LDL Oxidation in Patients With Severe Carotid Atherosclerosis
A Study of In Vitro and In Vivo Oxidation Markers

Elena Maggi, Roberto Chiesa, Germano Melissano, Renata Castellano, Domenico Astore, Adalberto Grossi, Giorgio Finardi, Giorgio Bellomo

Abstract Among the various risk factors involved in the development and progression of carotid atherosclerosis, the oxidation of LDL has been proposed to play a relevant role. LDL oxidation has been investigated in 94 patients with severe carotid atherosclerosis undergoing elective carotid artery endarterectomy and in 42 matched control subjects. LDL oxidation was evaluated in all patients as (1) the susceptibility to in vitro oxidation, (2) vitamin E concentration and its efficiency in LDL, and (3) the presence of autoantibodies against oxidatively modified lipoprotein to monitor the occurrence of the oxidative processes taking place in vivo. No difference was detected between control subjects and patients concerning vitamin E concentration and the kinetics of conjugated diene formation in isolated LDL exposed to CuSO₄. However, vitamin E efficiency was lower (9.6±4.2 versus 30.2±7.6 min/mmol vitamin E) and the duration of the vitamin E–independent lag phase was longer (105.5±16.5 versus 58±11.8 minutes) in the patient group. Autoantibodies against oxidatively modified lipoproteins were measured with an ELISA method using native LDL, Cu²⁺–oxidized LDL (oxLDL), or malondialdehyde-derivatized LDL (MDA-LDL) as antigens. To monitor cross-reactivity of the antibodies detected with other oxidatively modified proteins, human serum albumin (HSA) and MDA-derivatized HSA (MDA-HSA) were also employed. The antibody titer was calculated as the ratio of antibodies against modified versus native proteins. Patients with carotid atherosclerosis had an antibody ratio significantly higher than control subjects in regard to anti-oxLDL IgG (1.78±0.39 versus 1.05±0.3) and IgM (1.98±0.83 versus 1.40±0.09) and anti-MDA-LDL IgG (2.39±0.51 versus 2.04±0.11) and IgM (4.18±1.89 versus 2.9±0.15). The highest titers were found in patients with associated hyperlipidemia and hypertension, alone or in combination. On the other hand, the anti–MDA-HSA antibody titer did not differ between the two groups of patients investigated. These data indicate that patients with severe carotid atherosclerosis specifically develop autoantibodies against oxidatively modified LDL and, despite an apparently “normal” oxidation profile in vitro, provide support for the occurrence of an enhanced LDL oxidation in vivo. (Arterioscler Thromb. 1994;14:1892-1899.)

Key Words • low-density lipoprotein • free radicals • oxidation • antioxidants • atherosclerosis • anti-oxidized LDL autoantibodies • carotid endarterectomy

Carotid atherosclerosis, ranging from intima-media thickening to the appearance of more advanced lesions such as fibrotic and complicated plaques, affects about 25% of the whole population. Although most of the patients remain asymptomatic for years, the occurrence of severe complications such as stroke represents a frightening possibility. Various cardiovascular risk factors have been implicated in the pathogenesis and development of carotid atherosclerosis, including essential and renovascular hypertension, hyperlipidemia, elevated mean levels of lipoprotein (a), diabetes mellitus, and cigarette smoking. Recently, Salonen et al reported that the titer of autoantibodies to oxidatively modified LDL was an independent predictor of the progression of carotid atherosclerosis in Finnish men and suggested that oxidation of LDL may represent a crucial event of atherogenesis in the carotid wall.

LDL oxidation has been claimed to play a fundamental role in the formation and progression of early atherosclerotic lesions. Endothelial and smooth muscle cells as well as monocytes and macrophages are able to trigger the peroxidation of LDL lipids in the subendothelial space, leading to the derivatization of the apo-lipoprotein (Apo) B100 molecule with aldehydic products such as malondialdehyde (MDA) and 4-OH- nonenal.11 Oxidative modifications of LDL generate molecular epitopes that provide chemotactic stimuli for monocyte recruitment,12 are more avidly taken up by macrophages, thus forming foam cells,13 and are cytotoxic for endothelial cells.14 Oxidized lipoproteins (ox-LDL) are more atherogenic than their parent forms, and the demonstration that LDL oxidation does actually occur in vivo has promoted clinical investigations to ascertain the role played by this process in the progression of atherosclerosis in humans.

The methodologies employed to investigate LDL oxidation in single individuals or groups of patients can be divided into two major categories depending on their end points: (1) the evaluation of the oxidizability of isolated LDL in vitro and (2) the detection of oxidized LDL in vivo.

To investigate LDL oxidizability, lipoproteins are isolated from the plasma and then are challenged with...
pro-oxidant stimuli. The kinetics of LDL oxidation is then measured as the formation of conjugated dienes, 16 oxysterols, 17 and various aldehydic products (thiobarbituric acid–reactive substances) 18 by fluorescence development 19 and by changes of the electrophoretic mobility of Apo B 100. 20 With the exception of a few studies, the vast majority of clinical investigations of LDL oxidation have concerned the demonstration of a decreased resistance of LDL to in vitro oxidation in conditions associated with accelerated atherosclerosis, such as hypercholesterolemia, 19 coronary heart disease, 21 uremia, 22 and hypertension. 23 Moreover, since the susceptibility of LDL to oxidation depends on both the entity of pro-oxidant stimuli and antioxidant concentration within the LDL particle, the level of vitamin E (the most abundant antioxidant in LDL) has also often been measured. 22, 23

An unresolved and definitely critical issue for considering the reported changes indicative of a real risk for lipoprotein oxidation is the definition of how relevant a decrease in LDL resistance to in vitro oxidation is for the oxidative modifications occurring in vivo. Following such modifications, antigenic epitopes are generated and elicit an immune response. Among these modifications is the derivatization of the ε-amino groups of lysine in Apo B 100 with MDA 10 and the detection of anti–MDA-LDL autoantibodies has been proposed as the biological signature of LDL oxidation occurring in vivo. 9 However, since the advent MDA-lysine is the antigenic determinant of the immune reaction, MDA-derivatization of a variety of other proteins different from LDL would generate autoantibodies cross-reacting with MDA-LDL 24 and would give false-positive values.

The present study was performed to investigate the markers of in vitro and in vivo LDL oxidation in a population of patients with severe carotid atherosclerosis. LDL oxidizability risk was evaluated as both the susceptibility to in vitro oxidation and vitamin E concentration and efficiency. LDL oxidation in vivo was evaluated as the presence of autoantibodies against Cu 2+–oxidized and MDA-derivatized LDL. The absence of a significant cross-reactivity with MDA-derivatized HSA was employed as a criterion to assess the specificity of the autoantibodies detected for the LDL molecule.

Methods

Patients

Forty-two subjects (28 men and 14 women, 61.8±7.3 years old) without dyslipidemia, hypertension, diabetes mellitus, or any clinically evident sign of atherosclerosis were included in the control group (Table 1).

Ninety-four unselected patients (68 men and 26 women, 67.3±8.18 years old) scheduled for elective carotid artery endarterectomy were taken as a group of patients with severe carotid atherosclerosis. The initial diagnosis of carotid atherosclerosis was made with ultrasonography; however, the preoperative evaluation of all patients included either intrarterial digitized subtraction angiography or magnetic resonance angiography and cerebral computerized tomographic scan. The incidence of cardiovascular risk factors and the actual therapy in the patient group are reported in Table 1.

The two groups also differ in regard to plasma concentrations of total cholesterol and LDL cholesterol, which were significantly higher in the group of patients with carotid atherosclerosis (Table 1).

Table 1. Demographic and Biochemical Features of Control Subjects and Patients With Severe Carotid Atherosclerosis Undergoing Carotid Artery Endarterectomy

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control Subjects</th>
<th>Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>42</td>
<td>94</td>
</tr>
<tr>
<td>Age, y</td>
<td>61.8±7.3</td>
<td>67.3±8.18</td>
</tr>
<tr>
<td>Sex, M/F</td>
<td>28/14</td>
<td>68/26</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>82.1±9.8</td>
<td>102±37.3</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>183±9.8</td>
<td>222±42.8*</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dl</td>
<td>101±7.6</td>
<td>142±32.7*</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dl</td>
<td>50.5±2.7</td>
<td>44.1±15.6</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>91.8±37.2</td>
<td>150±64.7</td>
</tr>
<tr>
<td>Cardiovascular risk factors, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>0</td>
<td>65 (69)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>0</td>
<td>20 (21)</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>0</td>
<td>25 (28)</td>
</tr>
<tr>
<td>Obesity</td>
<td>4 (9.5)</td>
<td>7 (7)</td>
</tr>
<tr>
<td>Cigarette smoking</td>
<td>8 (19)</td>
<td>22 (23)†</td>
</tr>
<tr>
<td>Current therapy, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin</td>
<td>0</td>
<td>4 (4)</td>
</tr>
<tr>
<td>Ca 2+ channel blockers</td>
<td>0</td>
<td>47 (50)</td>
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<tr>
<td>β-Blockers</td>
<td>0</td>
<td>5 (5)</td>
</tr>
<tr>
<td>ACE inhibitors</td>
<td>0</td>
<td>12 (13)</td>
</tr>
<tr>
<td>Antiaggregating agents</td>
<td>0</td>
<td>35 (37)</td>
</tr>
<tr>
<td>No therapy</td>
<td>42 (100)</td>
<td>13 (14)</td>
</tr>
</tbody>
</table>

ACE indicates angiotensin-converting enzyme. Results concerning biochemical data are expressed as mean±SD.

*Significantly different from control subjects (P<.05).
† Twenty-three patients (24%) were ex-smokers.

Evaluation of Plasma Lipid Profile

Plasma obtained from EDTA-supplemented blood (1 mg EDTA/mL blood) was used for measuring lipid parameters. Cholesterol was assayed by an enzymatic method as total cholesterol or as HDL cholesterol in the supernatant obtained after centrifugation of dextran sulfate–precipitated plasma. Triglycerides were measured by an enzymatic-colorimetric method. LDL cholesterol concentration was calculated from total and HDL cholesterol and triglyceride levels by use of Friedewald’s formula.

LDL Isolation

The experimental protocol described by Esterbauer et al 10 was used. Briefly, venous blood was taken from each patient, after overnight fasting, in polypropylene tubes containing K 3-EDTA (final concentration, 1 mg EDTA/mL blood), and plasma was collected after centrifugation. The LDL fraction was isolated from the whole plasma by ultracentrifugation through a KBr discontinuous gradient and collected as the fraction floating at a density of 1.019 to 1.063 g/mL. EDTA was then removed by rapid filtration through disposable desalting columns (Econo-Pac 10 DG, Bio-Rad), and LDLs were resuspended in oxygen-saturated phosphate-buffered saline (PBS) (10 mmol/L Phosphate, pH 7.2) at a concentration of 0.25 mg LDL mass/mL buffer (50 μg LDL protein/mL=0.1 μmol/L).

LDL Oxidation

LDL oxidation was triggered by the addition of 2 μmol/L CuSO 4 and continuously monitored spectrophotometrically at...
234 nm to follow the formation of conjugated dienes. The oxidation curve obtained was characterized by three parameters (Fig 1). The lag phase (expressed in minutes), that is, the interval between the addition of CuSO₄ and the beginning of the extensive oxidation, was measured on the basis of the intercept between the baseline and the tangent to the rapid oxidation phase. The propagation rate (expressed in changes in absorbance per minute) is the maximal rate of LDL oxidation detected in the kinetic curve. The peak time (expressed in minutes) is the interval between the addition of CuSO₄ and the time at which the maximum absorbance at 234 nm is reached.

Vitamin E Determination

Vitamin E in LDL was determined as described.²⁵ Briefly, LDLs were precipitated with ethanol, and subsequently, vitamin E was extracted with hexane. The hexane phase was then evaporated, and the residue was dissolved in methanol and separated by high-performance liquid chromatography.

The concentration of an antioxidant is not sufficient to describe its efficiency in preventing oxidation. Esterbauer et al.²⁶ addressed this problem by correlating the duration of the lag phase with the concentration of vitamin E. The assumed linear correlation is described by the general equation

\[ \text{lag phase} = K \times \text{vitamin E} + a \]

where K (the slope of the correlation line) is the efficacy constant of vitamin E and a (the intercept of the correlation line with the y axis) is the vitamin E-independent variable in the lag phase. The same methodological approach was employed to measure the above-mentioned parameters (vitamin E efficiency and the duration of vitamin E-independent lag phase) for LDL from control subjects and patients with carotid atherosclerosis.

Antigen Preparation

Non-modified LDL and Human Serum Albumin

Native LDLs obtained after plasma ultracentrifugation were kept in saline phosphate (10 mmol/L, pH 7.2) buffer containing EDTA (1 mg/mL) and immediately used to coat ELISA plates. EDTA efficiently protected LDL from oxidation, since it completely prevented the formation of conjugated dienes and thiobarbituric acid–reactive substances. Human serum albumin (HSA) was used as a negative control. Each well was coated with 10 μg antigen (as revealing reagents). The absorbance was measured at 492 nm in an automatic microplate reader.

Oxidized LDL

Following EDTA removal, LDLs were oxidized with CuSO₄, as described above. After 18 hours at 30°C, EDTA (1 mg/mL, final concentration) was added to the incubation mixture to stop the oxidation process.

MDA-LDL and MDA-HSA

Freshly isolated LDL (2 mg/mL) or HSA (2 mg/mL) was incubated for 3 hours at 37°C with 0.5 mmol/L MDA obtained by acid hydrolysis of MDA-bisdimethyl acetal. Unbound MDA was then removed by rapid filtration through disposable desalting columns. Under these conditions, a large fraction of the e-amino group in lysine residues was derivatized.

Evaluation of MDA-Lysine Adduct in Modified Proteins

The presence of MDA adduct in modified proteins was detected by scanning fluorescence spectroscopy. Native or modified LDL (1 mg/mL) and HSA (1 mg/mL) were resuspended in 10 mmol/L phosphate buffer, pH 7.2, and analyzed with a Perkin-Elmer LS-5B Spectrofluorometer. The quantification of fluorescence intensity at 400 nm excitation/470 nm emission was taken as an indirect measure of the protein-MDA adduct formation.²⁷ Following Cu²⁺-induced LDL oxidation, the fluorescence intensity (arbitrary units) increased from 22.1±18 to 38.8±4.4 (P<.001). Following LDL derivatization with MDA, the fluorescence intensity (arbitrary units) increased from 22.1±18 to 93.4±5.6 (P<.001). Furthermore, Cu²⁺-oxidized LDL used for coating the microtiter plates was characterized as concerning the formation of conjugated dienes (measured as increase in the absorbance at 234 nm), the generation of thiobarbituric acid–reactive substances (which increased from 0.8±0.3 to 16.3±3.5 nmol MDA Eq/mg cholesterol), and the increase of the negative charge (as revealed by changes in the electrophoretic mobility).

Measure of Anti-oxLDL and Anti–MDA-Modified LDL and HSA Autoantibodies

The different antibodies were detected with an ELISA method. Disposable, 96-well, polystyrene plates (Corning) were used. Antigens for this assay included native LDL and underivatized HSA (protected against oxidation by EDTA), oxidized LDL (obtained after extensive oxidation with 2 μmol/L CuSO₄), and LDL and HSA modified with MDA as described above. Each well was coated with 10 μg antigen protein in PBS for 4 hours at 37°C. After removal of the antigen, the remaining binding sites were blocked by use of 3% fetal bovine serum in PBS (coating buffer) for 2 hours at 37°C.

In the present study, 1:11 dilution of serum from each subject was prepared, and 220 μL was added in duplicate to wells coated with native and modified proteins. After 2 hours of incubation at 37°C, wells were decanted and washed four times before an appropriate peroxidase-conjugated antibody specific for IgG or IgM (diluted 1:2000) was added. After 1 hour of incubation at 37°C and extensive washing, the peroxidase activity was developed with phenylenediamine dihydrochloride and H₂O₂ as revealing reagents. The absorbance was measured at 492 nm in an automatic microplate reader.

To calculate antibody titre, we used the ratio between the spectrophotometric reading of anti-modified and anti-native antigen wells, as previously described by Salonen et al.⁸ With this approach, the spectrophotometric readings of anti-native antigen wells represent the corresponding blank of anti-modified antigen wells and reduce the possible detection of false-positive values due to cross-reactivity with both epitopes.

Statistical Analysis

All data were analyzed with Student's t test, ANCOVA, and linear regression analysis using the CSS:Statistica program for personal computers. Results are expressed as mean±SD.

Results

In Vitro Oxidation of LDL From Patients With Carotid Atherosclerosis

An increased susceptibility of LDL to oxidation is basically mirrored by changes in the kinetics of conju-
 gated diene formation following incubation of isolated LDL with Cu^{2+} and is characterized by decreased lag phase and peak time and by an increase of the propagation rate. However, as reported in Table 2, these three parameters did not differ between LDL obtained from control subjects and from patients with carotid atherosclerosis.

**Vitamin E Content and Vitamin E Efficiency in LDL From Patients With Carotid Atherosclerosis**

The duration of the lag phase is a direct measure of the resistance of LDL to oxidation promoted by Cu^{2+} and basically depends on the antioxidant content of LDL. Vitamin E is the most abundant antioxidant present in LDL. Changes in vitamin E concentration result in changes in the susceptibility of LDL to oxidation. As expected, no difference was observed concerning vitamin E concentration in LDL from control subjects and from patients with carotid atherosclerosis (Table 2).

The analysis of the data obtained in patients with carotid atherosclerosis revealed a vitamin E efficiency of 9.59±4.2 minutes lag phase/nmol vitamin E (Table 2), significantly lower than that observed in control subjects (30.2±7.6 minutes lag phase/nmol vitamin E, P<.01) (Table 2). In other words, a change of 1 nmol vitamin E/mg LDL will result in a change of approximately 30 minutes in the lag phase of LDL in control subjects and of only 9 minutes in patients with carotid atherosclerosis (Table 2).

The parameters of lag phase, propagation rate, and peak time in vitro LDL oxidation are presented in Table 2. The propagation rate was 103±28.2 Abs/min x 1000 Abs/min/nmol vitamin E in control subjects and 88±16 Abs/min/nmol vitamin E in patients with severe carotid atherosclerosis. The peak time was 280±8.5 minutes in control subjects and 294±39.4 minutes in patients with severe carotid atherosclerosis. The vitamin E efficiency, defined as the intercept between the correlation line and they axis, was higher in patients with carotid atherosclerosis: that is, a 70% decrease in the efficiency of vitamin E in LDL is probably the most abundant molecular epitope present in LDL.

**Presence of Autoantibodies Against oxLDL, MDA-LDL, and MDA-HSA in Patients With Carotid Atherosclerosis**

As reported in Table 3, patients with carotid atherosclerosis had an autoantibody ratio significantly higher than control subjects in regard to anti-oxLDL IgG (1.78±0.39 versus 1.05±0.3, P<.01) and IgM (1.98±0.83 versus 1.40±0.09, P<.05) and anti-MDA-LDL IgG (2.39±0.51 versus 2.04±0.11, P<.01) and IgM (4.18±1.89 versus 2.9±0.15, P<.05).

Competitive immunoassay studies have revealed that monoclonal and polyclonal anti-MDA-LDL antibodies can cross-react with other substrates containing the MDA-lysine adduct. MDA-modified HSA was used as antigen to detect the presence of autoantibodies in plasma of control subjects and patients with carotid atherosclerosis. However, no significant difference was detected between the two groups (Table 3).

During LDL oxidation, the derivatization of Apo B100 with end products of lipid peroxidation such as MDA and 4-OH-nonenal takes place. Thus, it can conceivably be predicted that a correlation should exist between anti-MDA-LDL and anti-ox-LDL autoantibodies, and, as reported in Fig 2, this was actually the case (r=.81; P<.001). Values obtained in control subjects were clustered in the lower left corner of the scattergram and were encompassed by two lines that delimited four different fields. The spectrophotometric readings of anti-oxLDL and anti-MDA-LDL autoantibodies of the IgG type were distributed more than 60% in the upper right field, indicating the presence, in the plasma of the same subject, of antibodies recognizing both oxLDL and MDA-LDL. On the other hand, despite the presence of a significant correlation (r=.62; P<.001), the spectrophotometric readings of anti-oxLDL and anti-MDA-LDL were above the control limits in only 16% of the patients investigated. These findings suggest that MDA-lysine in LDL is probably the most abundant molecular epitope.
FIG 2. Scatterplots showing correlation between anti-MDA-LDL and anti-oxLDL IgG and IgM autoantibodies in patients with carotid atherosclerosis. The spectrophotometric readings of anti-MDA-LDL IgG (top) and IgM (bottom) were plotted against those of anti-Cu$^{2+}$-oxidized LDL IgG and IgM. The correlation coefficients were $r=0.81$ ($P<0.001$) and $r=0.62$ ($P<0.001$), respectively. The two lines present in the graphs encompass a box in the lower left corner that identifies the distribution of values from control subjects. MDA indicates malondialdehyde; oxLDL, oxidatively modified LDL.

Specificity of Oxidatively Modified LDL as the Molecule Triggering the Immune Response

Although in plasma of patients with carotid atherosclerosis the titer of autoantibodies reacting with MDA-HSA did not differ from that detected in control subjects (Table 3), a correlation was observed between the spectrophotometric readings of anti-MDA-LDL and anti-MDA-HSA samples for both IgG and IgM (Fig 3). This raises the question of which molecule is triggering the immune reaction in vivo. In other terms, the production of autoantibodies could occur in response to either specific LDL oxidation or nonspecific oxidative modifications of a variety of other molecules, including HSA. A useful parameter to discriminate between these two possibilities could be the ratio between the antibody titers against MDA-LDL and MDA-HSA (anti-MDA-LDL/anti-MDA-HSA specific ratio). As illustrated in Fig 4, this value was >1 for both IgG and IgM and significantly higher in patients with carotid atherosclerosis than in control subjects in regard to IgG. This suggests that MDA-LDL was the molecule preferentially recognized by the antibodies detected in patients with carotid atherosclerosis.

In addition, the detection of higher anti-MDA-LDL/anti-MDA-HSA specific ratios would imply that an enhanced oxidation of LDL has occurred in vivo. However, no correlation was found between this parameter and the duration of the lag phase of in vitro LDL oxidation. On the other hand, a highly significant negative correlation ($r=-0.33$, $P<0.017$) was detected between the anti-MDA-LDL/anti-MDA-HSA IgG specific ratio and vitamin E concentration in LDL from patients with carotid atherosclerosis.

Clinical Correlates With In Vitro and In Vivo Markers of LDL Oxidation in Patients With Carotid Atherosclerosis

No correlation was found between in vitro parameters of LDL oxidation (lag phase, propagation rate, peak time, and vitamin E concentration and efficiency) and any of the following cardiovascular risk factors: age, sex, and vitamin E concentration.
presence of hypertension, diabetes mellitus, dyslipidemia, obesity, and cigarette smoking habit. However, the presence of hypertension or dyslipidemia was associated with an anti-MDA-LDL/anti-MDA-HSA IgG specific ratio higher than that observed in control subjects. Furthermore, this parameter was even higher when the two risk factors were present simultaneously (Fig 5).

Discussion

This is the first study that compares in vitro and in vivo LDL oxidation markers in a group of patients with severe atherosclerosis. The results obtained demonstrate that, despite an apparently normal susceptibility of LDL to in vitro oxidation, patients with severe carotid atherosclerosis develop autoantibodies of the IgG and IgM type against oxidatively modified LDL (Cu²⁺-oxidized and MDA-derivatized LDL); the results also support the occurrence of an enhanced LDL oxidation in vivo.

The demonstration that LDL from patients with carotid atherosclerosis was as resistant to in vitro oxidation as was LDL from control subjects is apparently in contrast with previous findings obtained in different groups of patients with diseases associated with an enhanced risk for atherosclerosis, such as chronic renal failure, hypertension, hypercholesterolemia, and arteriogenic lipoprotein phenotype (pattern B) or in patients with overt atherosclerosis.

Studies performed in vitamin E–supplemented LDL or in LDL isolated from subjects who have received vitamin E supplementation have supported the assumption that vitamin E concentration and efficiency are among the most relevant factors controlling LDL oxidation. Although vitamin E concentration in LDL from patients with carotid atherosclerosis is comparable to that found in LDL from control subjects, its efficiency in preventing LDL oxidation is markedly reduced, as shown by its contribution to determining the length of the lag phase (about 84 minutes in control subjects versus 30 minutes in patients). In other words, additional factors other than vitamin E would make LDL from the patients with carotid atherosclerosis investigated in this study resistant to oxidation. It cannot be excluded that a selective increase in the concentration of the other lipid-soluble antioxidants present in LDL, such as ubiquinol-10 and carotenoids, would contribute to the increased resistance. However, none of the patients were taking pharmacological or dietetic supplements of either ubiquinol-10 or carotenoids. More likely, a direct effect of drugs such as calcium channel blockers, ACE inhibitors, β-blockers, and hypolipidemic agents (about 68% of the patients were taking these drugs; see Table 1) can be hypothesized because of their direct activity in preventing LDL oxidation in vitro.

In addition, the discrepancy could theoretically be linked to the unspecificity and lack of sensitivity of the methodology used here to evaluate the susceptibility of isolated LDL to oxidation (eg, conjugated diene formation following exposure to CuSO₄). Several other agents have been used to trigger LDL oxidation, including iron, hemin, the azo initiator 2,2'-azobis-(2-amidinopropane hydrochloride), and peroxidase(s). Although the biochemical mechanisms underlying the pro-oxidant effects of the conditions listed above are different, all the methodologies can be considered equally sensitive in detecting appreciable changes in the resistance of LDL to oxidation. Furthermore, Cu²⁺-induced formation of conjugated dienes has been successfully used by us and by other groups to detect modifications of the susceptibility to oxidation in LDL with different antioxidant concentrations or isolated from patients prone to atherosclerosis. Thus, it can conceivably be concluded that LDL from the patients with severe carotid atherosclerosis investigated in this study did not differ from those obtained from control subjects in regard to their resistance to in vitro oxidation.

The presence of high titers of autoantibodies against MDA-LDL in patients with severe carotid atherosclerosis confirms data obtained by Palinski et al, Tatzber et al, and Salonen et al and indicates the generation of MDA-LDL in vivo. Since MDA is one of the major end products of lipid peroxidation, the presence of autoantibodies against MDA-modified LDL can be considered a reliable signature of the peroxidation of LDL lipids that took place in vivo.

The finding of enhanced LDL oxidation in vivo together with an apparently normal susceptibility of LDL to in vitro oxidation raises nontrivial questions about the reliability of the different methodologies used to evaluate LDL oxidation and about the meaning of the results obtained. The available evidence linking a decreased resistance of LDL to oxidative modifications with oxidation actually occurring in various diseases associated with atherosclerosis is, with few exceptions, largely circumstantial. Thus, on the basis of the results reported in this study, it is advisable to restrict these widely used methods exclusively to the detection of modifications linked to variations in components intrinsic to LDL, such as saturated or polyunsaturated fatty acids and lipid-soluble antioxidants.

It has been proposed that oxidatively modified LDL could be critically involved in the recruitment of monocytes/macrophages and in the subsequent formation of foam cells. If this were the exclusive role played by oxidized LDL, the oxidative modifications might be relevant only to the generation of the fatty streaks and not necessarily to their evolution to the more compli-
icated and more clinically significant lesions. However, the detection of anti-MDA-LDL autoantibodies in patients with severe carotid atherosclerosis (this study) demonstrates that LDL undergoes oxidative modifications even in the presence of more advanced atherosclerotic lesions. Accordingly, oxidation-specific lipid-protein adducts have been detected in advanced and complicated atherosclerotic plaques from both Watanabe heritable hyperlipidemic rabbits and humans, and autoantibodies to MDA-LDL have been detected in patients with severe inflammatory reactions to already existing plaques (chronic periaortitis).

As discussed above, the presence of anti-MDA-LDL autoantibodies in plasma of the patients investigated has been taken as the biological signature of an increased LDL oxidation in vivo. However, under oxidative conditions, a variety of plasma proteins could interact with MDA, with the formation of a number of derivatized lysine residues eliciting the generation of anti--MDA-lysine autoantibodies cross-reacting with different carrier molecules. Thus, the specificity of MDA-derivatized LDL as the immune trigger would be at least questionable. Two lines of evidence suggest that, at least in the atherosclerotic patients investigated in this study, LDL is the real target for oxidative modifications. First, only 5% of the patients have autoantibodies exclusively reacting with MDA-HSA, 27% of them have autoantibodies specifically reacting with MDA-LDL, and 17% have autoantibodies cross-reacting with both derivatized proteins. In addition, the anti--MDA-LDL/anti--MDA-HSA ratio (a useful parameter to evaluate the specificity of LDL as the preferential immunogen) was >1 and, for IgG, was significantly higher than in control subjects. The use of this parameter could be criticized since MDA-derivatized lysine residues in LDL are more abundant than those present in HSA, thus favoring the preferential detection of anti-MDA-LDL antibodies. However, against this simplistic view is the demonstration that in patients with chronic renal failure under dialytic treatment (a condition associated with increased levels of plasma MDA resulting from the bioincompatibility of the dialyzer membrane), the anti--MDA-LDL/anti--MDA-HSA IgG specific ratio was 1.28, significantly lower than in control subjects and in atherosclerotic patients.

The demonstration of an inverse correlation between the anti--MDA-LDL/anti--MDA-HSA IgG specific ratio and vitamin E concentration in LDL confirms the effects of this antioxidant in protecting LDL from oxidation in vivo. On the other hand, the lack of correlation with the duration of the lag phase seems to suggest that the observed correlation with vitamin E has little to do with a vitamin E--dependent reduction of the susceptibility of LDL to in vitro oxidation. More likely, since LDL represents one of the physiological carriers of vitamin E to different cells and tissues, an augmented vitamin E concentration in LDL could just be a mirror of a more powerful antioxidant environment in various districts, including the subendothelial space, where LDLs are oxidized.

Among the various vascular risk factors investigated in the patients under study, hypertension and dyslipidemia are linked to an enhanced oxidation of LDL in vivo, as demonstrated by the increased anti--MDA-LDL/anti--MDA-HSA IgG specific ratio. Essential hypertension has previously been reported to be associated with both a decreased resistance of LDL to oxidation in vitro and an increased LDL oxidation in vivo. In addition, a shift of the pro-oxidant/antioxidant balance toward oxidation in essential hypertension has been reported in different animal models and in humans. Dyslipidemia, and in particular hypercholesterolemia, enhances the susceptibility of LDL to in vitro oxidation and the occurrence of vascular damage by the combination of hypercholesterolemia and increased levels of endogenous prooxidants or deficient levels of antioxidants has been reported in rats.

In conclusion, the data reported in this study provide strong evidence in favor of an enhanced and specific oxidation of LDL in patients with advanced carotid atherosclerosis and raise at least two essential questions concerning (1) the biological mechanisms promoting the oxidative process and (2) their relevance to the progression of the atherosclerotic disease. It is conceivable that inflammatory cells present within the advanced atherosclerotic lesions may represent the sources of oxygen-reactive species causing the peroxidation of LDL lipids, and the demonstrations that lymphocytes can oxidize LDL and that anti-oxLDL autoantibodies are present in patients with chronic aortitis are in agreement with this view. Furthermore, a correlation between the antioxidatively modified LDL autoantibody titer and the extent and severity of inflammatory reactions within the atherosclerotic plaque can be predicted, together with a possible beneficial effect of antioxidant supplementation in protecting against plaque complication. Studies are actually in progress to validate these hypotheses.

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