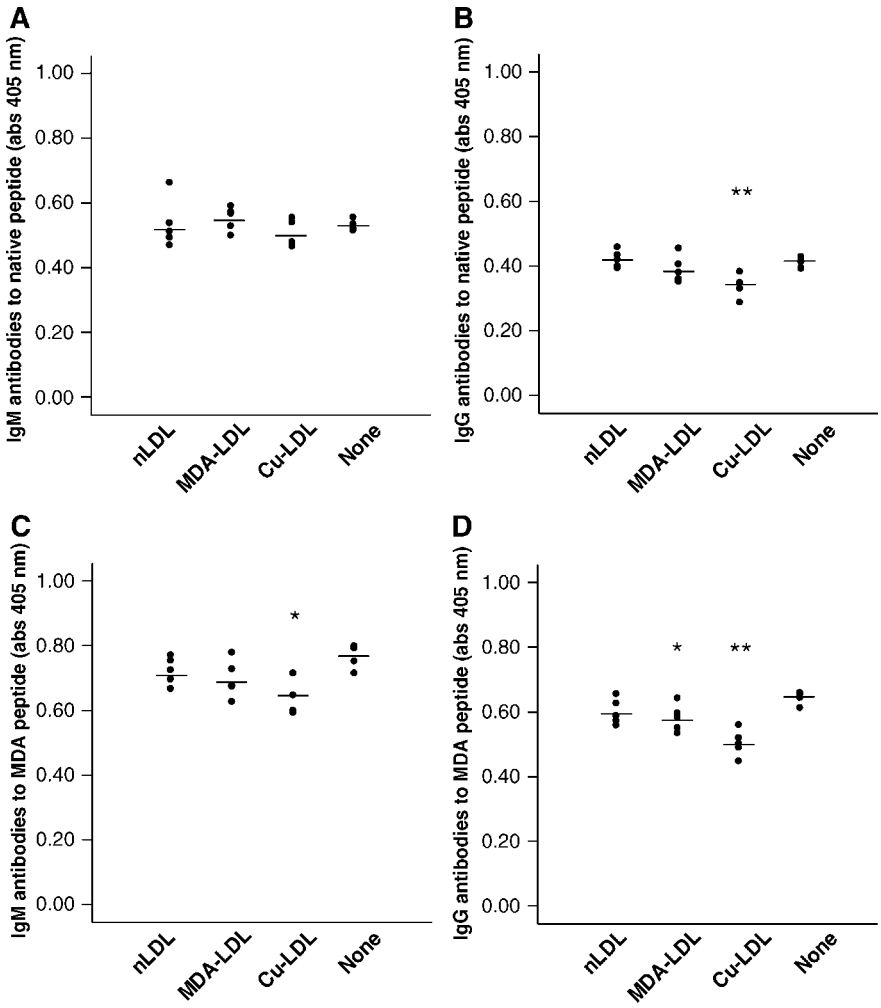

Fig. 3. Specificity tests with native LDL (nLDL), MDA-LDL, or copper-oxidized LDL (Cu-LDL). Levels of IgM and IgG recognizing the unmodified (A, B, respectively) or MDA-modified (C, D, respectively) LDL receptor binding peptide after preincubation of plasma with native LDL, MDA-LDL, or copper-oxidized LDL are shown. As a control, buffer was used for the preincubation (none). * $P < 0.05$, ** $P < 0.01$ versus none (n = 5 per group). abs, absorbance.

ingly, it cannot be excluded that autoantibodies against the LDL receptor binding site may influence lipoprotein metabolism in some individuals. This possibility will require further investigation.

In summary, our findings show that autoantibodies recognizing the unmodified and MDA-modified peptide corresponding to the LDL receptor binding site in apoB-100 are commonly expressed in humans. IgG recognizing the synthetic binding site peptide binds poorly to the corresponding sequence, as expressed in intact LDL, but oxidation appears to change the conformation of the binding site in a way that allows antibody binding. The inverse association with oxidized LDL in plasma also suggests that they may have a role in the clearance of these particles. However, it remains to be determined whether LDL receptor binding site autoantibodies play any significant role in cholesterol homeostasis and the development of atherosclerosis.

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