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Oxidized LDL Autoantibodies Are Related to Apolipoprotein E Phenotype, Independently of Postprandial Change in Plasma Triglycerides and LDL Size, among Patients with Angiographically Verified Coronary Artery Disease and Healthy Controls

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Key Words
Oxidized low-density lipoprotein · Apolipoprotein E · Triglyceride · Low-density lipoprotein size · Atherosclerosis

Abstract
Objective: Oxidized low-density lipoprotein (LDL) autoantibodies (oxLDLab), apolipoprotein E (apoE) phenotype, postprandial triglyceride changes and LDL size are suggested to be risk factors for coronary artery disease (CAD). Our aim was to study the interaction between these new risk factors among patients with CAD and healthy controls. ***Methods:*** oxLDLab from 31 men with angiographically verified CAD and 31 healthy men were analyzed by enzyme-linked immunosorbent assay. Isoelectric focusing and immunoblotting were used for apoE phenotyping. Triglyceride level was measured after 12 h of fasting and 3, 5 and 7 h after a high-fat meal. Nondenaturing gradient gel electrophoresis was used to separate LDL particles according to size. ***Results:*** oxLDLab levels increased according to apoE phenotype in the following order: E2 < E3 < E4 ($p = 0.004$, ANOVA). The postprandial response of triglycerides, the size of LDL particles and the concentration of LDL and high-density lipoprotein (HDL) cholesterol did not differ between apoE phenotypes, and the use of these variables as covariates did not change the statistically significant difference in oxLDLab levels between apoE phenotypes ($p = 0.01$, ANCOVA). oxLDLab levels did not differ between the patient and control groups. ***Conclusion:*** We found an association between apoE allele ε2 and decreased levels of oxLDLab, which was independent of the postprandial response of triglycerides, the size of LDL particles and plasma LDL and HDL cholesterol levels. The mechanism by which apoE affects oxidation of LDL remains unknown.

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Oxidized low-density lipoprotein (LDL) is present in the lesions of atherosclerosis in humans. Oxidative modification of LDL is believed to occur mostly in the subendothelial space, where the antioxidant defense is supposedly less effective and arterial wall cells can oxidize LDL [37]. The level of oxidized LDL autoantibodies (oxLDLab) has been suggested to reflect in vivo oxidation of LDL, since such modification of LDL results in the appearance of new epitopes that render LDL more antigenic [30].

oxLDLab, postprandial triglyceridemia, small dense LDL particles and apolipoprotein E (apoE) phenotype have been associated with the occurrence of coronary artery disease (CAD) [1, 4, 19, 34]. According to former literature, there might be an interaction between these new risk factors for CAD and the pathogenesis of atherosclerosis [1, 6, 14]. Namely, postprandial triglyceridemia, small dense LDL particles and apoE phenotype have all been associated with LDL oxidation [17, 19]. Small dense LDL is more susceptible to in vitro oxidation [2, 23] and associates with higher levels of IgM antibodies against malondialdehyde-modified LDL than large LDL [17]. The small size of triglyceride-enriched LDL enables it to infiltrate easily into the artery wall where it can be oxidized [19]. Serum lipid peroxide levels have also been reported to be higher in carriers of apoE allele ε4 than in carriers of other apoE alleles [35]. Furthermore, elevated postprandial triglyceride levels and small LDL particle size have been associated with both apoE alleles ε4 [6, 14] and ε2 [5, 7], although some studies did not find any association between apoE phenotype and LDL size [27, 38].

The aim of our study was to clarify the possible interaction between oxLDLab, apoE phenotype, the postprandial response of triglycerides and the size of LDL particles among patients with angiographically verified CAD and healthy controls.

Methods

Participants

The study group consisted of 31 men with stable CAD (age 45–69 years) and 31 healthy men (age 36–63 years). The patients with CAD were treated at the Central Hospital of Kanta-Häme in 1993–1994, and their CAD was verified by coronary angiography during the previous 36 months at the Tampere University Hospital. The controls were outpatients of the orthopedic clinic and medical personnel of the Central Hospital of Kanta-Häme. The controls had no symptoms of angina pectoris or other forms of atherosclerosis and no changes typical for myocardial ischemia on electrocardiogram. Each subject was questioned with regard to smoking habits, hypertension, diabetes mellitus, the family history of CAD and the usage of medication. Exclusion criteria were heavy drinking, liver disease, diabetes mellitus and a concentration of plasma cholesterol over 8 mmol/l or plasma triglycerides less than 4 mmol/l. All subjects gave written informed consent after explanation of the purpose and the nature of the study. The research was carried out in accordance with the Declaration of Helsinki (1989) of the World Medical Association, and the study protocol was approved by the Ethics Committee of the Tampere University Hospital and the Central Hospital of Kanta-Häme.

Blood Samples

The postprandial test was performed before starting medication affecting lipid metabolism and at least 6 months after myocardial infarction or major operations. After a 12-hour fast, blood samples were collected for baseline values of total cholesterol, high-density lipoprotein (HDL) cholesterol and triglycerides. At the same time, blood for determination of apoE phenotype, LDL size and oxLDLab was collected. Immediately after collection of baseline blood samples, the subjects consumed a high-fat meal, which consisted of 3.5 ml of cream (fat concentration 38 g/100 ml) per kg body weight and 45 g of glucose [27]. During the following 7 h, the subjects did not eat any food and drank only water. Blood samples for triglyceride measurements were taken 3, 5 and 7 h after the high-fat meal.

Lipid Analyses and Determination of LDL Size

The lipids were determined by enzymatic methods using reagents from Boehringer-Mannheim (Germany). LDL cholesterol was calculated according to the method of Friedewald et al. [10]. Nondenaturing gradient gel electrophoresis, which separates LDL particles by size, was used to identify distinct subpopulations of LDL particles, as described previously [27]. To determine the size of LDL, a mixture of globular proteins of known diameters, such as thyroglobulin dimer (23.6 nm), thyroglobulin (17 nm) and ferritin (12.2 nm), was run on each gel.

ApoE Phenotyping

ApoE phenotyping was performed using delipidated plasma, cysteamine treatment, isoelectric focusing and immunoblotting as described by Lehtimäki et al. [21].

Enzyme-Linked Immunosorbent Assay for Antibodies against Oxidized LDL

Plasma was separated by centrifugation (2,000 rpm, 20 min) and frozen (–20 °C) for up to 2 years until analyzed. oxLDLab levels were determined as previously described in detail [20]. In short, antigens for this assay included (1) native LDL prepared from the pooled plasma of 10 donors and protected against oxidation by 0.27 mmol/l EDTA and 20 μmol/l butylated hydroxytoluene (BHT) in phosphate-buffered saline (PBS), and (2) oxidized LDL obtained after 24 h of oxidation of native LDL with 2 μmol/l $CuSO_4$.

For enzyme-linked immunosorbent assay, half of the wells on a polystyrene plate (Nunc, Roskilde, Denmark) were coated with 50 μl of native LDL and the other half with 50 μl of copper-oxidized LDL antigen (both at a concentration of 5 μg/ml) in PBS for 16 h at 4 °C. After removal of the unbound antigen and washing of the wells, the remaining nonspecific binding sites were saturated using 2% human serum albumin in PBS and 20 μmol/l BHT for 2 h at 4 °C. After washing, 50 μl of the serum samples, diluted 1:50, were

added to wells coated with native LDL and oxidized LDL and incubated overnight at 4°C. After incubation, the wells were aspirated and washed six times before an IgG-peroxidase-conjugated rabbit anti-human monoclonal antibody (No. 55220 Cappel, Organon, USA), diluted 1:4,000 (vol/vol) in buffer (0.27 mmol/l PBS, 20 μmol/l EDTA, 1% BHT, 0.05% Tween human serum albumin), was added to each well for 4 h at 4°C. After incubation and washing, 50 μl of freshly made substrate [0.4 mg/ml *o*-phenylenediamine (Sigma) and 0.045% H_2O_2 in 100 mmol/l acetate buffer, pH 5.0] was added and incubated for exactly 5 min at room temperature. The enzyme reaction was terminated by adding 50 μl of 2 *M* H_2SO_4. Optical density (OD) was measured at 492 nm using a microplate reader (Multiskan MCC/340, Labsystems GmbH, Munich, Germany). All measurements were done in a blinded fashion and on coded serum samples. The results were expressed as the mean OD values from duplicate determinations. The level of anti-oxidized LDL antibody reactivity (oxLDLab) was calculated by subtracting the binding of antibodies to native LDL from that to oxidized LDL in order to reduce the possibility of obtaining false-positive values due to cross-reactivity with both LDL epitopes. The intraassay coefficient of variation for oxLDLab was 8.5%.

Statistical Methods

Normally distributed data are expressed as mean ± SD and skewed distributed data as median (range). Skewed distributed data, such as oxLDLab level and postprandial change in triglycerides, were analyzed in logarithmically transformed form, but the results are expressed in crude form. Differences between the patients with CAD and controls for numerical variables were compared with a t test. Differences between the patients with CAD and controls for categorical variables were tested with a χ^2 test. Associations between the classic and new risk factors of CAD were estimated with Spearman's rank correlation coefficients. ApoE phenotype groups were designated as E2 (E2/2 and E3/2), E3 (E3/3) and E4 (E4/3 and E4/4). Differences in the levels of oxLDLab and lipids between the apoE phenotype groups were compared with analysis of variance (ANOVA). We also analyzed the difference in oxLDLab levels between the different apoE phenotypes with ANOVA by using postprandial change in triglycerides (triglycerides 5 h postprandially minus fasting triglycerides), LDL particle size and LDL and HDL cholesterol as covariates (ANCOVA). A p value less than 0.05 was considered statistically significant. Statistical analysis was performed using Statistica for Windows Version 5.0 (Statsoft Inc., Tulsa, Okla., USA).

Results

The clinical characteristics of the patients with CAD and controls are presented in table 1. Mean oxLDLab level did not differ statistically significantly between the

Table 1. The clinical characteristics and oxLDLab levels of CAD patients and controls

	Patients (n = 31)	Controls (n = 31)	All (n = 62)	p value
Age, years	57±6	50±7	54±7	<0.001
BMI,kg/m^2	26.3±2.5	25.4±3.3	25.9±2.9	0.258
Total cholesterol, mmol/l	5.7±0.9	6.0±1.3	5.9±1.1	0.241
LDL cholesterol, mmol/l	3.8±0.8	4.0±1.3	3.9±1.1	0.45
HDL cholesterol, mmol/l	1.1±0.3	1.4±0.4	1.3±0.3	<0.001
Systolic BP, mm Hg	137±23	135±16	136±19	0.81
Diastolic BP, mm Hg	85±9	86±8	86±8	0.91
LDL size, nm	25.9±0.7	26.2±0.6	26.1±0.7	0.04
Triglycerides, mmol/l Range	1.60 1.00–3.60	1.19 0.50–3.02	1.39 0.50–3.60	0.007
oxLDLab, OD Range	0.073 0.0001–0.231	0.113 0.0001–0.263	0.082 0.0001–0.263	0.66
Smokers, %	23 (7)	10 (3)	16 (10)	0.20
Hypertension, %	32 (10)	6 (2)	19 (12)	0.01
Use of β-blockers, %	65 (20)	3 (1)	34 (21)	<0.001
Use of aspirin, %	77 (24)	0 (0)	39 (24)	<0.001

Figures in parentheses represent numbers of patients. BP = Blood pressure.

Table 2. Serum lipids, LDL size and oxLDLab level according to apoE phenotypes

	ApoE2 (n = 8)	ApoE3 (n = 41)	ApoE4 (n = 13)	p value
Total cholesterol, mmol/l	5.5 ± 0.9	6.0 ± 1.2	5.8 ± 1.1	0.51
LDL cholesterol, mmol/l	3.3 ± 0.9	4.1 ± 1.0	4.0 ± 1.1	0.14
HDL cholesterol, mmol/l	1.4 ± 0.4	1.2 ± 0.4	1.2 ± 0.2	0.40
LDL size, nm	26.1 ± 0.9	26.0 ± 0.6	26.1 ± 0.7	0.97
Change in triglycerides, mmol/l	1.46	1.23	0.88	0.61
Range	0.12–3.77	–0.16 to 5.63	0.25–3.84	
oxLDLab, OD	0.040	0.079	0.116	0.004
Range	0.0001–0.189	0.0001–0.231	0.029–0.263	

Change in triglycerides = triglycerides 5 h postprandially minus fasting triglycerides.

patients with CAD and the controls (p = 0.66, t test). The frequencies of apoE phenotypes among the patients with CAD and controls were as follows: apoE2, 3 (10%) versus 5 (16%); apoE3, 22 (71%) versus 19 (61%), and apoE4, 6 (19%) versus 7 (23%). The distribution of apoE phenotypes did not differ between the patients with CAD and controls (p = 0.72, χ^2 test). The patients with CAD were statistically significantly older (p < 0.001, t test) and had lower HDL cholesterol levels (p < 0.001, t test), smaller LDL particles (p = 0.04, t test) and higher triglyceride levels (p = 0.007, t test) than controls (table 1). The prevalence of hypertension and the usage of β-blockers and aspirin were higher among the patients with CAD than controls (p = 0.01, p < 0.001 and p < 0.001, respectively, with χ^2 test).

The concentrations of plasma oxLDLab and lipids according to different apoE phenotype groups in the whole population are presented in table 2. The subjects in the apoE2 phenotype group (n = 8) had statistically significantly lower oxLDLab levels than the subjects in the apoE3 (n = 41) and apoE4 (n = 13) phenotype groups (p = 0.004, ANOVA). Using mean LDL cholesterol, HDL cholesterol, postprandial change in triglycerides and LDL size as covariates did not change the statistically significant difference in oxLDLab levels between the apoE phenotypes (p = 0.01, ANCOVA).

When studied separately in the controls, the difference in oxLDLab levels between the apoE phenotypes was clear [the median oxLDLab levels (measured as OD) in the apoE phenotype groups were: apoE2, 0.0001; apoE3, 0.117, and apoE4, 0.116; p < 0.001, ANOVA]. However, in the patients with CAD, the difference in oxLDLab levels between the apoE phenotypes did not reach statistical significance [the median oxLDLab levels (measured as OD) in the apoE phenotype groups were: apoE2, 0.081; apoE3, 0.073, and apoE4, 0.097; p = 0.73, ANOVA].

Correlation coefficients between the classic and new risk factors for CAD are presented in table 3. There was no correlation between oxLDLab level and age, BMI, plasma total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, blood pressure or LDL size (table 3, p > 0.05). oxLDLab levels did not differ between subjects with and without hypertension (p = 0.53, t test), smokers and nonsmokers (p = 0.59, t test) or users and nonusers of β-blockers (p = 0.31, t test) and aspirin (p = 0.44, t test).

Discussion

The oxidation of LDL and the subsequent production of antibodies against oxidized LDL are believed to be useful in identifying those at high risk for atherosclerosis. However, the results of previous studies are obviously discrepant. Some studies indicate that oxLDLab level correlates positively with the occurrence and severity of CAD [3, 9, 13, 31], while others show a negative correlation between atherosclerosis and oxLDLab level [22, 28, 33]. Our findings are consistent with those reports where no difference in the level of oxLDLab between patients with CAD and controls could be shown [24, 29, 36]. In the present study, the patients with CAD were older, had a lower level of HDL cholesterol, smaller LDL particles, a higher level of triglycerides, a higher prevalence of hypertension and higher usage of β-blockers and aspirin

Table 3. Correlation between the classic and new risk factors for CAD in the whole study population

	BMI	LDL Chol	Total Chol	HDL Chol	Trigly-cerides	SBP	DBP	LDL size	oxLDLab
Age	0.09	0.26	0.17	−0.45*	0.29*	0.25	0.09	−0.26*	0.02
BMI		0.21	0.23	−0.31*	0.50*	0.23	0.35*	−0.26	0.12
LDL Chol			0.95*	0.00	0.09	0.03	0.30*	−0.17	0.04
Total Chol				0.20	0.22	0.08	0.31*	−0.15	−0.03
HDL Chol					−0.40*	−0.21	−0.16	0.52*	−0.18
Triglycerides						0.45*	0.30*	−0.56*	−0.06
SBP							0.65*	−0.23	0.08
DBP								−0.26	0.10
LDL size									−0.02

Chol = Cholesterol; SBP = systolic blood pressure; DBP = diastolic blood pressure. * $p < 0.05$ (statistically significant correlation between risk factors).

than the controls, which could have introduced bias. However, no associations between oxLDLab level and age, HDL cholesterol, LDL size, triglycerides, the prevalence of hypertension and the usage of β-blockers or aspirin were found. Our control group was a selection of healthy men without a history of CAD. Thus, participants with clinically nonmanifested CAD might have entered the control group. However, this would hardly explain the lack of difference in the oxLDLab level between the patients with CAD and the controls, because the prevalence of CAD in asymptomatic middle-aged subjects is only 4% [8].

In our earlier study, we found statistically significantly higher oxLDLab levels in the patients with CAD with stenosis in all three major vessels than that in the controls with no stenosis on coronary angiography by using the same method for measurement of oxidized LDL level as in the present study [20]. In the present study, the stable and mild stage of CAD might explain the lack of difference in oxLDLab level between the patients with CAD and healthy men. Thus, our results suggest that oxLDLab might be associated with atherosclerosis only at a late, accelerated stage of the disease. Atherogenesis progresses in a discontinuous fashion, and lipid oxidation product formation in the various stages of atherosclerosis is therefore likely to vary [30]. In addition, the presence of oxLDL ab reflects the capacity of the immune system to react to modified LDL [30]. The pathophysiological role of oxLDL ab may vary depending on the stage of atherosclerosis. According to experimental studies in immunomodulated mice, immune responses may protect from atherogenesis in early stages of the disease but result in accelerated atherosclerotic progression at later stages [12, 26]. Also, in humans, an inverse relationship between oxLDLab and carotid arterial intima-media thickness has been reported, suggesting that the immune response to oxidized LDL may even have a protective role at an early stage of human atherosclerosis [11, 16]. Furthermore, different epitopes of oxidized LDL may produce different kinds of antibodies, some of which might even protect from atherosclerosis [12].

The present study showed an association between apoE allele ε2 and decreased levels of oxLDLab. The relationship seemed to be independent of the plasma LDL cholesterol level, postprandial triglyceride response and LDL size. Interestingly, it has been proposed that isoforms of apoE possess antioxidant activity in vitro with an order of efficacy as follows: E2 > E3 > E4 [18, 25]. Plasma lipoproteins from apoE-deficient mice are more susceptible to in vitro oxidation than lipoproteins from wild-type mice [15]. Furthermore, serum lipid peroxide levels have been found to be higher in subjects with apoE allele ε4 [35], while apoE allele ε2 has been proposed to have an antioxidative effect [32]. In conclusion, apoE allele ε2 is associated with a decreased level of oxLDLab independently of plasma LDL and HDL cholesterol level, postprandial triglyceride response and LDL size, suggesting that apoE allele ε2 might have a favorable antioxidative effect.

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