Autoantibodies to malondialdehyde-modified low-density lipoprotein in patients with angiographically confirmed coronary artery disease

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Abstract

Oxidised low-density lipoprotein (LDL) has physicochemical properties that are pivotal in athero-sclerotic plaque formation. As a consequence, antioxidant regimens may prove an important therapy in the prevention and treatment of cardiovascular disease. Since oxidised LDL is immunogenic, the aims of our study were to measure serum IgG titres to malondialdehyde-modified LDL (MDA-LDL) in patients with coronary artery disease (CAD) and control subjects and assess their potential as a clinical marker for coronary atherosclerosis and, consequently, antioxidant intervention. Serum IgG titres to MDA-LDL were measured in patients with angiographically confirmed CAD (n = 40) and aged-matched controls (n = 40) by enzyme-linked immunosorbant assay (ELISA). Titres were calculated and expressed as both the difference and the ratio of blanked absorbance units (AU) for IgG binding to MDA-LDL and native LDL. For the control population, median IgG titres were 0.26 AU (interquartile range (IQR) 0.20–0.46 AU) and 5.34 (IQR 3.40–8.58), respectively, while the patient population had median IgG titres of 0.30 AU (IQR 0.20–0.47 AU) and 5.08 (IQR 3.30–9.66), respectively. Both sets of calculated titre values were not significantly different between the two groups (P = 0.60 and 0.82, respectively). In conclusion, this study could not establish any significant association between circulating autoantibody titres to MDA-LDL and coronary atherosclerosis. Therefore, the diagnostic value of autoantibodies to oxidised LDL remains unclear.

Introduction

Post-secretory oxidative modifications of low-density lipoprotein (LDL) play a critical role in the aetiology of atherosclerotic plaque development (Steinberg et al 1989; Aviram 1993). Oxidised LDL has a number of physicochemical properties important in atherogenesis, including cytotoxicity (Chisolm et al 1994), stimulation of specific cell-adhesion molecules (Berliner et al 1990) and growth factors within the artery wall (Ross 1993; Holvoet & Collen 1994), and the capacity to induce foam-cell formation via macrophage scavenger receptor uptake (Zingg et al 2000). Such observations have now raised the possibility that antioxidant intervention (e.g. vitamin E supplements) may help reduce the high level of morbidity and mortality that is frequently associated with vascular disease (Giugliano 2000; Pryor 2000). Supporting this concept have been studies where structurally unrelated lipid-soluble antioxidants inhibit the progression of atherosclerosis in hypercholesterolaemic rabbits and mice, although inhibition of LDL lipid peroxidation may not be the only mechanism by which antioxidants afford protection to vascular disease (see reviews by Daugherty & Roselaar 1995; Heinecke 1998). Despite the current inconclusive and somewhat confusing data from human clinical trials regarding the antagonising effects of antioxidant supplements on atherosclerosis, epidemiological studies do provide evidence of an inverse correlation between antioxidant intake and prevention of myocardial infarction, stroke and coronary artery disease (CAD) progression (Marchioli 1999; Meagher & Rader 2001). Furthermore, studies have shown that plasma concentrations of lipophilic antioxidants are significantly lower in CAD patients than in control subjects (Kontush et al 1999). As a consequence, the possibility that atherosclerotic plaque development and progression could be prevented, or reduced, by treatment with antioxidants could provide a

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potentially simple and relatively cost-effective strategy for the treatment of patients with CAD, as well as individuals at increased risk of the disease.

The peroxidation of polyunsaturated fatty acids in LDL generates highly reactive breakdown products – chiefly the aldehydes hexanal, 4-hydroxy-2-nonenal and malondialdehyde (MDA) (Esterbauer et al 1992; Jialal & Devaraj 1996) – which modify and cross-link amino groups in apolipoprotein (apo) B-100. These neoepitopes of oxidised LDL are immunogenic and stimulate autoantibody production (Palinski et al 1989, 1990). Although circulating autoantibodies to oxidised LDL are found in healthy individuals (Iughetti et al 1999; Vay et al 2001), significantly increased titres have been reported in patients with carotid (Salonen et al 1992; Maggi et al 1994), coronary (Bui et al 1996; Lehtimäki et al 1999; Inoue et al 2001) and early-onset peripheral (Bergmark et al 1995) atherosclerosis. In addition, Maggi et al (1993) reported an elevated IgG titre to oxidised LDL in asymptomatic subjects considered to be at increased risk of developing atherosclerosis. Antibodies against MDA-modified LDL have also been reported as being predictive of myocardial infarction (Puurunen et al 1994). Interestingly, an increase in the level of MDA-LDL has been observed in patients with acute myocardial infarction (and unstable angina) compared with patients with stable CAD (Holvoet et al 1998).

Against this background, the aims of this study were to measure serum IgG titres to MDA-LDL in angiographically confirmed CAD patients and control subjects and assess the potential of these antibodies as a clinical marker for coronary atherosclerosis and, consequently, antioxidant intervention.

Materials and Methods

Materials

All routine chemical reagents (Analar grade) were obtained from Merck (Poole, UK). Anti-human IgG-alkaline phosphatase (γ-chain specific), bovine serum albumin (BSA), butylated hydroxytoluene (BHT), 1,1,3,3-tetramethoxypropane and polyoxyethylene (20)-sorbitan monolaurate (Tween-20) were purchased from Sigma-Aldrich (Poole, UK). Bicinchoninic acid assay was from Pierce (Rockford, IL), while 96-well microtitre plates were obtained from Nunc (Polysorp; Roskilde, Denmark).

Subjects for study

This study was conducted in compliance with the Declaration of Helsinki (1996) and was approved by the Research Ethics Committee of Queen's University, Belfast. All patients recruited for the study gave consent for their blood to be taken. Blood samples were obtained from 40 patients (25 males, 15 females), aged 38–79 years, with angiographically confirmed CAD, and 70% or more stenosis in at least one coronary artery, and 40 control subjects (17 males, 23 females), aged 13–74 years, with no history or evidence of CAD upon clinical investigation. The characteristics and serum lipoprotein profile of both populations are shown in Table 1. Both groups were matched for age, concentrations of total cholesterol, LDL and HDL cholesterol and apo B-100. Triglyceride concentrations were significantly higher within the atherosclerotic population than the control group. The ratio of males to females was not statistically different between the two populations. None of the subjects were smokers.

Blood sample collection

For the preparation of plasma and serum, fasting blood samples were drawn into tubes containing disodium ethylenediaminetetraacetate (EDTA) or tubes with no anticoagulant, respectively. Plasma was quickly processed for the preparation of LDL, while serum was immediately frozen in aliquots at $-70°C$ in the presence of 100 mg mL^{-1} sucrose (Rumsey et al 1992).

Table 1   Characteristics of the control and atherosclerotic patient populations.

Parameter	Control subjects[a]	Patients[a]	P
Number	40	40	–
Age (years)	53±15	59±10	0.07†
Median age (years)	56	61	–
Age range (years)	13–74	38–73	–
Males/females	17/23	25/15	0.12‡
Total cholesterol (mg dL^{-1})	223±49	237±45	0.20§
LDL cholesterol (mg dL^{-1})	142±39	152±33	0.25§
HDL cholesterol (mg dL^{-1})	44±12	39±14	0.11§
Triglycerides (mg dL^{-1})	145 (97–217)	194 (143–294)	< 0.01¶
Apo B-100 (mg dL^{-1})	124±33	131±31	0.47§

[a]Age, total cholesterol, LDL and HDL cholesterol and apolipoprotein (apo) B-100 concentrations are presented as means±s.d., while triglyceride concentrations are presented as median values (IQR). Statistical analysis was performed using the †Welch unpaired two-tailed t-test, the ‡Chi-square test, §Student's unpaired two tailed t-test, and the ¶Mann–Whitney non-parametric test.

Blood lipoprotein profile

Serum concentrations of total cholesterol, LDL and HDL cholesterol, triglycerides and apo B-100 were measured using routine techniques previously described (McDowell et al 2001).

Preparation of LDL

Native LDL (density 1.019–1.063 g mL^{-1}) was rapidly prepared from freshly drawn plasma by ultracentrifugation (Havel et al 1955). To prevent modification of LDL during isolation, 1.0 g L^{-1} EDTA, 2 mM Pefablock (Roche Molecular Biochemicals, Lewes, UK) and 0.5 g L^{-1} sodium azide were added to the plasma sample. Protein concentrations were determined by the bicinchoninic acid assay, using BSA as a standard (Smith et al 1985). Purity of the LDL preparation was assessed by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Laemmlli 1970) and agarose gel electrophoresis (Noble 1968).

Modification of LDL

Purified LDL was modified with freshly prepared MDA following the method of Palinski et al (1990). The MDA-modified LDL sample was then dialysed at 4°C against deoxygenated 20 mM sodium phosphate, pH 7.2, containing 150 mM sodium chloride, 1 mg mL^{-1} EDTA and 0.2 mg mL^{-1} sodium azide. Modification of LDL was confirmed by agarose gel electrophoresis and the MDA content of the lipoprotein determined using the thiobarbituric acid reactive substances (TBARS) assay (Wallin et al 1993) with 1,1,3,3-tetramethoxypropane as a standard. Results were expressed as moles of MDA per mg of LDL protein. The modified LDL antigen was stored at −70°C in the presence of 100 mg mL^{-1} sucrose.

ELISA of IgG autoantibodies to MDA-modified LDL

All assays were performed in duplicate with volumes of 100 μL (unless stated otherwise). One-half of a 96-well microtitre plate (Polysorp) was coated with a 10 μg mL^{-1} solution of native LDL in 50 mM sodium carbonate buffer, pH 9.6, containing 1 mg mL^{-1} EDTA and 20 μM BHT. The other half of the plate was coated with a 10 μg mL^{-1} solution of MDA-LDL. Plates were incubated overnight at 4°C. Any unbound antigen was removed by three washes with 250 μL of 20 mM Tris-HCl, pH 7.4, containing 150 mM sodium chloride, 0.05% (v/v) Tween-20 and 2.0 mg mL^{-1} BSA (Grade V) (TBST-BSA). Wells were blocked for 2 h at 37°C with 200 μL of 20 mM Tris-HCl, pH 7.4, containing 150 mM sodium chloride and 30 mg mL^{-1} BSA. Plates were washed as described previously and each serum sample (diluted 1:20 in TBST-BSA) added before incubation overnight at 4°C. Plates were washed as before and goat anti-human IgG-alkaline phosphatase (γ-chain specific) (diluted 1:2000 in TBST-BSA) added before incubation for 3 h at 37°C. A final washing step was carried out and a

1 mg mL^{-1} solution of p-nitrophenyl phosphate in 10 mM diethanolamine buffer, pH 9.5, containing 0.5 mM magnesium chloride and 0.2 mg mL^{-1} sodium azide, added. After a 10-min incubation at room temperature, the reactions were stopped with 50 μL of 2 M sodium hydroxide and the absorbance of each well determined at 405 nm in a microtitre plate reader (Multiscan MS; Labsystems, Helsinki, Finland). Plates were blanked on a column of wells containing substrate only.

Expression of autoantibody titres

The autoantibody titres were expressed as both the difference and the ratio of blanked absorbance readings for IgG binding to MDA-modified and native LDL as previously described by Vaarala et al (1993) and Salonen et al (1992), respectively.

Statistical analysis

Data were analysed for a normal distribution using the Kolmogorov–Smirnov test. The distribution of males and females between the control and CAD groups was compared using the Chi-square test. The age of the control and atherosclerotic populations was compared using the Welch unpaired two-tailed t-test, due to unequal variance of the data. Total cholesterol, LDL and HDL cholesterol and apo B-100 concentrations were compared using the Student's unpaired two-tailed t-test, due to equal variance of the data. Triglyceride and autoantibody titres were analysed using the Mann–Whitney non-parametric test of significance, with the data expressed as the median (50th percentile) and interquartile range (IQR) for the 25th and 75th percentile values. Correlations were analysed by calculation of Spearman's rank order coefficients. Probability values (P) of < 0.05 were considered significant.

Results

Characterisation of native and MDA-modified LDL antigens

Freshly prepared native LDL was homogeneous by both polyacrylamide and agarose gel electrophoresis. Due to neutralisation of the positive charge on apo B-100, MDA-LDL demonstrated a 2.0-fold increase in mobility compared with native LDL upon agarose electrophoresis. Agarose electrophoresis indicated that all the LDL was modified. Analysis of the modified lipoprotein by the TBARS assay revealed approximately 270 nmol of MDA conjugated per mg of LDL protein (150 mol MDA/mol LDL protein).

Assay precision

Intra- and inter-assay precision of the ELISA was determined using three serum samples with low, medium and high IgG titres to MDA-LDL. Intra-assay s.d. was

determined by measuring each sample 12 times, in duplicate, during one run. Inter-assay s.d. was calculated by measuring each sample, in duplicate, on three different days. When measured as the difference in absorbance for IgG binding to MDA-modified and native LDL, the intra- and inter-assay coefficient of variation (CV) was 2.8–4.5% and 5.4–8.7%, respectively. When titres were calculated as a ratio of IgG binding to MDA-modified and native LDL, the intra- and inter-assay CV was 4.2–6.7% and 8.2–10.9%, respectively.

Autoantibody titres to MDA-LDL in patients with CAD

Autoantibody titres to MDA-LDL were initially measured as the difference in blanked absorbance readings for IgG binding to MDA-modified LDL and native LDL. Figure 1 shows the distribution of the titres for patients and age-matched control subjects. Values were in the range 0.02–0.85 AU for the control subjects and 0.04–1.43 AU for the CAD population, with median IgG titres of 0.26 AU (IQR 0.20–0.46 AU) and 0.30 AU (IQR 0.20–0.47 AU), respectively. Concentrations of anti-MDA-LDL IgG in the patient group were not statistically different from the age-matched controls ($P = 0.60$).

Figure 2 shows the distribution of autoantibody titres for the control subjects and patients when calculated as the ratio of blanked absorbance readings for IgG binding to MDA-modified and native LDL. Values were in the range 1.26–19.5 for the control subjects and 1.34–41.5 for the CAD population, with median ratio values of 5.34 (IQR 3.40–8.58) and 5.08 (IQR 3.30–9.66), respectively. As before, IgG autoantibody titres in the patient group were not
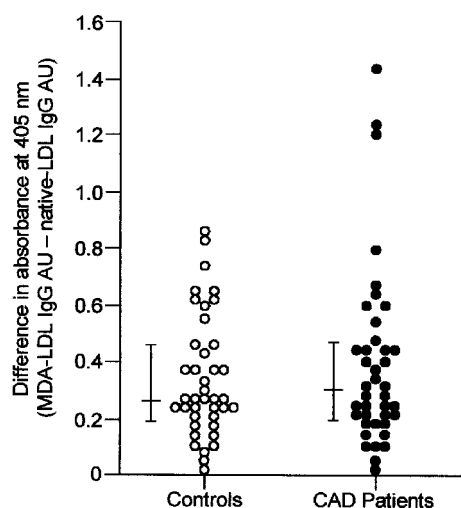

Figure 2 Autoantibody titres to MDA-modified LDL in the serum of control (○) and CAD patients (●). Titres are expressed as the ratio of blanked absorbance units (AU) for IgG binding to MDA- and native LDL. Values represent the mean of duplicate measurements. The population median and interquartile ranges (IQR) are shown as horizontal and vertical lines, respectively.
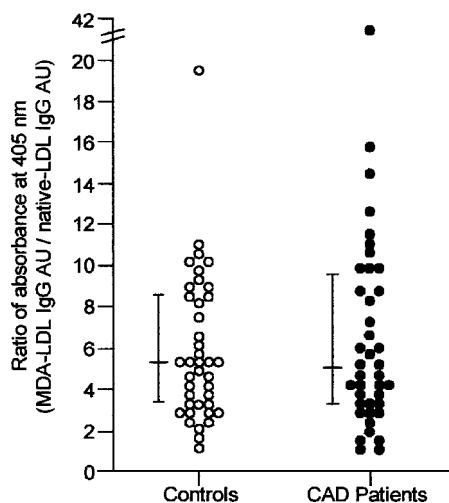
statistically different from the age-matched controls ($P = 0.82$).

Two serum samples were observed to have particularly high autoantibody titres when the data were expressed both ways. These samples came from a 53-year-old hypercholesterolaemic female with angina pectoris (titres of 1.20 AU and 15.8, respectively) and a 46-year-old hypercholesterolaemic male with angiographically confirmed CAD (titres of 1.24 AU and 41.5, respectively).

Autoantibody titres in relation to other variables

No correlations were established between autoantibody titres and age, concentrations of total, LDL and HDL cholesterol, triglycerides or apo B-100, for either the CAD or control populations (Table 2).

Discussion

Pharmacological intervention has served to significantly reduce the morbidity and mortality frequently associated with cardiovascular disease. In particular, the development of lipid-lowering drugs has helped to normalise LDL cholesterol concentrations, one of the most important risk factors for CAD (Vaughan et al 2000). The discovery that oxidative modifications of LDL play a critical role in the initiation and progression of atherosclerotic lesions has now raised the interesting possibility that antioxidant therapy might prevent, or at least reduce, the clinical sequelae of atherosclerosis. The aim of our study was to investigate whether IgG autoantibodies to MDA-LDL were significantly elevated in the serum of CAD patients

Figure 1 Autoantibody titres to MDA-modified LDL in the serum of control (○) and CAD patients (●). Titres are expressed as the difference in blanked absorbance units (AU) for IgG binding to MDA- and native LDL. Values represent the mean of duplicate measurements. The population median and interquartile ranges (IQR) are shown as horizontal and vertical lines, respectively.

Table 2 Correlation coefficients between anti-MDA-LDL titres and age, serum lipid and apo B-100 concentrations.

Subjects		Age (years)	Total cholesterol (mg dL^{-1})	LDL cholesterol (mg dL^{-1})	HDL cholesterol (mg dL^{-1})	Triglycerides (mg dL^{-1})	Apo B-100 (mg dL^{-1})
Controls[a]	I	−0.02	0.15	0.20	0.04	−0.16	0.20
	II	−0.24	0.16	0.25	0.27	−0.24	0.23
Patients[a]	I	0.01	0.01	0.03	0.18	−0.13	0.06
	II	0.02	0.09	0.09	0.28	−0.15	0.19

[a]Spearman's rank order correlation coefficients were calculated using titre values measured as the difference (I) and ratio (II) of absorbance values for IgG binding to MDA-modified and native LDL.

living in Northern Ireland (where levels of cardiovascular disease are very high) so their potential diagnostic value as a clinical marker for coronary atherosclerosis and subsequent antioxidant intervention could be assessed.

To measure IgG autoantibody titres to oxidised LDL, we utilised an MDA-modified LDL antigen. This particular form of oxidised LDL was selected since MDA is a major breakdown product of lipid peroxidation, and MDA-lysine adducts on apo B-100 are one of the primary neoepitopes generated during the oxidation process (Esterbauer et al 1992). Modification with MDA also renders homologous LDL immunogenic within the host, eliciting a strong autoantibody response that is easily detectable (Salmon et al 1987; Palinski et al 1990). Furthermore, since the density of MDA-lysine epitopes on immobilised MDA-LDL is high, the sensitivity of the autoantibody assay is increased. This is important, since studies by Mironova et al (1996) demonstrated that human autoantibodies to oxidised LDL were predominantly of moderate-to-low affinity. To eliminate the possibility of false-positive results, serum samples were also incubated with immobilised LDL so that any non-specific binding of the antibodies with the native lipoprotein could be detected. Autoantibody titres were therefore expressed as both the difference and ratio of IgG binding to MDA-modified and native LDL.

Although the age range and clinical characteristics of the subjects used in this investigation were similar to previously described studies, we could not detect any significant difference between CAD patients and control subjects with respect to oxidised LDL autoantibody titres. Also, we did not establish any correlation between autoantibody titres and age or blood lipid parameters, thus confirming other reports. Consequently, for this population at least, IgG titres to oxidised LDL appeared to have little diagnostic value. However, a number of similar investigations (using both Cu^{2+}-oxidised and MDA-modified LDL) have also been unable to establish any statistically significant difference between CAD patients and control subjects with respect to oxidised LDL autoantibody titres (Virella et al 1993; Boullier et al 1995; van de Vijver et al 1996; Halevy et al 1997). Studies have also found that antibodies against oxidised LDL are not significantly elevated, or associated with the intima-media thickness of the carotid or femoral arteries, in patients with familial hypercholesterolaemia compared with control subjects (Hulthe et al 1998; Paiker

et al 2000). The apparent discord between different investigations is difficult to fully assess. One potential factor that may contribute to the current confusion in results is the different ELISA methodologies adopted by various groups, especially the nature of the oxidised LDL antigen used (Närvänen et al 2001). For example, although Cu^{2+} oxidation of LDL creates epitopes that mimic those found within the arterial wall, the very complex cascade of reactions that occur during lipid peroxidation may lead to batches of oxidised LDL antigens that show significant heterogeneity between studies. Also, LDL prepared from different donors is likely to vary with respect to both polyunsaturated fatty acid and antioxidant content, which may further affect the course of the oxidation reactions. To reduce variation between LDL preparations and increase assay stability, the use of a pooled LDL antigen source has now been recommended (Craig et al 1999). The modification of LDL with MDA may also help to generate more reproducible batches of oxidised LDL for use in autoantibody assays. However, unless such oxidised forms of LDL are standardised with respect to MDA content, methodological variations may still occur due to differences in the level of modification and, thus, antibody binding.

In addition to the caveats associated with the use of different ELISA protocols, other factors are also likely to explain the inconsistent nature of the results between studies. For example, the detection of free autoantibodies against oxidised LDL may not accurately reflect the overall immune response since many autoantibodies will undoubtedly form complexes with the oxidised LDL antigen in-vivo. As a consequence, the measurement of oxidised LDL-immune complexes may have greater diagnostic utility. Indeed, previous studies have detected increased levels of LDL-containing immune complexes in the serum of CAD patients compared with healthy control subjects (Tertov et al 1990a, b), although investigations by Boullier et al (1995) did not confirm this observation. Studies by Lopes-Virella et al (1999), which concluded that oxidised LDL-immune complexes were a risk-factor for the development of macrovascular disease in patients with diabetes mellitus, have further brought the potential diagnostic significance of oxidised LDL-immune complexes in atherosclerosis into focus.

More recently, Palinski & Witztum (2000) raised a number of theoretical considerations that may further help

to explain the differences between studies. They suggest current risk factors, clinical events and non-invasive diagnostic methods, such as angiography and ultrasound, may not provide an accurate picture of the overall burden of atherosclerotic disease. Furthermore, Boullier et al (1995) highlighted that atherogenesis develops in a discontinuous fashion. Consequently, secretion of autoantibodies may follow the evolution of the atheromatous plaque with fresh lesions, containing nascent foam cells and oxidised LDL particles, providing a more potent immunogenic milieu than older calcified plaques. Autoantibodies against oxidised LDL may therefore prove to be a more appropriate marker for active atherogenesis rather than the extent of atherosclerosis. Unfortunately, information on the change in coronary artery stenosis over recent years was not available for our patient population. It is therefore a possibility that some of our patients may have been experiencing a stable period of coronary atherosclerosis at the time of analysis.

To conclude, we have not been able to establish any significant elevation in autoantibody titres to MDA-LDL in patients with CAD compared with control subjects. Further studies will clearly be required to finally resolve the outstanding issues surrounding the measurement of oxidised LDL autoantibodies and their role in atherosclerotic disease. With such data, the diagnostic potential of such antibodies should become clear.

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