

Relationships Between Serum Levels of Autoantibodies Against Oxidized Low Density Lipoproteins, Lipid-soluble Antioxidants and Apolipoprotein B in Patients with Coronary Heart Disease

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Abstract. High affinity IgG autoantibodies against oxidized low density lipoproteins (oxLDLs), apolipoprotein B and lipidsoluble antioxidants – alpha-tocopherol and beta-carotene, were tested in patients with coronary heart disease. Correlation relationships between these parameters were analysed.

Fifty one patients with coronary heart disease (37 males/14 females) defined as Q-wave myocardial infarction and/or stenosis of more than 50%, and 51 healthy blood donors (34 males/17 females) as controls participated in this study. LDLs were isolated by density gradient ultracentrifugation and oxidized with Cu^{2+} . OxLDLs or native LDLs (nLDLs) were used as antigens in enzyme immunoassay (ELISA) to detect IgG autoantibodies in the serum. The contents of alpha-tocopherol and beta-carotene were measured by HPLC. Apolipoprotein B was determined by immunoturbidimetry. Correlation analysis of the parameters was carried out by Spearman's test.

Alpha-tocopherol was decreased significantly in the serum of patients with coronary heart disease (2.96 ± 1.63 nmol/mg serum protein vs 6.23 ± 2.28 nmol/mg serum protein in Control group) ($p < 0.01$). Also, the serum level of beta-carotene was decreased in patients with coronary heart disease (174.0 ± 95.7 pmol/mg serum protein vs 313.2 ± 141.5 pmol/mg serum protein in Control group) ($p < 0.01$), while apolipoprotein B was increased significantly (1.20 ± 0.34 g/l in patients with coronary heart disease vs 0.86 ± 0.23 g/l in Control group) ($p < 0.001$). In a previous study we established that the mean serum level of IgG autoantibodies against oxLDLs (expressed in optical density units) was about 2.5 times higher in patients with coronary heart disease as compared to control subjects ($p < 0.001$).

A good positive linear correlation was observed between alpha-tocopherol and apolipoprotein B levels in Control group ($r = 0.78$, $p < 0.001$), as well as in the group of patients with coronary heart disease ($r = 0.42$, $p < 0.001$). Poor non-significant correlations were established between all another measured parameters.

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In conclusion, the lipid-soluble antioxidants – alpha-tocopherol and beta-carotene, are not informative with respect to the susceptibility of the serum to oxidative modifications and as to the extent of the subsequent humoral immune response. Presumably, the reduction of the correlation coefficient between apolipoprotein-B and alpha-tocopherol in patients with coronary heart disease in comparison with control subjects could provide indirect information on modifications of apolipoprotein-B and on a decrease of its susceptibility to interact with this major lipid-soluble antioxidant in atherogenesis.

Key words: Autoantibodies against oxLDL — Lipid-soluble antioxidants — Apolipoprotein B — Coronary heart disease

Introduction

Free radical processes play a major role in coronary heart disease (CHD) (Heinecke 1994; Penn and Chisolm 1994; Segrest and Anantharamaiah 1994; Losonzy et al. 1996). It has been established that in this case oxidatively modified low density lipoproteins (oxLDLs) are produced, leading to enhanced uptake in macrophages, cholesterol accumulation and foam cell formation (Packer 1992; Regnstrom et al. 1992; Parthasarathy and Santanam 1994).

Free radical oxidation products in blood (aldehydes, alkenals) have been observed to react with amino acid chains of apolipoprotein B (apo-B) (Kayden and Traber 1993; Lyons 1993; Penn and Chisolm 1994) with the resulting fragmentation of apo-B molecules and formation of new epitopes (Palinski et al. 1989; Regnstrom et al. 1992; Salonen et al. 1992). It is assumed that this is one of the major reasons for the immunogenicity of oxLDLs. Autoantibodies against oxLDLs (Ab-oxLDLs) are detected in the serum of patients with carotid atherosclerosis, CHD, diabetes (Palinski et al. 1989; Regnstrom et al. 1992; Salonen et al. 1992; Puurunen et al. 1994; Goudev et al. 1995, 1996; Bellomo et al. 1996; Bui et al. 1996). Those autoantibodies have a predictive value for further ischemic events.

Therefore, disturbed regulation of free radical oxidation of serum and LDLs is considered to be an important factor in the development of CHD.

Epidemiological and prospective studies have suggested an inverse correlation between serum antioxidant vitamins, antioxidant uptake and incidence of heart disease (Gey et al. 1991; Riemersma et al. 1991; Stampfer et al. 1991, 1993; Lin 1993; Segrest and Anantharamaiah 1994; Ohrvall et al. 1996). Reduction of coronary events was observed in people taking antioxidant vitamins (Dieber-Rotheneder et al. 1991; Esterbauer et al. 1992a; Lyons 1993; Heinecke 1994; Maggi et al. 1994). Therefore, it is important to study the molecular mechanism(s) by which antioxidants may protect against CHD, such as: inhibition of free radical oxidation of LDLs, suppression of apo-B fragmentation, and decreased serum levels of autoantibodies against oxLDLs.

The aim of the present study was to establish the relationships between serum levels of IgG autoantibodies against oxLDLs, lipid-soluble antioxidants and apo-B

in patients with CHD, as well as to estimate the informative role of these antioxidants for the susceptibility of the serum to oxidative modifications and the extent of the consequent humoral immune response.

Materials and Methods

Chemicals

All reagents of analytical grade, were obtained from Aldrich Chem. Co. Steinheim, Germany, Henkel Co., Merck Rahway, USA and Sigma Chem. Co. St. Louis, USA

Study Design

Fifty-one patients with CHD (37 males and 14 females, aged 52.0 ± 10.5 years) determined as Q-wave myocardial infarction and/or stenosis of more than 50% from coronarography, and 51 healthy blood donors (34 males and 17 females, aged 41.0 ± 6.7 years; free of CHD, hypertension, diabetes, renal and thyroid dysfunction) as controls participated in this study. None of the patients was on lipid-lowering therapy and antioxidant supplementation. Venous blood was taken from the cubital vein of each person, after overnight fast, into Vacutainer tubes for serum isolation.

Isolation of LDLs

LDLs were isolated by sequential ultracentrifugation in a Beckman L8 55 ultracentrifuge. Briefly, venous blood was taken from the cubital vein of 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) as described in Bakalova et al. (1999).

Immunoenzyme assay (ELISA)

LDL-fractions oxidized by CuSO_4 or lipoxygenase were used as antigens. The serum levels of IgG autoantibodies against oxLDLs were determined as described in Bakalova et al. (1999). Levels of IgG autoantibodies are expressed as optical density (OD) units at $\lambda = 492$ nm.

HPLC analysis of alpha-tocopherol and beta-carotene contents

Alpha-tocopherol and beta-carotene were extracted from serum as described by Lang et al. (1986) and were assayed by HPLC using a C-18 column (25×4.1 mm) (Perkin-Elmer apparatus). The eluent was methanol-ethanol 1:9 (v/v), 20 mmol/l lithium perchlorate. The flow rate was 1 ml/min, and the injected volume was 20 μl . The eluate was monitored by spectrofluorimetric detector – $\lambda_{\text{ex}} = 292$ nm, $\lambda_{\text{em}} = 325$ nm.

Serum beta-carotene concentration was assayed by HPLC using an ODS reverse phase column and an elution solvent mixture of methanol, toluene, and

ammonium acetate (82.5:17.5:0.5) (Perkin-Elmer apparatus). The flow rate was 1 ml/min, and the injected volume was 20 μ l. The eluate was monitored by spectrophotometric detector at $\lambda = 450$ nm (Kagan et al. 1992).

Other measurements

Apo-B was determined by immunoturbidimetry. Serum protein concentration was measured by the method of Lowry et al. (1951).

Statistical analysis

The results were expressed as mean \pm SD. The data were analyzed by ANOVA with statistical significance of differences between the groups determined by Dunnett's test. Statistical significance was assessed at $p < 0.05$. Spearman's correlation method was used for determination of correlation coefficients. The degree of correlation was estimated as described in Snedecor and Cochran (1989): $r < 0.25$ – lack of correlation; $r > 0.26 < 0.49$ – poor correlation; $r > 0.50 < 0.69$ – moderate correlation; $r > 0.70$ – good correlation.

Results

Figure 1 shows the serum levels of IgG autoantibodies against Cu^{2+} -oxidized LDLs in the two analysed groups. In patients with CHD the levels of Ab-oxLDLs (expressed as OD units) were 0.590 ± 0.330 – about 2.5 times higher than in the controls (0.244 ± 0.200) ($p < 0.001$) (Bakalova et al. 1999). Nine patients in the CHD-group were with familial hypercholesterolemia (total cholesterol > 7.2 mmol/l) and hard atherosclerotic disorders (marked with stars in Fig. 1). The average of Ab-oxLDLs in these nine patients was 0.722 ± 0.350 , much higher than the average level of OD units in the CHD group. There is a tendency to increased serum Ab-oxLDLs levels in patients with familiar hypercholesterolemia. The same tendency was observed by Raal et al. (1995) and Bergmark et al. (1995).

Similar results were found by using lipoxygenase-oxidized LDLs as antigen; the levels of Ab-oxLDLs were 0.683 ± 0.298 in patients with CHD and 0.290 ± 0.168 in healthy subjects.

There was a good correlation between the serum levels of IgG autoantibodies in the two cases of LDL-modification, by Cu^{2+} or lipoxygenase ($r = 0.95$, $p < 0.001$). It may be assumed that the generated epitopes in these two kinds of LDL-oxidation are close or identical.

Against native LDLs very low levels of IgG autoantibodies were established in CHD patients and in controls (Palinski et al. 1989; Bakalova et al. 1999).

The relationship between the serum level of Ab-oxLDLs and the risk of CHD was characterized by multiple logistic regression (odds ratio) with admitting sex and age. The odds ratio was 2.4 with 95% confidence interval of 1.35–4.28 and at $p < 0.01$. The increase of the serum level of Ab-oxLDLs by one unit enhances the risk of CHD 2 times in the upper interval. This suggests that Ab-oxLDLs could be

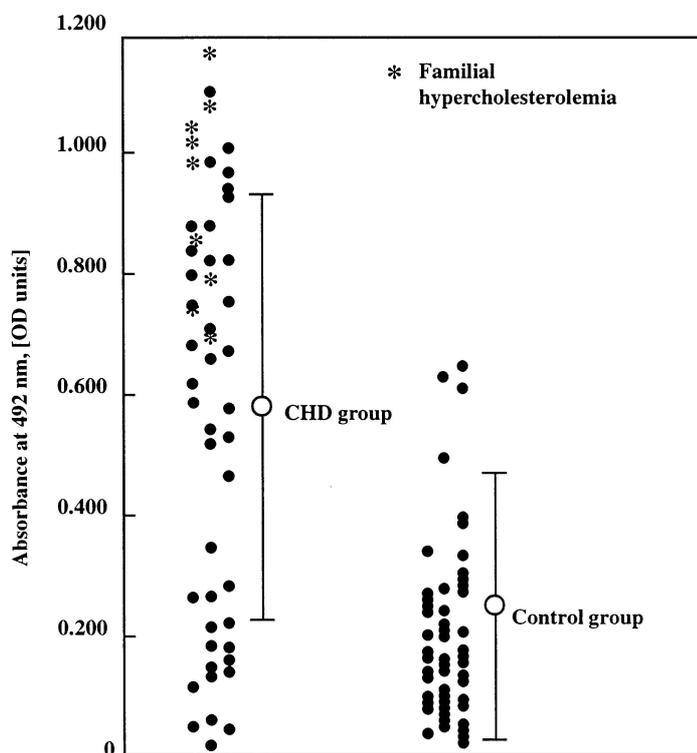


Figure 1. Serum level of IgG autoantibodies against oxLDLs (Ab-oxLDLs) in CHD patients (CHD group) and in clinically healthy blood donors (Control group) ($p < 0.001$).

Table 1. Serum levels of apo-B and lipid-soluble antioxidants-alpha/tocopherol and beta-carotene, in healthy blood donors (Control group) and in patients with CHD (CHD group).

Parameter	Control group	CHD group	<i>p</i>
Alpha-tocopherol, [nmol/mg protein]	6.23 ± 2.28	2.96 ± 1.63	$p < 0.01$
Beta-carotene, [pmol/mg protein]	313.2 ± 141.5	174.0 ± 95.7	$p < 0.01$
Apo-B [g/l]	0.86 ± 0.23	1.20 ± 0.34	$p < 0.001$

used as a marker of atherosclerotic damage of coronary arteries, and may have a predictive value for further ischemic events.

As can be seen from Table 1 alpha-tocopherol and beta-carotene levels were decreased significantly in CHD patients. The levels of alpha-tocopherol and beta-carotene in the sera of patients with CHD were about 2.5 and 2 times lower,

Table 2. Correlation coefficients between IgG autoantibodies against oxLDLs, alpha-tocopherol, beta-carotene and apo-B in patients with CHD (CHD group) and in healthy blood donors (Control group).

			Control group		
Apo-B	-0.12 ($p > 0.05$)				
Alpha-tocopherol	-0.10 ($p > 0.05$)	0.78	$p < 0.001^*$		
Beta-carotene	-0.14 ($p > 0.05$)	0.21 ($p > 0.05$)	-0.11 ($p > 0.05$)		
	Ab-oxLDLs	Apo-B	Alpha-tocopherol		
			CHD group		
Apo-B	-0.08 ($p > 0.05$)				
Alpha-tocopherol	0.04 ($p > 0.05$)	0.42	($p < 0.001$)[*]		
Beta-carotene	-0.07 ($p > 0.05$)	0.19 ($p > 0.05$)	-0.06 ($p > 0.05$)		
	Ab-oxLDLs	Apo-B	Alpha-tocopherol		

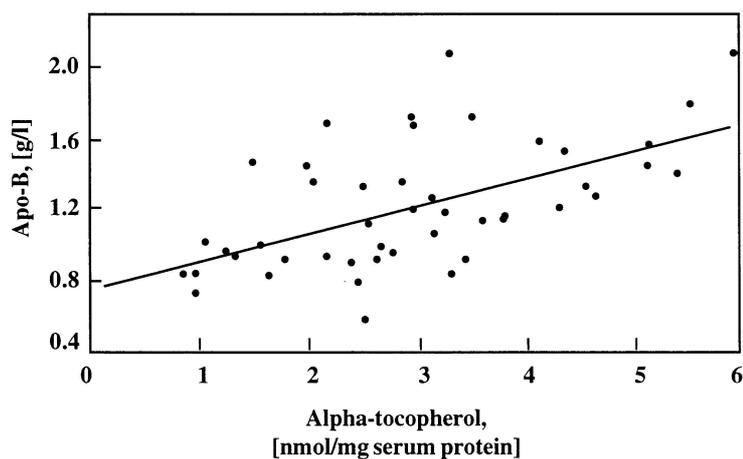


Figure 2. Positive linear correlation between serum levels of alpha-tocopherol and apo-B in clinically healthy blood donors ($r = 0.78$, $p < 0.001$). Spearman's correlation method was used.

respectively, as compared to healthy blood donors ($p < 0.01$). On the other hand, apo-B levels were increased significantly in patients with CHD – about 1.4 times vs control subjects ($p < 0.001$).

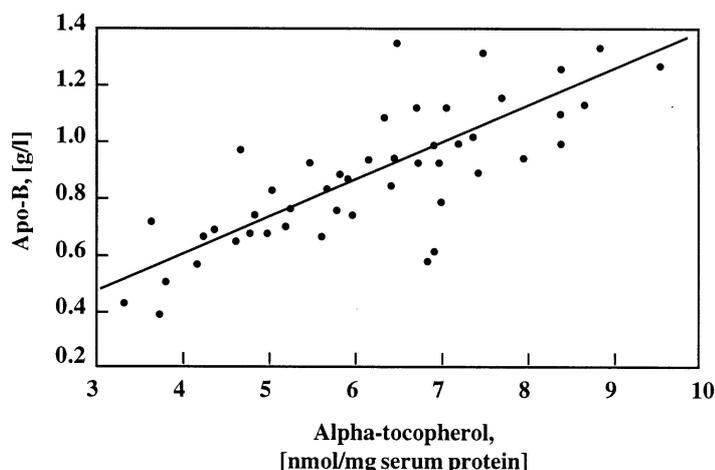


Figure 3. Positive linear correlation between serum levels of alpha-tocopherol and apo-B in CHD patients ($r = 0.42$, $p < 0.001$). Spearman's correlation method was used.

Table 2 shows the correlation coefficients between all analysed parameters: Ab-oxLDLs, apo-B, alpha-tocopherol and beta-carotene. The age/sex differences were accounted for. A poor nonsignificant correlation was observed between the following variables: Ab-oxLDLs – alpha-tocopherol, Ab-oxLDLs – beta-carotene, Ab-oxLDLs – apo-B, beta-carotene – apo-B, beta-carotene – alpha-tocopherol.

However, a statistically significant correlation could be established between alpha-tocopherol and apo-B in both groups. A good positive correlation was found between alpha-tocopherol and apo-B in Control group ($r = 0.78$, $p < 0.001$) (Fig. 2). In this case, the correlation coefficient was much higher than in the CHD group. A poor, but significant correlation was also observed for the CHD group ($r = 0.42$, $p < 0.001$) (Fig. 3).

Discussion

High serum levels of IgG autoantibodies against oxLDLs were observed in patients with CHD (Bakalova et al. 1999). It is accepted that the autoantibodies are a result of secondary immune response and they are characterized by a high specificity in relation to changes of serum proteins. It is well known that in the process of oxidative modification of LDLs apo-B gets fragmented (Kayden and Traber 1993; Lyons 1993; Penn and Chisolm 1994; Witztum 1994). Therefore, the changes in the apo-B molecule could be one reason for the autoimmune response. However, in the present work no significant correlation was found between the serum levels of Ab-oxLDLs and apo-B. The increased levels of apo-B and/or LDLs in patients with CHD are not a marker for increased levels of modified apo-B (or oxLDLs) and

for high serum levels of Ab-oxLDLs, respectively. Probably, the increased levels of apo-B in patients with CHD are a result of a compensation mechanism, leading to accelerated synthesis of new apo-B molecules and to formation of new non-modified native LDL-particles, replacing oxLDLs.

Neither, a significant correlation between serum levels of Ab-oxLDLs and lipid-soluble antioxidants (alpha-tocopherol and beta-carotene) could be found. The autoantibody formation is a complex process and it is affected by many different factors, serum lipid-soluble antioxidants being only one of them. Therefore, the lack of correlation between serum levels of Ab-oxLDLs and alpha-tocopherol or beta-carotene, observed in our study, is not surprising. Obviously, alpha-tocopherol and beta-carotene are not factors essentially influencing autoantibody formation in CHD. This assumption does not speak against the role of alpha-tocopherol and beta-carotene as major lipid-soluble antioxidants in the serum and as basic protectors of LDLs against oxidation (Esterbauer et al. 1989; 1992a,b; Packer 1992). In our previous work, we could establish a good inverse correlation between serum levels of Ab-oxLDLs and the lag-phase of serum oxidation by Cu^{2+} ($r = -0.69$, $p < 0.001$ for patients with CHD and $r = -0.62$, $p < 0.001$ for controls) (Bakalova et al. 1999). It is recognized that the lag-phase of serum or LDL oxidation *in vitro* is a good criterion for the assessment of the pro-/antioxidant balance (Esterbauer et al. 1989; Regnstrom et al. 1992; Maggi et al. 1994; Zeicher et al. 1995; Zeicher 1996). Therefore, the antioxidant status of the serum takes place in the formation of Ab-oxLDLs.

Probably, the relationship between Ab-oxLDLs and lipid-soluble antioxidants is indirect and mediated by other factors. Apo-B could be one of them.

In the present study no correlation was observed between serum levels of apo-B and beta-carotene in both groups. However, there was a high positive correlation between the serum levels of apo-B and alpha-tocopherol in clinically healthy blood donors ($r = 0.78$, $p < 0.001$). A similar relationship between both parameters was found in patients with CHD, but the correlation coefficient was lower ($r = 0.42$, $p < 0.001$). Whereas LDLs are responsible for the transport of alpha-tocopherol, it may be speculated that alpha-tocopherol is bound to LDLs (oxLDLs) to a smaller extent in patients with CHD as compared to control subjects. Yokota and Hanesan (1995) and Yokota et al. (1996) described a mutation in alpha-tocopherol-binding protein, resulting in a very low serum levels of alpha-tocopherol. Similar results were published by Gotoda et al. (1995).

It may be suggested that derivatization of apo-B in patients with CHD influences LDL-supplementation with alpha-tocopherol *in vivo*. In our previous study we found that *in vitro* oxidation of LDLs leads to disturbance of their binding to alpha-tocopherol (Bakalova et al. 1996). Native LDLs were able to bind much more exogenous alpha-tocopherol as compared to oxLDLs. Many authors have found that LDL-particles in patients with CHD also have a lower concentration of alpha-tocopherol in comparison with clinically healthy subjects (Gey et al. 1991; Riemersma et al. 1991; Stampfer et al. 1991, 1993; Segrest and Anantharamaiah 1994; Ohrvall et al. 1996).

Apo-B may be assumed to be one of the factors mediating the relationship between Ab-oxLDLs and lipid-soluble antioxidants. Probably, oxidative modifications of LDLs in CHD, leading to fragmentation of apo-B molecules, result in:

- a decrease of their capacity to bind lipid-soluble antioxidants;
- an increase of their susceptibility to oxidation, and
- an increase of IgG autoantibody formation in the serum.

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