Serum Level of IgG Autoantibodies Against Oxidized Low Density Lipoproteins and Lag-phase of Serum Oxidation in Coronary Heart Disease – Inverse Correlation

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Abstract. High affinity IgG autoantibodies (ABs) against oxLDLs and lag-phase of serum oxidation were tested in patients with coronary heart disease (CHD)

Fifty one (37M/14F) patients with CHD defined as Q-wave myocardial infarction and/or stenosis of more than 50% and 51 (34M/17F) healthy blood donors as controls participated in this study LDLs were isolated by gradient ultracentrifugation and oxidized with $CuSO_4$ The modified LDLs (oxLDLs) or native LDLs (nLDLs) were used as antigens in an enzyme immunoassay (ELISA) to detect IgG ABs in both groups The serum was oxidized by $CuSO_4$ and the oxidation was monitored spectrophotometrically at $\lambda = 234$ nm to follow the formation of conjugated diens The lag-phase (in minutes) is the interval between the addition of $CuSO_4$ to the serum and the beginning of extensive oxidation (increasing absorbance at 234 nm) The concentrations of total cholesterol, triglycerides, HDL-cholesterol, apo-A and apo-B were measured as well

The mean level of ABs against oxLDLs (expressed as optical density units) was 0 590 ± 0 330 in CHD-patients vs 0 244 ± 0 200 in controls ($p < 0\ 001$) The lag-phase in minutes was 47 00 ± 27 19 in CHD-patients and 80 23 ± 26 30 in controls ($p < 0\ 001$) A negative correlation between ABs levels and lag-phase was established in CHD-patients ($r = -0\ 69$, $p < 0\ 001$) and controls ($r = -0\ 62$, $p < 0\ 001$) A poor correlation was established between ABs levels or lag-phase, on one hand, and other measured parameters In conclusion, the lag-phase of serum oxidation by Cu^{2+} could be informative for LDL susceptibility to modification and the extent of consequent humoral immune response

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Indroduction

Oxidative modification of low density lipoproteins (LDLs) is a crucial step in atherogenesis (Lyons 1993; Segrest and Anantharamaiah 1994; Witztum 1994; Stephens et al. 1996). Oxidation of LDLs is a process of lipid peroxidation, and it is assumed that peroxidation products formed within LDL particles react with amino acid chains of apolipoprotein B (apo-B) to form new epitopes (Kayden and Traber 1993; Lyons 1993; Penn and Chisolm 1994). The oxidized LDLs (oxLDLs) are immunogenic and IgG autoantibodies against oxLDLs are detected in sera of patients with carotide atherosclerosis and coronary heart disease (CHD) (Palinski et al. 1989; Salonen et al. 1992; Heinecke 1994; Goudev et al. 1995). Such autoantibodies have a predictive value for further ischemic events. Predictors of the levels of IgG autoantibodies against oxLDLs are not clearly determined.

Observational studies showed a reduction in coronary events in people taking antioxidant vitamins (Gey et al. 1991; Esterbauer et al. 1992; Lyons 1993; Segrest and Anantharamaiah 1994; Ohrvall et al. 1996; Stephens et al. 1996). The molecular mechanism of this protection is prevention of LDL oxidation. Low serum antioxidant capacity is one of the discussed mechanisms for accelerated atherosclerosis (Esterbauer et al. 1992; Lyons 1993; Segrest and Anantharamaiah 1994; Witztum 1994; Stephens et al. 1996). It is accepted that the lag-phase of lipid peroxidation (LPO) induced *in vitro* by Cu^{2+} or diazocompounds is a good criterion for the assessment of the resistance of blood serum or LDL-suspension to LPO inducers as well as for the balance between prooxidants and antioxidants in these samples (Esterbauer et al. 1992; Rengstrom et al. 1992; Maggi et al. 1994).

The aim of this study was to establish the relationship between the serum level of IgG autoantibodies against oxLDLs and the lag-phase of serum oxidation by Cu^{2+} in CHD-patients and in clinically healthy blood donors.

Materials and Methods

Chemicals

All reagents of analytical grade were obtained from Aldrich Chem. Co., Henkel Co., Merck, Sigma Chem. Co.

Study design

A case-control study in 51 CHD-patients (case-group – 37 males and 14 females, age – 52.0 ± 10.5 years) diagnosed as Q-wave myocardial infarction and/or stenosis

of more than 50% from coronarography. None of the patients was on lipid-lowering therapy or antioxidant supplementation. The control group consisted of 51 clinically healthy blood donors (34 males and 17 females, age – 41.0 ± 6.7 years) free of CHD, hypertension, diabetes, renal or thyroid dysfunctions.

Oxidation of blood serum by Cu^{2+}

$CuSO_4$ was used as hydrophilic free radical initiator. One ml of blood serum was diluted in PBS (125 mmol/l NaCl:10 mmol/l sodium phosphate, pH 7.4) to yield 1 mg protein/ml, and was preincubated at 37 °C for 5 min. $CuSO_4$ (final concentration 46 mmol/l) was added to the incubation medium. The serum oxidation by Cu^{2+} was monitored spectrophotometrically at $\lambda = 234$ nm to follow the formation of conjugated diens in the serum. The lag-phase of the reaction was calculated as described in Fig. 1. The lag-phase in minutes is defined as the interval between the addition of $CuSO_4$ and the beginning of absorbance at 234 nm.

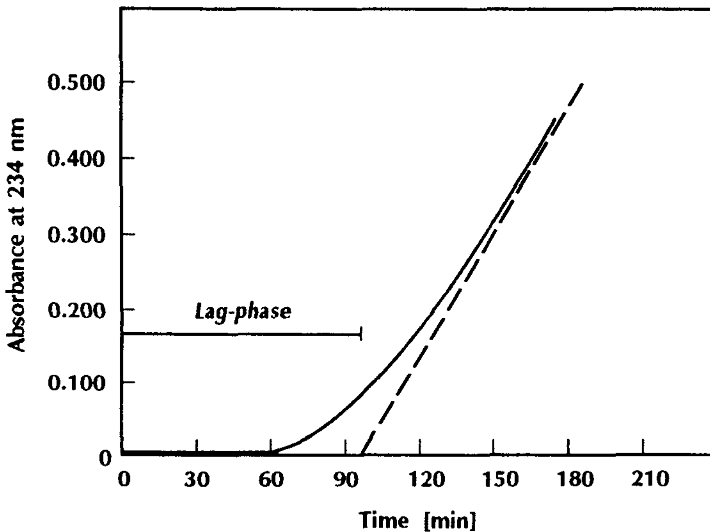

Figure 1. Estimation of the lag-phase of serum oxidation by Cu^{2+} The lag-phase in minutes is the interval between the addition of $CuSO_4$ to serum and the beginning of the absorbance at 234 nm (accumulation of conjugated diens in sera)

Isolation of LDLs

LDLs were isolated by ultracentrifugation in a Beckman L8 55 ultracentrifuge. Briefly, venous blood was taken from each person, after overnight fast, into Vacutainer tubes containing K-EDTA (1 mg/ml blood, final concentration). The plasma

was collected after centrifugation and was dialyzed against PBS (10 mmol/l, pH 7.4, 4°C, for 6 h). LDL fraction was isolated by ultracentrifugation in a density gradient (KBr). The solvent density interval used to purify LDLs was $d = 1.019 - 1.063$ g/ml. The isolated LDL fraction was dialysed extensively against PBS (10 mmol/l, pH 7.4, 4°C, for 6 h with 2 changes of the buffer). The purity of the LDL fraction was checked by electrophoresis. The isolated LDLs gave a single band on 1% agarose gel electrophoresis, and contained only intact apoprotein B. LDLs were used in experiments immediately after the isolation. LDLs were resuspended in PBS (10 mmol/l, pH 7.4).

Immunoenzyme assay (ELISA)

Polyvinylchloride microtitre 96-well plates were coated with 2 mg/ml nLDLs or oxLDLs by incubating them overnight at 4°C, and washed with PBS containing Tween 20 (T-PBS). Free binding sites were blocked with 10% inactivated calf serum in 0.5 mol/l sodium chloride for 1 hour at room temperature (RT). Human sera (diluted 1/100) were applied to each well and incubated for 2 h at RT. After extensive washings with T-PBS, anti-human IgG serum conjugated with peroxidase was added for 1 h at RT. Subsequently, the enzyme reaction was developed with 0.4 mg/ml *o*-phenylendiamine and 0.05% H_2O_2 for 20 min in the dark. The color reaction was stopped by adding 10% H_2SO_4 and the optical density at $\lambda = 492$ nm was read at microELISA reader (Dynatech Switzerland). It was demonstrated that autoantibodies against oxLDLs (Ab-oxLDLs) from patients with CHD react in a similar way with oxLDLs and MDA-lysine. LDLs isolated from human blood plasma and modified by treatment with MDA was used as the target antigen; MDA-lysine alone was the coating antigen in ELISA in our experiments (Palinski et al 1989). Levels of IgG autoantibodies are expressed as optical density (OD) units.

Other measurements

Total cholesterol, LDL-cholesterol and triglycerides were determined by enzymatic methods (COBAS-MIRA). HDL-cholesterol was determined by the phospho-tungstate-magnesium precipitation procedure. LDL-cholesterol was calculated by the Friedewald formula. Apo-B and apo-A1 were determined by immunoturbodimetry. Serum protein concentration was measured by the method of Lowry et al. (1951).

Statistical analysis

The results were expressed as mean ± SD. Differences between the groups were assessed by Dunnett's test. Statistical significance was assessed at $p < 0.05$. Spearmann's correlation method was used for the determination of correlation coefficients. All tests were performed using SPSS package.

Results

The results of laboratory serum tests on lipid metabolism are presented in Table 1. Statistically significant differences were observed in all analysed parameters between control- and case-groups. The group of patients with CHD showed a statistically significant increase of total cholesterol (TC), about 1.5 times that of the control group ($p < 0.001$); triglycerides (TG), about 2 times that of the control group ($p < 0.01$); LDL-cholesterol (LDL-C), about 1.5 times that of the control group ($p < 0.001$); VLDL-cholesterol (VLDL-C), about 2 times that of the control group ($p < 0.001$); apoprotein B (apo-B), 1.4 times that of the control group ($p < 0.001$); and TC/HDL ratio ($p < 0.001$). A statistically significant decrease of HDL-cholesterol (HDL-C) ($p < 0.05$) and apoprotein A1 (apo-A1) ($p < 0.001$) in CHD-patients was also observed.

Table 1. Clinical characteristic of the patients with CHD (case-group) and the clinically healthy blood donors (control group)

Parameter	Case-group	Control group	p
Sex (M/F)	37/14	34/17	
Age (years)	52 0 ± 10 5	41 0 ± 6 7	
TC [mmol/l]	6 54 ± 1 95	4 76 ± 1 00	$p < 0\ 001$
TG [mmol/l]	2 26 ± 2 00	1 30 ± 1 10	$p < 0\ 001$
LDL-C [mmol/l]	4 47 ± 2 00	2 74 ± 1 00	$p < 0\ 01$
HDL-C [mmol/l]	1 22 ± 0 45	1 41 ± 0 54	$p < 0\ 001$
VLDL-C [mmol/l]	0 92 ± 0 55	0 49 ± 0 30	$p < 0\ 05$
Apo-A1 [g/l]	1 17 ± 0 34	1 54 ± 0 65	$p < 0\ 001$
Apo-B [g/l]	1 20 ± 0 34	0 86 ± 0 23	$p < 0\ 001$
TC/HDL	6 27 ± 3 96	4 00 ± 2 45	$p < 0\ 001$

The results in Table 2 show the serum levels of IgG autoantibodies against oxLDLs (Ab-oxLDLs) or native LDLs (Ab-nLDLs). In CHD-patients, the levels of Ab-oxLDLs were 0.590 ± 0.330, about 2.5 times higher than in the control group (0.244 ± 0.200) ($p < 0.001$).

Both groups were also tested for IgG autoantibodies against native LDLs (Ab-nLDLs). As can be seen from Table 2, in this case very low levels were established in CHD-patients (0.086 ± 0.045) as well as in controls (0.077 ± 0.048) ($p > 0.05$). Probably, this is not a result of the antibody-antigen reaction but the real background of the immunoenzyme test.

Table 2. Levels of IgG autoantibodies against oxidized LDLs(Ab-oxLDLs) or native LDLs (Ab-nLDLs) in CHD-patients (case-group) and in clinically healthy blood donors (control group)

Parameter	Case group	Control group	p
Ab-nLDLs [OD units]	0 590 ± 0 330	0 244 ± 0 200	$p < 0\ 001$
Ab-nLDLs [OD units]	0 086 ± 0 045	0 077 ± 0 048	ns

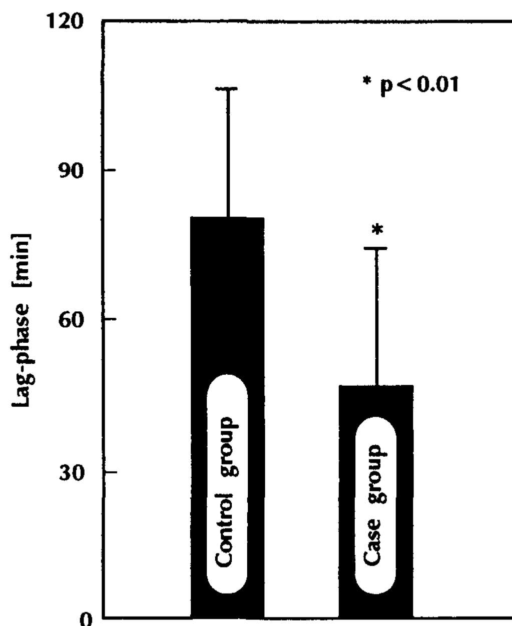

Figure 2. Lag-phase of serum oxidation by Cu^{2+} in CHD-patients (case-group) and in clinically healthy blood donors (control group) Incubation conditions 1 ml blood serum was diluted in PBS to yield protein concentration 1mg/ml $CuSO_4$ (46 mmol/l) was added to the serum and the incubation was carried out at 37 °C The lag-phase of serum oxidation was calculated as described in Fig 1

The relationship between the serum Ab-oxLDLs levels and the risk of CHD was characterized by multiple logistic regression (odds ratio, OR) with admitting accounting for sex and age. The odds ratio was 2.4 at 95% confidential interval 1.35–4.28 and $p < 0.01$. An increase of the serum Ab-oxLDLs level of one unit increases the risk of CHD 2 times.

In the second step, the lag-phase of serum oxidation by Cu^{2+} was studied. We used the same serum samples as for Ab-oxLDLs determination.

The results in Fig. 2 illustrate the duration of the lag-phase of serum oxidation by Cu^{2+} in CHD-patients and in clinically healthy blood donors. It is well known that the duration of the lag-phase of serum oxidation *in vitro* depends on the balance between endogenous prooxidants and antioxidants in the serum (Esterbauer et al. 1992; Rengstrom et al. 1992; Maggi et al. 1994). Therefore, this parameter could provide information on the antioxidative status of the serum, and indirectly on LDL-susceptibility to oxidation as well as on the LDL-oxidative modification. In our experiments a statistically significant decrease of the lag-phase of serum oxidation in CHD-patients could be measured as compared to the control group

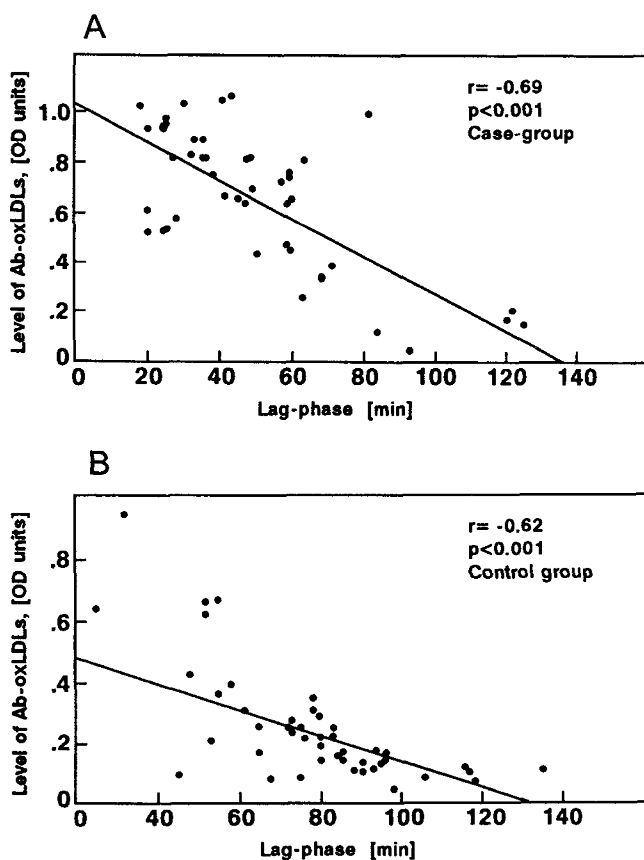

Figure 3. Negative linear correlation between serum levels of IgG autoantibodies against oxLDLs (Ab-oxLDLs) and the lag-phase of serum oxidation by Cu^{2+} in CHD-patients (A) and in clinically healthy blood donors (*B*). Spearmann's correlation method was used.

($p < 0.001$). In control subjects, the lag-phase was 80.23 ± 26.30 min, about 2 times higher than in CHD-patients (47.00 ± 27.19 min).

The correlation relationship between serum IgG autoantibody levels and the lag-phase of serum oxidation is shown in Fig. 3*A* (for the experiments) and in Fig. 3*B* (for the control group). Negative linear correlation between the two investigated parameters was established for both groups ($r = -0.69$, $p < 0.001$ for CHD-patients, and $r = -0.62$, $p < 0.001$ for clinically healthy blood donors). No significant correlations could be established between the level of IgG autoantibodies and sex, age and routine lipid parameters (TC, LDL-C, HDL-C, TG, apo-B, apo-A1) in either group.

Discussion

It is well known that the oxidative modification of LDLs affects the lipid components as well as the apo-B molecule (Lyons 1993; Kayden and Traber 1993; Penn and Chisolm 1994). The lipid peroxidation of LDLs leads to the formation of highly reactive substances, such as aldehydes and alkenals, which react with apo-B (Jurgens et al. 1987; Kayden and Traber 1994). The result of this process is derivatization of the apo-B molecule and formation of new epitops (Lyons 1993; Kayden and Traber 1993; Penn and Chisolm 1994). It is assumed that the derivatization of apo-B is one of the major reasons for the immunogenic properties of oxLDLs.

We could observe an increased serum level of IgG autoantibodies against oxLDLs in CHD-patients in comparison to clinically healthy blood donors (Table 2). The serum of both groups was also tested for autoantibodies against native LDLs. We detected very low levels of Ab-nLDLs in the sera of both groups (case and control) (Table 2). Probably, the levels of Ab-nLDLs are not a result of the antibody-antigen reaction but the real beckground of the ELISA-test.

Nine patients from the case-group had familiarly hypercholesterolemia (TC > 7.2 mmol/l) and serious atherosclerotic disorders. The average levels of Ab-oxLDLs in these nine CHD-patients were 0.722 ± 0.350, much higher than the average levels of the case-group. The number of patients with familiarly hypercholesterolemia was too low ($n = 9$) to make them a separate group. However, we can suppose that there is a tendency to increased serum Ab-oxLDLs levels in these patients. The same tendency was observed by Raal et al. (1995).

Our observations are in agreement with the data reported recently (Palinski et al. 1989; Salonen et al. 1991; Maggi et al. 1994; Puurunen et al. 1994; Bui et al. 1996). Salonen et al. (1991) have shown increased titers of autoantibodies against MDA-modified LDLs in patients with carotid atherosclerosis. These authors have reported that Ab-oxLDLs titers are an independent predictor of the progression of carotid atherosclerosis in males.

The results of multiple logistic regression of our experimental data (Odds ra-

tio, serum Ab-oxLDLs levels and risk of CHD) suggest that Ab-oxLDLs have a predictive value with respect to risk of CHD The odds ratio was 2 4 at 95% confidential interval (1 35–4 28 and $p < 0\ 01$) An increase of the serum Ab-oxLDLs levels of one unit increases the risk of CHD 2 times

Bergmark et al (1995) have established an increased titer of Ab-oxLDLs in patients with peripheral vascular disease High serum levels of autoantibodies against modified LDLs were also found in patients with insulin-independent diabetes (Rosen et al 1992) However, in this case the autoantibodies are not a predictor of coronary events Probably, this is due to the specificity of the atherosclerotic disorders in diabetic patients In this case, glycated LDLs are predominantly involved in the pathogenesis of atherosclerosis (Schmidt et al 1994)

In spite of the fact that oxidative modification of LDLs affects apo-B molecule and lipid components of LDL particles we could not establishe a significant correlation between serum Ab-oxLDLs levels and the serum lipid parameters studied (TC, TG, LDL-C, VLDL-C, HDL-C) or apo-A1 and apo-B Similar results were published by other authors (Salonen et al 1991, Maggi et al 1994) A positive correlation has been observed between serum Ab-oxLDLs levels and copper (Salonen et al 1991) Processes of free radical oxidation may be assumed to take place in Ab-oxLDLs formation as copper is one of the major *in vivo* prooxidants, responsible for LDL-oxidation

Ab-oxLDLs accepted as indirect marker for the degree of LDL-oxidative modification *in vivo* (Palinski et al 1989, Salonen et al 1992, Heinecke 1994,) Therefore, the serum level of Ab-oxLDLs could be dependent on the degree of the oxidative processes in the blood The factors influencing Ab-oxLDLs formation are unclear Consumption of endogenous antioxidants could be an important factor triggering Ab oxLDLs accumulation and autoimmune response This raise the question whether there is a parameter which would sufficiently indicate the serum susceptibility to oxidation and the balance between prooxidants and antioxidants in sera?

There are many data indicating that the lag-phase of LDL-oxidation *in vitro* provides information about the antioxidative status of lipoproteins and about LDL susceptibility to oxidation (Esterbauer et al 1992, Rengstrom et al 1992, Maggi et al 1994) On the basis of these data we suppose that the lag-phase of serum oxidation *in vitro* could suggest the susceptibility of sera to oxidation and the balance between prooxidants and antioxidants In CHD-patients, a significant shortering of the lag-phase was observed in comparison to control group (Fig 2) This suggests an increased susceptibility of the serum to oxidation in CHD-patients and its decreased antioxidative status A decrease of the serum antioxidative status will undoubtedly influence the degree of oxidative modification of LDLs and indirectly, serum Ab-oxLDLs levels

To illustrate this assumption, we conducted a correlation analysis of the two

investigated parameters, serum Ab-oxLDLs levels and duration of the lag-phase of serum oxidation *in vitro*

We observed a good inverse correlation between the duration of the lag-phase and serum Ab-oxLDLs levels ($r = -0\ 69$, $p < 0.001$ for CHD-patients, $R = -0\ 62$, $p < 0\ 001$ for controls)

It may be assumed that the lag-phase of serum oxidation *in vitro* could provide information about the LDL-susceptibility to oxidative modification and about the extent of the associated humoral immune response

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