Increased levels of high-density lipoprotein cholesterol are ineffective in inhibiting the development of immune responses to oxidized low-density lipoprotein and atherosclerosis in transgenic rabbits expressing human apolipoprotein (apo) A-I with severe hypercholesterolaemia

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ABSTRACT

High levels of high-density lipoprotein (HDL) cholesterol have been reported to protect against the development of atherosclerosis in humans by increasing reverse cholesterol transport and inhibiting the oxidation of low-density lipoprotein (LDL) due to the paraoxonase content of HDL. The purpose of the present study was to assess if there are any relationships between in vivo increases in serum levels of immunological LDL oxidation markers [autoantibodies against oxidized LDL, autoantibodies against malondialdehyde-modified LDL, LDL immune complexes and anti-cardiolipin autoantibodies], paraoxonase activity and the development of atherosclerosis in control rabbits and in transgenic rabbits expressing human apolipoprotein (apo) A-I. A total of 13 apo A-I transgenic rabbits and 18 non-transgenic littermates were fed on a cholesterol-rich diet (0.4%, w/w) for 14 weeks, and were monitored at weeks 0, 2, 6, 10 and 14. Aortic atherosclerotic lesions were measured at the end of this period. Human apo A-I transgenic rabbits with high HDL cholesterol levels were not protected against the development of atherosclerosis when they were fed on a cholesterol-rich diet which induced dramatic hypercholesterolaemia. Immunological markers of LDL oxidation increased and serum paraoxonase activity decreased similarly in control and transgenic rabbits. In conclusion, the present study demonstrates that high HDL cholesterol levels are ineffective in inhibiting increases in immunological markers of LDL oxidation and the development of atherosclerosis in a mammal with severe hypercholesterolaemia.

Key words: anti-cardiolipin autoantibodies, anti-(oxidized LDL) autoantibodies, apolipoprotein A-I, atherosclerosis, IgG-bound LDL, paraoxonase, rabbit, transgenesis.

Abbreviations: apo, apolipoprotein; Ab-C, anti-cardiolipin autoantibodies; LDL, low-density lipoprotein; MDA, malondialdehyde; Ab-LDL, autoantibodies against native LDL; Ab-MDA-LDL, autoantibodies against MDA-modified LDL; Ab-OxLDL, autoantibodies against Cu²⁺-oxidized LDL; HDL, high-density lipoprotein; IgG-LDL, circulating immune complexes measured as IgG-bound LDL; MDA-LDL, MDA-modified LDL; OxLDL, oxidized LDL; PON-1, paraoxonase-1; TBARS, thiobarbituric acid-reacting substances.

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INTRODUCTION

Substantial evidence indicates that oxidized low-density lipoprotein (OxLDL) contributes to atherogenesis through a number of mechanisms [1]. One of the critical steps in the development and progression of atherosclerosis is the enhanced uptake of OxLDL by macrophages, which results in the formation of lipid-laden foam cells, an early step in the development of atherosclerosis [1].

Even minor modifications to LDL render it immunogenic [2], and circulating autoantibodies that recognize several forms of OxLDL, in particular that containing malondialdehyde (MDA)-modified lysine, are prevalent in humans and other species [3–5]. Plasma levels of autoantibodies directed against MDA-modified LDL (MDA-LDL) may be valuable in predicting the progression of atherosclerosis in humans [3]. Anti-OxLDL autoantibodies are present in lesions, partly as immune complexes with OxLDL [6], and immunoglobulin-bound lipoprotein (circulating immune complexes) is a marker of familial hypercholesterolaemia and atherosclerosis [7–9].

The anti-phospholipid antibody syndrome is characterized by the presence of circulating autoantibodies that bind in vitro to moieties formed by negatively charged phospholipids, such as cardiolipin (anti-cardiolipin autoantibodies; Ab-C). The presence of a high level of Ab-C has been reported to be an independent risk factor for myocardial infarction [10]. A strong cross-reaction has been reported between antibodies to OxLDL and those to cardiolipin [11,12]. Hörrkö et al. [13] demonstrated that Ab-C are directed against epitopes of oxidized phospholipids, and cloned a monoclonal antibody that binds identically to OxLDL and to oxidized cardiolipin.

Epidemiological studies in humans have suggested that high levels of high-density lipoprotein (HDL) and apolipoprotein (apo) A-I protect against the progression of atherosclerosis [14]. This hypothesis was confirmed in transgenic mice overexpressing human apo A-I [15]. However, most of the plasma cholesterol in mice is carried in HDL and not in LDL, unlike in humans. On the other hand, rabbits, like humans, are ‘LDL mammals’, in that the LDL fraction is the major carrier of cholesterol in plasma [16]. Furthermore, rabbits develop a human type of atherosclerotic lesion [17]. In these animals, overexpression of human apo A-I induces a rise in HDL cholesterol [18] and a decrease in the development of atherosclerosis [19]. This protection is probably related to an increase in so-called ‘reverse cholesterol transport’ in transgenic rabbits, since we reported that the first step (i.e. cellular cholesterol efflux) in this metabolic pathway is increased in these animals [18].

Recent experimental evidence has suggested that HDLs inhibit atherogenesis not only through their capacity to induce reverse cholesterol transport, but also because they inhibit LDL oxidation [20–22] through their paraoxonase-1 (PON-1) content [21]. PON-1 is a calcium-dependent HDL-associated ester hydrolase [23]. Low levels of HDL-associated PON-1 are correlated with hypercholesterolaemia [24] and susceptibility to myocardial infarction [25,26]. Watson et al. [27] suggested that PON-1 in HDL may protect against the induction of inflammatory responses in artery wall cells by destroying biologically active lipids in mildly oxidized LDL. Recently it was reported that HDL isolated from PON-1-deficient mice was unable to prevent LDL oxidation [28,29], and that PON-1-null mice were more susceptible to atherosclerosis than their wild-type littermates [28] or apo E-null littermates [29].

PON-1 is not the only protein of HDL with antioxidant activity, as human apo A-I possesses antioxidant properties that might neutralize LDL lipid peroxidation [30]. We have recently demonstrated that PON-1 activity is reduced by a pro-atherosclerotic diet in control rabbits and in transgenic rabbits expressing human apo A-I [31]. Therefore the present study was undertaken with the following aims. (1) To determine if, in cholesterol-fed rabbits, the concentrations of immunological LDL oxidation markers [autoantibodies against Cu²⁺-oxidized LDL (Ab-OxLDL), autoantibodies against MDA-LDL (Ab-MDA-LDL), Ab-C and circulating immune complexes measured as IgG-bound LDL (IgG-LDL)] are correlated with the extent of development of aortic atherosclerosis. (2) To evaluate if the transgenesis of human apo A-I to the rabbit modifies the changes in the concentrations of these markers in proportion to the expected decrease in the development of atherosclerosis during cholesterol feeding. (3) To determine possible relationships between serum HDL levels, serum PON-1 activity, serum concentrations of HDL immunological oxidation markers and the development of atherosclerosis during cholesterol feeding in control and human apo A-I transgenic rabbits.

MATERIALS AND METHODS

Animal model

Groups of 13 New Zealand White rabbits transgenic for human apo A-I (line 8 [19]) (four males, nine females) and 18 non-transgenic littermates (10 males, eight females) (age 7 months) were used. Animals were housed individually in the Charles River Centre (Saint Aubin les Elbeuf, France). Atherosclerosis was induced by feeding the animal on a cholesterol-rich (0.4 %, w/w) diet for 14 weeks (120 g per day). The atherogenic diet was prepared by spraying normal chow (NIH-09) with cholesterol.

Blood was collected after 0, 2, 6, 10 and 14 weeks of the high-cholesterol diet, from animals that had been starved.
oxidative markers in human apolipoprotein A-I transgenic rabbit

overnight. Serum was separated by low-speed centrifugation and kept either at 4 °C until lipid and lipoprotein analysis (< 1 week) or frozen at −20 °C until measurement of anti-OxLDL markers and PON-1 activity (< 6 months).

The experimental procedures were carried out in accordance with United States NIH guidelines [32].

Lipid measurements

Serum lipids (cholesterol, phospholipids and triacylglycerols) and HDL cholesterol were measured colorimetrically using a microtitre plate reader and commercially available reagents (Boehringer Mannheim). Cholesterol was measured in the HDL-containing supernatant after precipitating apo B-containing lipoproteins with sodium phosphotungstate/magnesium chloride (Boehringer Mannheim). Non-HDL cholesterol values were calculated by subtracting HDL cholesterol from total cholesterol.

Measurement of immunological OxLDL markers

LDL isolation and modification

LDL (1.019 < d < 1.063) was isolated by sequential ultracentrifugation [33] from the pooled serum of five control rabbits. The protein content of LDL was measured [34]. LDLs were oxidized (OxLDL) by incubation for 24 h at 30 °C with CuSO₄ (0.100 mg/ml protein and 5 μmol/l CuSO₄). MDA-LDL was produced by adding freshly prepared MDA, as described by Palinski et al. [2].

The extent of oxidation was determined by analysing various markers of lipoprotein peroxidation: dienes were measured at 234 nm in solution containing 0.1 mg/ml protein, using ε₂₃₄ = 2.95 × 10⁴ M⁻¹ cm⁻¹ [35]. Thiobarbituric acid-reacting substances (TBARS) were measured using fluorimetric detection [36]. Lipid peroxides were measured using the lipid-peroxide-dependent oxidation of iodoide to iode [37]. The fluorescence intensity of modified LDL, corresponding to apolipoproteins modified by lipid peroxidation products (in particular 4-hydroxynonenal) [38], were measured at λₐ = 360 nm/λₑ = 430 nm and at λₐ = 400 nm/λₑ = 470 nm. The electrophoretic mobility of modified LDL relative to native LDL was measured using agarose gels and barbital buffer (Sebia, Issy-les-Moulineaux, France). Free amino groups were measured using trinitrobenzenesulphonic acid [39].

Measurement of autoantibodies against native LDL (Ab-LDL), Ab-OxLDL and Ab-MDA-LDL

Autoantibodies were measured by non-competitive ELISA. A 96-well microtitre plate (Costar, Cambridge, MA, U.S.A.) was divided into four quarters, and one quarter was coated with 200 μl of 2% (w/v) BSA, one quarter with native LDL (100 μl; 5 μg/ml), one quarter with OxLDL (100 μl; 5 μg/ml) and the final quarter with MDA-LDL (100 μl; 5 μg/ml) in PBS containing 2.7 mmol/l EDTA and 20 μmol/l butylated hydroxytoluene to prevent LDL oxidation. The plates were incubated for 16 h at 4 °C. The plates were then washed four times with 0.1 mol/l PBS containing 0.05% Tween 20, 0.1% BSA and 0.001% aprotinin, and then blocked with 200 μl of 2% BSA in 0.1 mol/l PBS for 1 h at room temperature. Plates were subsequently washed an additional four times with 0.1 mol/l PBS, and then serum diluted 1:20 (v/v) in buffer A (0.1 mol/l PBS containing 0.05% Tween 20 and 0.2% BSA) was incubated for 2 h at room temperature. Plates were then washed four times with buffer A. Peroxidase-conjugated anti-(rabbit IgG) prepared from goat IgA (Sigma) and diluted 1:3000 in buffer A was added (100 μl), and the mixture was left for 2 h at 37 °C. After four successive washings with buffer A, peroxidase substrate (o-phenylenediamine; 100 μl, 1.5 g/l) in solution in phosphate citrate buffer (pH 5.5) containing hydrogen peroxide (3.5 mmol/l) was added to each well. The reaction, carried out in the dark, was stopped with 100 μl HCl (1 mol/l) after 30 min; absorbance was measured at 492 nm. Autoantibody titres were expressed in absorbance units. The within-run and between-run coefficients of variation were 6% and 9% respectively. All samples from one animal (i.e. weeks 0, 2, 6, 10 and 14) were analysed on the same plate in duplicate, and samples from one control rabbit were paired with samples from one transgenic rabbit on the same plate.

Measurement of Ab-C

A 96-well microtitre plate was coated with cardiolipin (100 μl, 10 μg/ml; Sigma E-5646; diphasphatidylglycerol) in solution in 0.1 mol/l PBS and incubated overnight at 4 °C. After four washings with PBS containing 0.1% fatty acid-free BSA (Sigma) and 0.05% Tween 20, non-specific binding sites were saturated by addition of 200 μl of 2% fatty acid-free BSA for 2 h at room temperature. The plate was washed another four times with 0.1 mol/l PBS containing 0.05% Tween 20 and 0.1% fatty acid-free BSA. Then serum diluted 1:100 in buffer A (in which the BSA was fatty acid-free) was incubated for 2 h at room temperature; the blank consisted of 100 μl of buffer A. The plate was washed four times with buffer A, and peroxidase-conjugated goat anti-(rabbit IgG) (Sigma), diluted 1:5000 in buffer A, was added (100 μl), followed by incubation at 37 °C for 2 h. Peroxidase-conjugated anti-(rabbit IgG) activity was measured as described above. Ab-C titres were expressed in absorbance units. All samples from one animal (weeks 0, 2, 6, 10 and 14) were analysed on the same plate in duplicate, and samples from one control rabbit were paired with samples from one transgenic rabbit on the same plate.
Measurement of IgG-LDL
Goats were immunized with rabbit LDL, and immunoglobulins (IgG) were isolated by precipitation. IgG-LDL was measured using a bi-site ELISA method. Microtitre plates were coated overnight at room temperature with goat immunoglobulins in solution (10 μg/ml; 100 μl) in 0.1 mol/l PBS. These immunoglobulins contain antibodies that specifically recognize rabbit apo B that will capture rabbit LDL and IgG-LDL. Each plate included negative controls without capture antibodies. After washing four times with 0.1 mol/l PBS containing 0.05 % Tween 20, 0.1 % (w/v) BSA and 0.001 % aprotinin, the solid-phase antibody was saturated with 5 % BSA/0.1 mol/l PBS for 2 h at room temperature. Plates were then washed four times with 0.1 mol/l PBS. After washing, the solid-phase antibody was incubated in duplicate with antigen (10 μl of serum diluted 1:500 in buffer A). After 2 h of incubation at room temperature, wells were washed four times with buffer A. Then peroxidase-conjugated goat anti-(rabbit IgG) diluted 1:5000 in buffer A was added (100 μl), and the mixture was left for 2 h at 37 °C. This antibody does not cross-react with the goat capture antibodies. After washing four times with 0.1 mol/l PBS, enzymic substrate (o-phenylenediamine) was added to develop the reaction as described above, and absorbance was read at 492 nm. The IgG-LDL titre was expressed in absorbance units. All parameters at every point of the protocol between the two groups were evaluated by the Mann–Whitney U-test.

Measurement of PON-1 activity
PON-1 activity was measured using the method of Mackness et al. [21]. Substrate (paraoxon; Sigma) was diluted in Tris buffer (100 mmol/l Tris and 2 mmol/l CaCl₂, pH 8) at a concentration of 2 mmol/l. The reaction was initiated by adding 10 μl of serum diluted previously 1:10 (v/v) in 10 μmol/l eserine (Sigma) in 1 ml of substrate, and p-nitrophenol formation was quantified by monitoring the absorbance at 405 nm at 25 °C for 5 min.

Evaluation of atherosclerosis
Animals were killed by an overdose of intravenous ketamine (80 mg/kg) and carotid exsanguination. The thoracic aorta from the aortic valves to the diaphragm, above the coeliac artery, and the abdominal aorta from the diaphragm to the iliac bifurcation were removed and stripped of adventitial fat and tissue. Then aortas were cut lengthwise into right and left halves by dorsal and ventral incisions, and fixed in 10 % buffered formalin. The fixed aortas were stained with Oil Red O to reveal fatty deposits. Morphometric assessment of the percentage of the total aorta covered by lipid deposits was determined by computerized planimetry. For light microscopy observations, segments of the Red O-stained aortas were embedded in paraffin and then stained with haematoxylin/eosin.

Statistical analysis
All data are expressed as means ± S.D. Serum data were evaluated by the Mann–Whitney U-test, to compare each parameter at every point of the protocol between the two groups. Changes in each of these parameters, from week 0 through weeks 2, 6, 10 and 14, were tested by the paired Wilcoxon t test. Differences in atheroma area between the two groups were evaluated by Student’s t test. Coefficients of correlation between the different variables were calculated. P < 0.05 was considered as significant.

RESULTS
Lipoprotein profiles
The basal (week 0) levels of total cholesterol, triacylglycerols and phospholipids were all significantly higher in transgenic rabbits than in controls (Table 1). The increase in total cholesterol corresponded to a 3-fold higher level of HDL cholesterol (P < 0.001), while...
Figure 1  Serum total cholesterol (a), non-HDL cholesterol (b), HDL cholesterol (c), triacylglycerols (d) and phospholipids (e) in control (●) and human apo A-I transgenic (▲) rabbits fed on a 0.4% (w/w) cholesterol diet. All values (means ± S.D.) are in g/l. Significance of differences between transgenic rabbits and controls: *P < 0.05, **P < 0.01, ***P < 0.002, ****P < 0.0001 (Mann–Whitney U-test). Significance of differences compared with week 0: (o) P < 0.05, (oo) P < 0.01, (ooo) P < 0.001.

Table 2  Markers of modified LDL

<table>
<thead>
<tr>
<th>Marker</th>
<th>Cu^{2+}-oxidized LDL</th>
<th>MDA-LDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diene conjugates (nmol/mg of protein)</td>
<td>0.00</td>
<td>743</td>
</tr>
<tr>
<td>Hydroperoxides (nmol/mg of protein)</td>
<td>0.00</td>
<td>553</td>
</tr>
<tr>
<td>TBARS (nmol/mg of protein)</td>
<td>0.00</td>
<td>36</td>
</tr>
<tr>
<td>Fluorescence (λ_{exc} 340/λ_{em} 430 nm) (a.u.)</td>
<td>100</td>
<td>290</td>
</tr>
<tr>
<td>Fluorescence (λ_{exc} 400/λ_{em} 470 nm) (a.u.)</td>
<td>48</td>
<td>110</td>
</tr>
<tr>
<td>Free NH_{2} groups (%)</td>
<td>95.3</td>
<td>62</td>
</tr>
<tr>
<td>Electrophoretic mobility (mm)</td>
<td>23</td>
<td>—</td>
</tr>
<tr>
<td>Electrophoretic mobility (mm) cf. native LDL</td>
<td>1</td>
<td>—</td>
</tr>
</tbody>
</table>

λ_{exc}, excitation wavelength; λ_{em}, emission wavelength; a.u., arbitrary units.
Table 3 Basal levels of immunological markers of LDL oxidation

<table>
<thead>
<tr>
<th>Marker</th>
<th>Controls (n = 18)</th>
<th>Transgenics (n = 13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab-LDL</td>
<td>158 ± 62</td>
<td>199 ± 81</td>
</tr>
<tr>
<td>Ab-OxLDL</td>
<td>221 ± 125</td>
<td>290 ± 155</td>
</tr>
<tr>
<td>Ab-MDA-LDL</td>
<td>522 ± 162</td>
<td>710 ± 309*</td>
</tr>
<tr>
<td>IgG-LDL</td>
<td>427 ± 80</td>
<td>495 ± 104</td>
</tr>
<tr>
<td>Ab-C</td>
<td>164 ± 71</td>
<td>218 ± 100</td>
</tr>
</tbody>
</table>

Figure 2 Serum levels of immunological markers of LDL oxidation in control (●) and human apo A-I transgenic (▲) rabbits fed on a 0.4% (w/w) cholesterol diet

All values (means ± S.D.) are in 1000 × absorbance (A). Significance of differences between transgenic rabbits and controls: * P < 0.05, ** P < 0.01. Significance of differences compared with week 0: (o) P < 0.05, (oo) P < 0.01, (ooo) P < 0.001.

Non-HDL cholesterol levels were similar between the groups. The serum level of human apo A-I in the transgenic rabbits was 1.50 ± 0.31 g/l.

During cholesterol feeding, total cholesterol levels increased progressively and dramatically in both groups (Figure 1a), and the significant difference in levels between the groups had disappeared at weeks 6 and 10, but not at week 14. This increase in total cholesterol was mediated by a rise in the non-HDL fraction (Figure 1b), while HDL cholesterol levels were maintained at their

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Table 4 Correlation coefficients between the different immunological markers of LDL oxidation at week 0 (a) and at week 14 (b)

Significance: *P < 0.05; **P < 0.02; ***P < 0.001. C, controls; T, human apo A-I transgenic rabbits.

(a) Week 0

<table>
<thead>
<tr>
<th></th>
<th>Ab-LDL</th>
<th>Ab-Ox-LDL</th>
<th>Ab-MDA-LDL</th>
<th>IgG-LDL</th>
<th>Ab-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab-LDL</td>
<td>C</td>
<td>I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ab-Ox-LDL</td>
<td>C 0.613***</td>
<td>I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T 0.642**</td>
<td></td>
<td>I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ab-MDA-LDL</td>
<td>C 0.617***</td>
<td>0.627***</td>
<td>I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T 0.643**</td>
<td>0.657**</td>
<td>I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG-LDL</td>
<td>C 0.375</td>
<td>0.493*</td>
<td>0.775***</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>T 0.780***</td>
<td>0.499</td>
<td>0.636**</td>
<td>I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ab-C</td>
<td>C 0.592***</td>
<td>0.636*</td>
<td>0.696***</td>
<td>0.559**</td>
<td>I</td>
</tr>
<tr>
<td>T 0.887***</td>
<td>0.631**</td>
<td>0.680***</td>
<td>0.759***</td>
<td>I</td>
<td></td>
</tr>
</tbody>
</table>

(b) Week 14

<table>
<thead>
<tr>
<th></th>
<th>Ab-LDL</th>
<th>Ab-Ox-LDL</th>
<th>Ab-MDA-LDL</th>
<th>IgG-LDL</th>
<th>Ab-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab-LDL</td>
<td>C</td>
<td>I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ab-Ox-LDL</td>
<td>C 0.715***</td>
<td>I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T -0.476</td>
<td></td>
<td>I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ab-MDA-LDL</td>
<td>C 0.537**</td>
<td>0.880***</td>
<td>I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T -0.507</td>
<td>-0.325</td>
<td>I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG-LDL</td>
<td>C 0.168</td>
<td>0.493*</td>
<td>-0.537**</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>T -0.449</td>
<td>-0.290</td>
<td>-0.026</td>
<td>I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ab-C</td>
<td>C 0.471*</td>
<td>0.470*</td>
<td>-0.396</td>
<td>-0.044</td>
<td>I</td>
</tr>
<tr>
<td>T 0.730***</td>
<td>-0.370</td>
<td>-0.143</td>
<td>0.640**</td>
<td>I</td>
<td></td>
</tr>
</tbody>
</table>

Immunological markers of LDL oxidation

Chemical characteristics of rabbit LDLs used as antigens

Cu²⁺-oxidized LDL and MDA-LDL were used as antigens after extensive dialysis for 24 h at 4 °C to remove EDTA, metals and water-soluble aldehydes. Dialysed OxLDL showed signs of lipid peroxidation, such as increases in diene conjugates and hydroperoxide content (Table 2). Levels of TBARS were very slightly increased in dialysed OxLDL, indicating that only very small amounts of MDA were tightly bound to apo B. On the other hand, the fluorescence intensity of these lipoproteins was greatly increased, while levels of free NH₂ residues were decreased (−40%), suggesting that other lipid peroxidation products besides MDA (e.g. 4-hydroxynonenal) were bound to apo B. The decrease in the free NH₂ residue content was concomitant with an increase in electrophoretic mobility (1.30-fold compared with that of native LDL). Lipids of dialysed MDA-LDL were hardly oxidized at all (absence of diene conjugates and of hydroperoxides), although they contained many TBARS (MDA) molecules bound to apo B, which dramatically increased their fluorescence intensity and their electrophoretic mobility (1.60-fold compared with native LDL), and concomitantly decreased their content of free NH₂ residues (−70%).

Serum levels of immunological markers of LDL oxidation

These are reported in Table 3. Before cholesterol feeding was started (week 0), serum levels of Ab-LDL, Ab-Ox-LDL, IgG-LDL and Ab-C were similar in the control.
Table 5 Correlation coefficients between plasma lipids and immunological markers of LDL oxidation at week 0 (a) and week 14 (b)

<table>
<thead>
<tr>
<th></th>
<th>Ab-LDL</th>
<th>Ab-Ox-LDL</th>
<th>Ab-MDA-LDL</th>
<th>IgG-LDL</th>
<th>Ab-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDL cholesterol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.152</td>
<td>0.372</td>
<td>0.041</td>
<td>0.163</td>
<td>0.203</td>
</tr>
<tr>
<td>T</td>
<td>0.666**</td>
<td>0.670**</td>
<td>0.326</td>
<td>0.517</td>
<td>0.619**</td>
</tr>
<tr>
<td>Non-HDL cholesterol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>—0.136</td>
<td>0.106</td>
<td>—0.298</td>
<td>—0.110</td>
<td>—0.006</td>
</tr>
<tr>
<td>T</td>
<td>0.347</td>
<td>0.444</td>
<td>0.192</td>
<td>0.142</td>
<td>0.264</td>
</tr>
</tbody>
</table>

(a) Week 0

(b) Week 14

Figure 2 reports the time course of changes in serum immunological OxLDL markers during cholesterol feeding. Ab-LDL levels were not modified during the entire period in either group (Figure 2a), while Ab-OxLDL levels increased significantly from week 10 in the control group and non-significantly in the transgenic group (Figure 2b). At all time points there were no significant differences in serum levels of Ab-LDL or Ab-OxLDL between control and transgenic rabbits.

Ab-MDA-LDL serum levels decreased significantly from week 2 in both groups (Figure 2c). These levels were significantly higher in the transgenic animals than in the controls until week 10.

IgG-LDL serum levels increased significantly from week 2 in the control group and from week 6 in the transgenic group (Figure 2d), but there was never any significant difference between the two groups.

Ab-C serum levels increased significantly from week 2 in controls and from week 6 in transgenic rabbits (Figure 2e). These levels were higher in the transgenic group than in the control group, but the differences were not significant.

Before cholesterol feeding was started (week 0), all immunological OxLDL markers were correlated positively, except for IgG-LDL with Ab-LDL in the control group and with Ab-OxLDL in the transgenic group (Table 4a). At the end of the experiment (week 14), Ab-C levels were no longer correlated with Ab-MDA-LDL or IgG-LDL levels in the control group (Table 4b). In the transgenic group there was no longer any correlation between the immunological markers of LDL oxidation, with the exception of positive correlations of Ab-C with Ab-LDL and IgG-LDL (Table 4b).

Ab-LDL, Ab-OxLDL and Ab-C levels were highly positively correlated with HDL cholesterol at week 0 in the human apo A-I transgenic rabbit group (Table 5a). There was a negative correlation at week 14 between Ab-LDL and HDL cholesterol in the control group (Table 5b).

Changes in serum PON-1 activity during cholesterol feeding, and relationships with HDL cholesterol and immunological OxLDL markers

Before cholesterol feeding was started (week 0), serum PON-1 activity was higher in the transgenic rabbits than in the controls (3608 ± 912 and 3097 ± 653 nmol·min⁻¹·ml⁻¹), but, due to the high S.D.s, the difference was not significant (P = 0.09) (Figure 3). During cholesterol feeding, PON-1 activity decreased progressively in both groups; differences between the groups remained non-significant. After 14 weeks on the diet, PON-1 activity was decreased by 43 ± 16% and 50 ± 11% in control and transgenic rabbits respectively.

At weeks 0 and 14, PON-1 activity was significantly and positively correlated with the HDL cholesterol level in control rabbits, but not in transgenic animals (Table 6). At week 0, PON-1 activity was positively correlated with Ab-MDA-LDL in the transgenic group, while at week 14 there was no correlation between any immunological marker of LDL oxidation and PON-1 activity in either group (Table 6).
Relationship between immunological OxLDL markers, lipoprotein concentrations, PON-1 activity and the development of atherosclerosis

After 14 weeks of cholesterol feeding, the percentage area of the aorta covered by lipid deposits was similar in control and transgenic rabbits (23 ± 14% and 26 ± 11% respectively; \( P = 0.52 \)). Table 7 shows that these surface areas were not significantly correlated with the serum levels of cholesterol, nor with the immunological markers of LDL oxidation or PON activity.

DISCUSSION

The present study does not confirm our preliminary results showing that human apo A-I transgenic rabbits (line 20) developed less atheroma when they were fed on...
a cholesterol-rich diet [19], but confirms our data from human apo A-I transgenic rabbits of line 8 [31] showing that these animals were not protected against atherosclerosis when they were fed a cholesterol-rich diet. However, there were some differences between the experimental protocols of these studies. In the earliest study [19], transgenic rabbits belonged to line 20 of transgenesis, and expressed five copies of the human apo A-I transgene; in the other studies they belonged to line 8, and expressed only two copies of this transgene. Nevertheless, basal HDL cholesterol and human apo A-I serum levels were similar in the transgenic groups of the two lines. One major difference between the three studies is that, in the earliest study [19], transgenic rabbits were given a 0.3% (w/w) cholesterol diet after an initial 2 weeks of supplementation with a 0.4% (w/w) cholesterol diet, while control rabbits remained on a diet supplemented with 0.4% (w/w) cholesterol during the entire 14 weeks of treatment. In the present study, and in the study by Mackness et al. [31], control and transgenic rabbits were fed a 0.4% (w/w) cholesterol diet during the entire 14 weeks of the protocol. In the earliest study [19], the goal of this adjustment was to produce similar levels of non-HDL cholesterol in the two groups of rabbits. These non-HDL cholesterol levels did not actually differ between the two groups during the 14 weeks of treatment, whereas in the present study non-HDL cholesterol levels were significantly higher in the transgenic group than in the control group at week 14.

As oxidation of LDL is the primary step in the development of atherosclerosis [1] one goal of this study was to determine the evolution in the serum concentrations of immunological markers of LDL oxidation during a cholesterol feeding period that induces atheroma formation in control and human apo A-I transgenic rabbits. Ab-MDA-LDL serum levels were significantly higher in apo A-I transgenic rabbits than in controls because of the higher triacylglycerol and phospholipid plasma concentrations. This increase in phospholipid concentration was linked largely to the 3-fold higher level of HDL in transgenic animals, but the increase in triacylglycerols was due partly to increases in HDL triacylglycerol and in non-HDL triacylglycerol (results not shown).

The LDL of patients with diabetes, who have higher serum triacylglycerol and phospholipid concentrations, is more susceptible to lipid oxidation than the LDL of control patients [38]. Therefore, by analogy with the human diabetic condition, we speculate that the degree of LDL oxidation in vivo is higher in apo A-I transgenic rabbits than in controls because of the higher triacylglycerol and phospholipid plasma concentrations. This would lead to the formation of more oxidized immunogenic LDL and to the production of more Ab-MDA-LDL.

We have already shown that endothelial-derived vasorelaxation is impaired in human apo A-I transgenic rabbits fed on a normal chow diet [40], and we have suggested that increases in triacylglycerols and phospholipids in the lipoproteins of these animals would exacerbate lipoprotein oxidation, which would in turn inhibit endothelial-dependent vasorelaxation [41–49]. Increases in LDL oxidation might not be the only cause of lipid oxidation in human apo A-I transgenic rabbits fed a standard chow diet (and of the resulting increase in Ab-MDA-LDL plasma levels), because the possibility cannot be excluded that high plasma levels of HDL cholesterol (and HDL phospholipid) may induce HDL oxidation [50] and the synthesis of autoantibodies against oxidized HDL that would also recognize OxLDL. The strong positive correlation between HDL cholesterol and Ab-LDL, Ab-OxLDL and Ab-C at week 0 in the human apo A-I transgenic rabbit group supports the hypothesis that high plasma levels of HDL cholesterol would stimulate the immunological response against lipids.

We made the unexpected observation that Ab-MDA-LDL titres decreased progressively in both groups of animals during the cholesterol feeding period, while the titres of other immunological markers increased. Ab-MDA-LDL titres have been reported to be higher in patients suffering from carotid atherosclerosis [3,51] and in hypercholesterolaemic apo E-deficient mice which develop atherosclerosis [52]. In order to explain our observation, we speculated on the heterogeneity of anti-OxLDL autoantibodies. Mironova et al. [4] have isolated human anti-OxLDL autoantibodies. All purified IgG antibodies showed some cross-reactivity with MDA-LDL, native LDL and cardiolipin, and had moderate-to-low affinity for OxLDL. We hypothesize that, in the present study, Ab-MDA-LDL had high affinity, and Ab-OxLDL and Ab-C had low affinity, for in vivo OxLDL. We speculate that MDA-LDL is present at high concentrations in rabbits during the cholesterol feeding period and that, due to their high affinity, Ab-MDA-LDL bind to the MDA-LDL to constitute LDL immune complexes. This mechanism would explain why IgG-LDL concentrations increased progressively while Ab-MDA-LDL levels decreased progressively during the cholesterol feeding period. Because of their putative low affinity for OxLDL, Ab-OxLDL and Ab-C would not bind to the circulating OxLDL and would remain free in the serum and not associated with IgG-LDL, which accounts for the standard increases reported in the serum concen-
trations of Ab-OxLDL and Ab-C during the development of atherosclerosis. Recently, it has been reported that autoantibodies against MDA-LDL are reduced after acute feeding in patients with coronary artery disease [53]. This suggests that binding of MDA-LDL epitopes to circulating Ab-MDA-LDL would account for the observed postprandial decrease in Ab-MDA-LDL.

We have shown in the present study that increases in Ab-C levels occur before increases in Ab-OxLDL. We may therefore conclude that, in human patients free of anti-phospholipid syndrome, it would be more sensible to measure Ab-C rather than anti-OxLDL titres in order to evaluate atheroma development [10,54].

Changes in Ab-OxLDL, Ab-C and IgG-LDL concentrations during the cholesterol feeding period showed no differences between apo A-I transgenic and control rabbits, in accordance with the identical development of atherosclerosis in the two groups. Furthermore, there was no correlation between serum levels of these markers of LDL oxidation and atherogenic lipoprotein concentrations (non-HDL cholesterol) in either group of rabbits, as has been reported previously in humans [54,55].

There was no correlation between titres of immunological markers of LDL oxidation and the extension of aortic atheroma in rabbits. These data are in contrast with the report of a correlation between the development of atherosclerosis and titres of autoantibodies against OxLDL in humans [3,54]. Nevertheless, the putative correlation between plasma levels of immunological markers of LDL oxidation and the development of atherosclerosis in humans is highly controversial [55].

Duverger et al. [18,19] reported that the reduced development of atherosclerosis in human apo A-I transgenic rabbits was due to their high HDL cholesterol levels and to a subsequent increase in reverse cholesterol transport. However, it has been proposed that an increase in reverse cholesterol transport is not the only mechanism of inhibition of atheroma development induced by HDL, because HDL also inhibits the oxidation of LDL [20–22,28,30]. PON-1 is located in HDL, and may be the component of HDL responsible for decreasing the accumulation of lipid peroxidation products in LDL [27]. As PON-1 is linked exclusively to HDL, its activity tended to be higher (without the difference reaching significance) in the apo A-I transgenic rabbits, which had a 3-fold higher level of HDL cholesterol than the controls. (In a subsequent protocol carried out with 99 controls and 92 transgenic rabbits, PON-1 activity was significantly higher in the transgenic animals before starting cholesterol feeding [31].) In the present study there were positive correlations between HDL cholesterol levels and PON-1 activity in control rabbits at week 0 and at week 14. During the cholesterol feeding period, PON-1 activity decreased progressively and similarly in both groups (−43 ± 16% and −50 ± 11% at week 14 in comparison with week 0 in control and transgenic rabbits respectively). During this period the decrease in PON-1 activity was correlated with the decrease in HDL cholesterol levels. There was no negative correlation between serum levels of immunological markers of LDL oxidation and PON-1 activity in either group before starting cholesterol feeding (week 0) or after 14 weeks of treatment. We may speculate that a negative correlation would have been apparent between PON-1 activity and in vivo markers of LDL oxidation if PON-1 had been effective in reducing LDL oxidation in vivo. In the present study we show that PON-1 activity decreased similarly in cholesterol-fed control and human apo A-I transgenic rabbits, and that increases in serum immunological markers of LDL oxidation and the development of atherosclerosis were similar in the two groups. These data suggest that, under these experimental conditions, high serum HDL cholesterol levels are ineffective in inhibiting the development of atherosclerosis in human apo A-I transgenic rabbits when compared with control rabbits, perhaps because their associated PON-1 activity does not induce greater inhibition of LDL oxidation than does the HDL-associated PON-1 activity in control rabbits.

The high-cholesterol-fed rabbit is probably not a convenient model in which to study the mechanisms of atheroprotection in relation to the antioxidative properties of HDL, because of the extravagantly high levels of atherogenic lipoproteins present in the serum of these animals in comparison with the levels of anti-atherogenic lipoproteins (control rabbits after 14 weeks of cholesterol feeding: non-HDL cholesterol, 17.27 g/l; HDL cholesterol, 0.14 g/l). In this case the inhibition of LDL oxidation by HDL and the associated PON-1 activity is a marginal phenomenon; however, this does not exclude its possible relevance in humans, where LDL cholesterol levels are often below 1.90 g/l and HDL cholesterol levels are over 0.30 g/l. In normolipidemic patients, the molar ratio between HDL and LDL could be instrumental in the inhibition of LDL oxidation by HDL. Nevertheless, our study suggests that, in patients with severe heterozygous or homozygous familial hypercholesterolaemia, as well as in those with severe mixed dyslipidaemia, an isolated increase in HDL cholesterol levels would not be effective in inhibiting the development of atherosclerosis, since the lack of a significant increase in PON-1 activity means that there would be no corresponding inhibition of LDL oxidation.

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Oxidative markers in human apolipoprotein A-I transgenic rabbit

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