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Autoantibody against oxidised LDL and progression of carotid atherosclerosis

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Abstract

Oxidative modification of LDL renders it immunogenic and autoantibodies to epitopes of oxidised LDL, such as malondialdehyde (MDA)-lysine, are found in serum and recognise material in atheromatous tissue. However, there has been no prospective study to assess the importance of oxidised LDL among patients with vascular disease.

We compared the titre of autoantibodies to MDA-modified LDL and native LDL in baseline serum samples of 30 eastern Finnish men with accelerated two-year progression of carotid atherosclerosis and 30 age-matched controls without progression. Neither group had specific antibody binding to native LDL. A titre was defined as a ratio of antibody binding to MDA-LDL/binding to native LDL. Cases had a significantly higher titre to MDA-LDL (2.67 vs 2.06, $p=0.003$). Cases also had a greater proportion of smokers (37% vs 3%), higher LDL cholesterol (4.2 mmol/l vs 3.6 mmol/l), and higher serum copper concentration (1.14 mg/l vs 1.04 mg/l). Even after adjusting for these variables and the severity of baseline atherosclerosis, the difference in antibody titre remained significant in a multifactorial logistic model ($p=0.031$).

Thus, the titre of autoantibodies to MDA-LDL was an independent predictor of the progression of carotid atherosclerosis in these Finnish men. Our data provide further support for a role of oxidatively modified LDL in atherogenesis.

Introduction

Data now support the notion that oxidative modification of low-density lipoprotein (LDL)^{1,2} takes place in vivo: antibodies against epitopes of oxidised LDL recognize material in atherosclerotic lesions of rabbit and man, but not in normal arteries³⁻⁵ LDL extracted from human and atherosclerotic lesions contains nearly all of the physicochemical and

immunological properties of in-vitro oxidised LDL⁶⁻⁸ autoantibodies to epitopes of oxidised LDL exist in human serum⁴ immunoglobulins specific for oxidised LDL are present in atherosclerotic lesions of rabbits and man⁸ and antioxidant drugs, such as probucol, can inhibit atherosclerosis in hypercholesterolaemic rabbits.⁹

We^{4,10} and others¹¹ have reported that autoantibodies against oxidised LDL exist in serum of patients with coronary artery disease and controls. However, there have been no cross-sectional or prospective studies among patients with atherosclerosis. In this study, we investigated the association between autoantibodies to epitopes of oxidised LDL and progression of carotid atherosclerosis by assessing titres of autoantibodies in baseline serum samples drawn two years before subsequent follow-up. Our results show that the titre of autoantibodies against a prominent epitope of oxidised LDL-namely, malondialdehyde (MDA)-lysine, was an independent predictor of progression of carotid atherosclerosis in Finnish men.

Materials and methods

Study design

We conducted a nested case-control study, in which both cases and controls were selected from the same population-based cohort. The Kuopio ischaemic heart disease risk factor study (KIHD) is a population study of risk factors for extracoronary atherosclerosis.¹² The study sample consisted of a randomly selected third of men living in the city of Kuopio, Eastern Finland, and five neighbouring rural communities, aged 42, 48, 54, or 60 years at examination. A total of 2682 men (82.9% of those invited and eligible) were examined between 1984 and 1989. The details of the examination have been described elsewhere.^{12,13}

A subsample of 146 men examined in the KIHD in 1987 were invited to a two-year reassessment of carotid atherosclerosis in 1989. Of these men, 128 were examined. The mean age at baseline was 54.0 years. Other details of this two-year cohort have been presented elsewhere.¹⁴ None of the men had either symptomatic cerebrovascular disease or were taking lipid-lowering drugs. For this study, 30 men with the greatest and 30 men with the smallest increase during two years in the intima-media thickness of the common carotid artery were specifically selected by age stratification from the larger cohort to be cases and controls. An equal number of cases and controls were taken from each of the four age groups: 2 men aged 42, 7 men aged 48, 9 men aged 54, and 12 men aged 60 years at baseline. Controls were not paired with cases; no other selection criteria were used.

Assessment of atherosclerosis

Details of ultrasound scanning of carotid arteries have been presented elsewhere.¹³ The ATL UM 4 Duplex Ultrasound System (Advanced Technology Laboratories, Bothel, Washington, USA), with a mechanical sector transducer and a 10 MHz transducer frequency in B-mode and 5 MHz in pulsed doppler-mode, was used. The intima-media thickness was measured from videorecordings of the scanings by the scanning physician. The baseline and follow-up videotapes were read in random order. The reader was blinded to all risk factor values and, when she was reading the follow-up tapes, to the baseline intima-media thickness values. Three measurements of intima media thickness were completed of the far wall of both the right and left common carotid artery, at the site of the greatest intima-media thickness in each recording. The mean of these six thicknesses was used in the present study. Interobserver and intraobserver variabilities of these measurements have been described elsewhere.^{15,16} In addition, the same observer repeated all intima media thickness measurements for the study subjects from videorecordings. She was blinded to the originally measured values and all risk factor values. The two-year increase of carotid intima-media thickness ranged from 0.05 mm to 1.00 mm among cases and from about 0.08 mm to 0.03 mm among controls. Thus, on the basis of the remeasurements, all cases and controls were classified identically as in the original categorisation.

Other measurements

All risk factor measurements were done in the 1987 baseline examination. Fasted blood samples were drawn on the same day as the ultrasound scan after an abstinence from smoking of 12 h and from alcohol of three days. The subjects rested in supine position for 30 min before uncuffed blood sampling. The main lipoprotein fractions (VLDL, LDL, and HDL) were separated weekly from unfrozen serum samples by ultracentrifugation and precipitation methods. The cholesterol content of all lipoprotein fractions was measured enzymatically (CHOD-PAP method, Boehringer Mannheim); the between-batch coefficient of variation during 1987-88 was 2.2% for total cholesterol and 5.2% for low-density lipoprotein cholesterol (n = 76).

Serum copper and selenium concentrations were measured by atomic absorption spectrometry. Serum samples had been kept frozen at -20°C for approximately two years before analysis. A Seronorm control serum was included in all daily batches. For copper, a Perkin-Elmer (Norwalk, CT, USA) 306 atomic absorption spectrometer with flame technique was used against standards made in 5% glycerol. The between-batch coefficient of variation was 4.0% (n = 12). Selenium concentrations were measured with a Perkin-Elmer 5000 atomic absorption spectrophotometer by the graphite furnace technique with Zeeman background correction. The between-batch coefficient of variation was 5.1 % (n = 20). Plasma ascorbic acid concentrations were determined daily from unfrozen samples by high-

performance liquid chromatography.¹⁷ White cell counts were measured with the Coulter Model Dn leucocyte counter. Cigarettes, cigar, and pipe tobacco consumption, history of myocardial infarction, angina pectoris, and other ischaemic heart disease, as well as hypertension and anti-hypertensive medication, were recorded by questionnaire, which was checked by an interviewer. The medical history was also cross-checked by a physician.

Autoantibody titres were measured by solid-phase radioimmunoassay techniques.^{4,18} For these assays, 96-well polyvinylchloride microtitre plates (Dynatech Lab, Chantilly, VA) were coated with 50 μ l antigen (5 μ g/ml) in phosphate-buffered saline (PBS) for 2 h at 37°C. To prevent oxidation of native LDL, PBS contained 0.27 mmol/l edetic acid and 20 μ mol/l butylated hydroxytoluene. LDL was incubated in wells for 16 h at 4°C.¹⁸ Each well was washed four times with PBS (containing 0.02% NaN₃, 0.05% Tween 20, and 0.001% aprotinin) by microtitre plate washer (BioRad Model 1550, New York, USA). The remaining binding sites were then blocked by incubation with 2% bovine-serum albumin (BSA) in PBS for 2 h at room temperature. Wells were aspirated and washed four times as described above. One set of plates was prepared, which contained only the post-coat (BSA) and no added antigen.

Antigens for this assay included native LDL (protected from oxidation as noted above) prepared from the pooled plasma of ten donors.¹⁸ Because MDA-lysine is a prominent epitope of oxidised LDL that is found in atherosclerotic lesions,¹⁸ we also prepared MDA-LDL as an antigen. LDL contains about 360 moles of lysine per mole apo B and, since we combined 60%-70% of lysine residues with MDA,¹⁸ MDA-LDL was an antigen with a high density of the MDA-lysine epitope. We also prepared oxidised LDL by incubating LDL with Cu²⁺ for 18 h.¹⁸

In preliminary studies to determine the titre of autoantibody, we found that the antibody binding dilution curves of selected sera were parallel over several dilutions tested. Therefore, for these studies 1 in 8 dilution of serum from each subject was prepared and 50 μ l added in duplicate to wells coated with native LDL, oxidised LDL, MDA-LDL, or post-coat only. After overnight incubation at 4°C, wells were aspirated and washed four times before an appropriate iodinated monoclonal antibody specific for IgG, IgA, or IgM was added. Monoclonal antibodies were obtained from Zyrned laboratories (South San Francisco, California, USA) and iodinated with lactoperoxidase (Enzymobeads, BioRad, Richmond, California, USA) to yield an average specific activity of 10⁴ counts/min per ng.¹⁸ Each monoclonal was diluted with buffer and approximately 4 x 10⁵ counts/min were added to each well. Plates were incubated for 4 h at 4°C, each well was aspirated and washed four times, and the amount of bound radioactivity was counted in a gamma counter (Model 1282, LKB, Stockholm, Sweden).

To calculate antibody titres, we determined the absolute counts/min of bound anti-IgG, anti-IgA, or anti-IgM second antibody for each serum added to the different plated antigens and postcoat wells. We have previously defined a titre as the reciprocal of the greatest dilution of serum that gave binding twice that of pre-immune controls. In this study, there was no pre-immune control. We adopted binding to native LDL as the non-specific control and defined a titre as the ratio of binding to modified LDL to that of native LDL. In a preliminary analysis, we determined that the mean (SD) antibody titre to native LDL (ie, binding to native LDL/binding to post-coat only) was not different between cases and controls [1.40 (0.660 vs 1.26 (0.32), $p = 0.26$]. We have presented data in the text as the absolute value for the ratio of antibody binding to modified LDL/native LDL.

Statistical methods

Our statistical analysis concerned associations of antibody titres assayed from baseline serum samples and risk factors measured before follow-up with the probability of being a case – i.e., having experienced accelerated progression of carotid atherosclerosis during two-year follow-up. The statistical significance of differences between cases and controls was tested with logistic modelling. The logistic regression procedures of 'SAS' statistical software for the Vax 11/785 computer were used. The models were fitted both with antibody titres as single independent variables and with covariates. These were chosen on the basis of relative differences in group means between cases and controls.

Results

The mean two-year increase of carotid intima-media thickness was 0.26 mm (range 0.06 to 0.73 mm) among cases and -0.004 mm (range -0.05 to 0.02 mm) among controls (table I). Cases had 12% greater average carotid intimamedia thickness at baseline, but this difference was not statistically significant. The mean, minimum, and maximum values of the standard risk factors for atherosclerosis in the cases and the controls are shown in table I. The greatest differences between cases and controls were in the proportion of current smokers, serum LDL cholesterol, copper, and selenium concentrations. Cases and controls did not differ in either blood pressure or serum HDL-cholesterol concentration.

The mean (SD) of IgG autoantibodies to MDA-modified LDL was 2.67 (0.75) in cases and 2.06 (0.61) in controls ($p = 0.001$, t-test; $p = 0.003$, univariate logistic model; table I). There were no statistically significant differences in titres of either IgA or IgM autoantibody to MDA-LDL, although there was a trend towards a greater titre in cases for IgA to MDA-LDL (1.98 [0.44] vs 1.79 [0.54], $p = 0.159$).

There was no significant difference in titre of autoantibodies to copper-oxidised LDL for any immunoglobulin.

TABLE I - BASELINE CHARACTERISTICS AND TWO-YEAR CHANGE IN CAROTID INTIMA-MEDIA THICKNESS IN CASES AND CONTROLS

	Cases			Controls			P
	Mean	Minimum	Maximum	Mean	Minimum	Maximum	
Age (yr)	54.4	42.2	60.3	54.4	42.2	60.3	0.993
Cigarettes smoked daily*	7.3	0-0	4-0-0	0.0	0.0	0.0	0.002
Serum LDL cholesterol (mmol/l)	4.2	2-20	6-88	3.6	2.19	5.55	0.020
Serum copper (rng/l)	1-14	0.88	1.71	1.04	0.81	1.30	0.030
Serum selenium (µg/l)	112	86	135	119	79	144	0.040
Plasma vitamin C (mg/l)	6-6	0.3	14.2	8.7	0.7	16.7	0.053
Baseline carotid IMT (mm)	1.03	0.67	1-4-0	0.92	0.62	1.50	0.058
Body-mass index (kg/m ²)	27-2	22.7	34-0	26.5	20.9	35.6	0.373
Serum HDL cholesterol (mmol/l)	1-30	0.92	1.76	1.34	0.82	2.39	0.613
2-yr increase in IMT (mm)	0.26	0.06	0.73	-0.004	-0.05	0.02	<0.001
Antibody titre to MDA-LDL #	2.67	1-42	4.69	2.06	1.04	3.95	0.001

* Overall proportion of smokers: 37% cases vs 3% controls (p < 0.001).

Ratio of binding of antibody to MDA-LDL/binding to native LDL. IMT = intima-media thickness.

The antibody titre to MDA-LDL correlated significantly (table II) with the number of cigarettes smoked daily ($r = 0.35$, $p = 0.006$), with blood haemoglobin ($r = 0.27$, $p = 0.037$), and with serum copper concentration ($r = 0.26$, $p = 0.046$). There was no significant correlation with age, blood pressure, serum LDL cholesterol, plasma ascorbate, or serum selenium concentration. The two-year increase in carotid intima-media thickness had the strongest correlations (table II) with the number of cigarettes smoked daily ($r = 0.52$, $p < 0.001$), titre of antibodies to MDALDL ($r = 0.41$, $p = 0.001$), serum LDL-cholesterol concentration ($r = 0.37$, $p = 0.004$), and serum copper concentration ($r = 0.26$, $p = 0.045$). The association between the MDA-LDL antibody titre and the two-year increase in carotid intima-media thickness is shown in the figure.

TABLE II -CORRELATION OF ANTIBODY TITRE AND INTIMA-MEDIA THICKNESS WITH RISK FACTORS FOR PRO-OXIDANTS

	MDA-LDL antibody titre	2-year increase of carotid IMT
Cigarettes/day	0.35	0.52
Blood haemoglobin (g/l)	0.27	-0.10
Serum copper (mg/l)	0.26	0.26
Serum selenium (µg/l)	-0.22	-0.23
Serum LDL cholesterol (nmol/l)	0.10	0.37
MDA-LDL antibody titre		0.41

Correlation coefficients of 0.26 or higher are statistically significant at two-sided $p < 0.05$ (cases and controls pooled. $n = 60$).

Two-year increase in carotid intima-media thickness and autoantibody titre to MDA-LDL.

○ controls; ▲ cases.

The difference between cases and controls was also tested in a multivariate logistic model controlling for potential confounding factors - namely, variables that were associated with both the antibody titre and the increase in intima-media thickness (table III). Even in a

model allowing for smoking, serum LDL-cholesterol concentration, baseline carotid intima-media thickness, history of ischaemic heart disease, and serum copper concentration, the difference in MDA-LDL antibody titre between cases and controls was still significant ($p = 0.031$). Antibody titre, smoking, and serum LDL cholesterol had very similar standardised logistic regression coefficients (data not shown).

TABLE III - MULTIFACTORIAL LOGISTIC REGRESSION ANALYSIS OF MDA-LDL ANTIBODY TITRE AND RISK FACTORS WITH TWO-YEAR INCREASE IN CAROTID INTIMA_MEDIA THICKNESS

	Regression coefficient (SE)	Wald X^2	p
MDA-LDL antibody titre	1.26 (0.58)	4.68	0.031
Smoking (yes vs no)	2.87 (1.29)	4.98	0.026
Serum LDL cholesterol (mmol/l)	0.77 (0.43)	3.30	0.069
Baseline carotid IMT (mm)	1.79 (1.52)	1.38	0.240
History of IHD (yes vs no)	0.93 (0.94)	0.98	0.322
Serum copper (mg/l)	1.83 (2.24)	0.67	0.414
Wholmodel		21.25	<0.001

IHD, ischaemic heart disease

Discussion

We have shown that the titre of autoantibodies to MDA-modified LDL in baseline serum samples was an independent predictor of progression of carotid atherosclerosis over two years. This association was equally strong as that for smoking and serum LDL-cholesterol concentration. Part of the effect of MDA-LDL antibodies on atherosclerotic progression was explained by smoking and serum copper concentration, factors that may promote oxidation of LDL.^{2,19} The association persisted even after controlling for baseline severity of atherosclerosis; this may indicate that increased MDA-LDL antibody titres are associated with active atherogenesis rather than simply reflecting more advanced atherosclerotic lesions.

The titre of antibodies to MDA-modified LDL was associated positively with known promoters of lipid oxidation such as smoking, serum copper concentration, and haemoglobin, a measure of iron status. Antibody titres also had a weak inverse association with serum selenium, an indicator of antioxidative defence against lipid oxidation. In the cohort from which the cases and controls of the present study were drawn, increased serum LDL-cholesterol values were associated with accelerated atherosclerotic progression only in men with high serum copper concentrations.¹⁴ These observations support the importance of pro-oxidants and antioxidants in atherogenesis.

One qualification to our study of autoantibodies against MDA-modified LDL is that these antibodies could recognise MDA-lysine epitopes on other MDA-modified proteins in either serum or tissue. In conditions of accelerated lipid peroxidation, not only LDL but also many other proteins are susceptible to oxidative modification and MDA-lysine adduct formation.²⁰

Extensively modified MDA-LDL was used in our study as a target antigen because the antigen density was high – i.e., the high number of MDA-lysine residues on a given LDL particle (about 200) provided a more sensitive assay to detect low amounts of autoantibody. By contrast, for copper-oxidised LDL there are far fewer MDA-lysine residues per LDL particle. Since MDA is one of the major end-products of lipid peroxidation,² the presence of autoantibodies against MDA modified proteins is, at the very least, an indicator of lipid peroxidation that took place in vivo before blood sampling.

We failed to find a significant difference in the titre of antibodies to the copper oxidised LDL antigen in cases, possibly reflecting a low density of immunodominant epitopes of oxidised LDL-eliciting autoantibodies. We have seen great variability in the apparent titre of antibodies from the same serum sample when different oxidised LDL preparations were used, presumably because of the presence of different concentrations of epitopes in different preparations. Other researchers have previously reported increased autoantibody binding to oxidised LDL in patients with coronary artery disease, although patient populations were poorly defined.⁹ We did not find significant antibody binding to native LDL in either controls or cases, in contrast to a recent report in patients with coronary disease.²²

The titre of circulating autoantibodies reflects a balance between the amount of antibody generated and released into the circulation and the consumption of that antibody, either specifically (e.g., by binding to specific antigens in the atherosclerotic lesion) or non-specifically. In addition, antibody production is under genetic control. Several factors may therefore affect serum antibody titres. Nevertheless, by establishing an association in a random population cohort between baseline values of serum autoantibodies against MDA-modified LDL and carotid artery atherosclerotic progression, our data provide the first prospective evidence in humans for an in-vivo role of lipid peroxidation in atherogenesis. Although our study concerns early atherosclerotic progression, the role of lipid peroxidation in the formation of more complicated atherosclerotic plaques is unknown.

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