Malondialdehyde-modified Low Density Lipoproteins in Patients with Atherosclerotic Disease

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Abstract

The murine monoclonal antibody mAb-1H11 raised against malondialdehyde (MDA)-modified LDL, was used to detect cross-reacting material in human atheromatous tissue and in plasma. MDA-modified LDL levels in plasma were 0.19±0.02 mg/dl (mean±SEM) in 44 control subjects, 0.24±0.02 mg/dl in 15 patients with chronic stable angina pectoris (P = NS vs LDL cholesterol matched controls), 1.4±0.1 mg/dl in 60 patients with acute myocardial infarction ($P < 0.001$ vs controls), and 0.86±0.11 mg/dl in 22 patients with carotid atherosclerosis ($P < 0.001$ vs controls). Modified LDL, isolated from pooled LDL of 10 patients, showed a higher electrophoretic mobility on agarose gels, a higher content of thiobarbituric acid reactive substances, and a higher cholesterol/protein ratio than native LDL and had a similar reactivity (antigen/protein ratio) in the assay as the in vitro MDA-modified LDL used for calibration. Its apo B-100 moiety was not fragmented. Uptake of this modified LDL by macrophages resulted in foam cell generation. In conclusion, elevated plasma levels of atherogenic MDA-modified LDL may be a marker for unstable atherosclerotic cardiovascular disease. (*J. Clin. Invest.* 1995. 95:2611–2619.)
Key words: atherosclerosis • malondialdehyde-modified LDL • foam cell generation

Introduction

Subendothelial accumulation of foam cells, primarily derived from monocytes/macrophages by uptake of LDL, plays a key role in the initiation of atherosclerosis (reviewed in reference 1). The LDL receptor (2), which is down-regulated when the intracellular cholesterol levels are increased, is not involved in intracellular cholesterol accumulation in foam cells. However, macrophages avidly accumulate oxidized LDL, by uptake via scavenger receptors that are unresponsive to intracellular cholesterol levels (2, 3). Oxidative modification of LDL may be obtained in vitro by incubation with cultured cells (4), with purified lipoxygenase in the presence of phospholipase A_2 (5), or with Cu^{2+} (6). In vitro oxidation occurs in three phases; a lag, a propagation, and a decomposition phase. During the lag phase, endogenous LDL antioxidants (e.g., vitamin E) are consumed. During the propagation phase, unsaturated fatty acids are rapidly oxidized to lipid hydroperoxides, which are converted during the decomposition phase to a variety of other products including reactive aldehydes (e.g., malondialdehyde [MDA][1]) (7). These aldehydes may react with lysine residues in the LDL apo B-100 moiety, resulting in a decreased affinity of apo B-100 for the LDL receptor and an increased affinity for scavenger receptors. Oxidatively modified LDL has previously been demonstrated in atherosclerotic lesions, but not in normal arteries (8–10), whereas LDL eluted from atherosclerotic lesions, but not from normal arteries, is rapidly taken up by macrophages (11, 12). Furthermore, autoantibodies against oxidized LDL have been demonstrated in plasma (13, 14) and in atherosclerotic lesions (15). However, direct evidence for the occurrence of oxidized LDL in circulating blood is lacking.

In the present study, we produced and characterized a murine monoclonal antibody (mAb-1H11) cross-reacting with MDA-modified apo B-100, but not with native apo B-100, that allowed the detection of modified LDL in atheromatous lesions and in plasma of patients with atherosclerotic disease. Modified LDL isolated from plasma had a reactivity in the immunoassay similar to that of in vitro MDA-modified LDL used for production of the antibody and calibration of the assay.

Methods

Lipoproteins: preparation and modification. LDL were isolated from pooled sera of fasting normolipidemic donors by density gradient ultracentrifugation in a tabletop ultracentrifuge (TL-100, Beckman Instruments, Inc., Fullerton, CA) for 2 h (16), and were dialyzed at 4°C against 0.15 M NaCl containing 0.01% EDTA (pH 7.5). The lipoproteins were used within 2 wk after isolation. All lipoprotein preparations were sterilized by filtration, using a 0.45-μM low-protein binding filter (Millex; Millipore Corp., Brussels, Belgium) and stored at 4°C under nitrogen in the presence of 20 μM vitamin E and 10 μM butylated hydroxytoluene to prevent in vitro oxidation, and of 10 IU/ml aprotinin to prevent protein degradation. MDA-modified albumin, MDA-modified polylysine, MDA-modified LDL, and copper-oxidized LDL were prepared as described elsewhere (17–19). Delipidation of LDL was performed as previously described (20). Sodium deoxycholate was used to substitute the naturally occurring lipid environment of LDL particles for the production of apo B-100 detergent complexes which are stable and soluble in aqueous buffer. Aldehydes were assayed as thiobarbituric acid reactive substances according to Sparrow et al. (5) and were expressed as mole MDA per mole apo B-100. Electrophoretic mobility of LDL preparations was determined by one-dimensional electrophoresis, at 90 V for 75 min on 1% agarose gels and lipid staining with oil red O. Results were expressed as relative electrophoretic mobilities by comparison with the migration of native LDL. The levels of free cholesterol and cholesterol esters were quantified by HPLC on a Nova-Pak C-18 reversed phase column (Waters Associates, Milford, MA) (21). The

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Received for publication 11 July 1994 and in revised form 9 December 1994.

J. Clin. Invest.

0021-9738/95/06/2611/09 $2.00
Volume 95, June 1995, 2611–2619

1. *Abbreviation used in this paper:* MDA, malondialdehyde.

levels of phospholipids were analyzed by two-dimensional chromatography on silica G 60A TLC plates. Lipids were separated in the first dimension in chloroform/methanol/25% ammonium hydroxide (60:30:5, vol/vol), dried in a forced air oven at 50°C followed by development in chloroform/methanol/formic acid (65:25:10, vol/vol) in the second dimension (22). Lipids were transiently visualized by iodine and their identity was confirmed by specific staining (amino and phosphate groups) and comparison of R_f values with those of standards. Identified spots were scraped into Pyrex tubes (Corning Inc., Corning, NY) and their phosphate content was determined after wet ashing (23). Plasma proteins were separated by gel-exclusion chromatography on a Superose 6HR 10/30 column (24) in a fast protein liquid chromatography system (Pharmacia Diagnostics AB, Uppsala, Sweden). Modified LDL was purified from the isolated LDL fractions of patients with acute myocardial infarction by ion-exchange chromatography on a mono Q-Sepharose column in the fast liquid chromatography system (25, 26). Alternatively, modified LDL was purified from the isolated LDL fractions of patients with acute myocardial infarction by affinity chromatography on immobilized mAb-1H11 (27).

Characterization of the monoclonal antibody mAb-1H11. The monoclonal antibodies mAb-1H11 and mAb-13F6 were obtained after immunization of Balb/c mice with MDA-modified LDL and native LDL, respectively, according to previously published procedures (10). Specificity was derived from the inhibition of the binding of these antibodies to immobilized MDA-modified LDL with different competing soluble ligands including native LDL, MDA-modified LDL, copper-oxidized LDL, MDA-modified albumin, and MDA-modified polylysine, and delipidated apo B-100 and MDA-modified apo B-100. The binding constant for immobilized MDA-LDL and the maximal number of binding sites per MDA-modified apo B-100 molecule were calculated from the amount of bound ^{125}I-labeled antibodies to immobilized MDA-modified LDL (10).

Immunocytochemistry. Endarterectomy specimens, obtained from patients with established carotid atherosclerosis, were treated as described elsewhere (10). The specimens were collected in PBS (pH 7.4) containing 4% sucrose, 20 μM vitamin E as an antioxidant, and 1 mM EDTA, and were stored at −80°C. Serial 3-μm thick sections were cut and stained with hematoxylin and eosin. Duplicate slides, containing six sections each, were developed either with mAb-1H11 or with the control antibody mAb-17C8 (10) (final concentration 1 μg/ml), with PG-M1, a murine monoclonal antibody specific for human macrophages, or with 1A4, a murine monoclonal antibody specific for human smooth muscle α-actin (both from Dako SA, Glostrup, Denmark). Specificity of binding of mAb-1H11 was confirmed by inhibition of binding with an excess MDA-modified LDL but not with native LDL.

Patients and blood samples. Venous blood samples were collected on 0.1 vol of 0.1 M citrate containing vitamin E (final concentration 20 μM) as an antioxidant. The samples were centrifuged at 3,000 *g* at 4°C, immediately frozen and kept at −70°C until use. Blood samples were obtained from 44 control subjects, from 15 patients with chronic stable angina pectoris, from 22 patients with established extensive carotid atherosclerosis which were scheduled for carotid endarterectomy, and from 60 patients with confirmed evolving acute myocardial infarction. Plasma samples from the latter patients were collected within the first 4 h after the onset of the symptoms and before the start of the thrombolytic therapy. The ELISA was performed on fresh samples or on samples that were kept at −80°C for ≤ 72 h. Furthermore, a set of 10 plasma samples of patients were thawed, assayed, and frozen again. This freezing and thawing was repeated three times.

Immunoassay for MDA-modified LDL in plasma. The immunoassay was carried out according to published principles (28). Briefly, equal volumes of diluted purified mAb-1H11 solution (competing ligand at a final concentration 0.05 nM) and of diluted MDA-modified LDL solution (final concentration of MDA-modified apo B-100 ranging between 0.1 and 10 nM) were mixed and incubated for 30 min at room temperature. Then, 200-μl aliquots of the mixtures were added to wells of polyvinylchloride microtiter plates, coated with MDA-modified LDL. Samples were incubated for 2 h at room temperature. After washing,

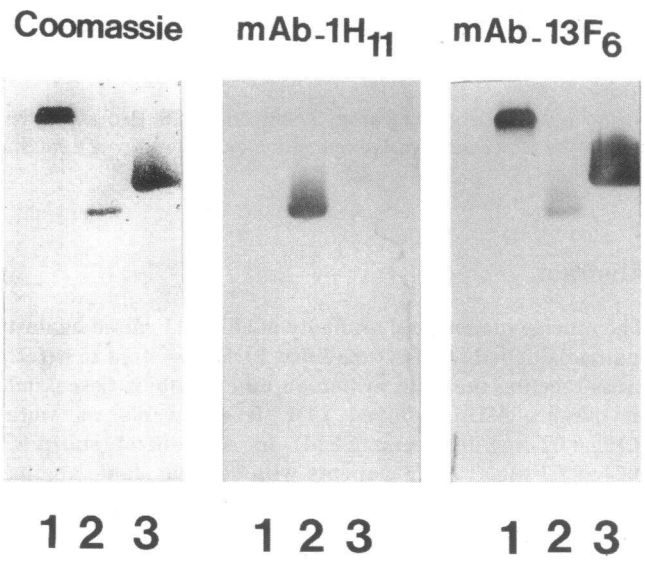

Figure 1. Agarose gel electrophoresis and Western blot analysis of native LDL (lane *1*), MDA-modified LDL (lane *2*), and copper-oxidized LDL (lane *3*). Lipoproteins were separated in a 1% agarose gel and either stained with Coomassie brilliant blue or transblotted to a nitrocellulose membrane. Western blots were performed with mAb-1H11 (dilution 1:1,000) or mAb-13F6 (dilution 1:1,000). Protein contents in the gel were 15 μg in lanes *1* and *3* and 2 μg in lane *2*.

the wells were incubated for 1 h with horseradish peroxidase–conjugated rabbit IgG raised against mouse immunoglobulins and washed again. The peroxidase reaction was then performed by addition of 100 μg/ml *o*-phenylenediamine and 0.003% hydrogen peroxide. After 30 min, the reaction was arrested with 50 μl of 4 M sulfuric acid, and the absorbance was read at 492 nm. Controls without competing antigen and blanks without antibody were included routinely. The percent inhibition of binding of mAb-1H1 to the immobilized MDA-modified LDL was calculated as

$$\frac{A^{492nm}_{control} - A^{492nm}_{sample}}{A^{492nm}_{control} - A^{492nm}_{blank}} \quad (1)$$

and standard curves were obtained by plotting the percentage of inhibition vs the concentration of competing ligand.

Measurement of IgG autoantibody titers in ELISA. Titers of IgG autoantibodies against MDA-modified LDL were measured according to Salonen et al. (14). Immobilized antigens for this assay included native LDL prepared from pooled plasma (protected against in vitro oxidation as described above), MDA-modified LDL (prepared as described above starting from the same native LDL preparation) and HSA. The plasma samples were diluted 100-fold, and incubated for 2 h at room temperature in the microtiter plates. The wells were then washed and incubated for 1 h with horseradish peroxidase–conjugated goat IgG raised against human immunoglobulins and washed again. The peroxidase reaction was then performed by addition of 100 μg/ml *o*-phenylenediamine and 0.003% hydrogen peroxide. After 30 min, the reaction was arrested with 50 μl of 4 M sulfuric acid, and the absorbance was read at 492 nm. The IgG antibody titers for native LDL were expressed as the ratios of the absorbance in wells coated with native LDL vs wells coated with serum albumin. The IgG antibody titers for MDA-modified LDL were expressed as the ratios of the absorbance in wells coated with MDA-modified LDL vs wells coated with native LDL.

Measurement of plasma cholesterol and apo B-100 levels. Plasma levels of total cholesterol and triglyceride were determined using standard enzymatic colorimetric assays (Boehringer Mannheim, Meylan, France). The HDL cholesterol levels were determined after precipitation

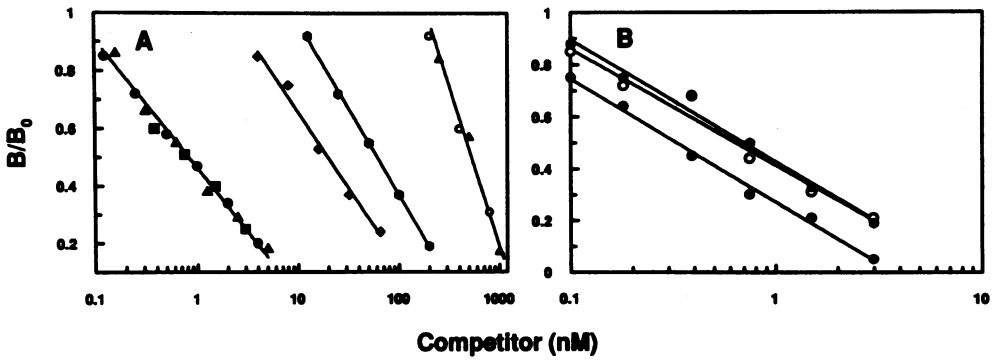

Figure 2. Interaction of mAb-1H11 (*A*) or mAb-13F6 (*B*) with various competing ligands. In vitro human MDA-modified LDL (1 μg/ml) was the plated antigen. mAb-1H11 (18 ng/ml) was added with and without competitor. Results are expressed as B/B_O where B is the amount of mAb-1H11 bound in the presence and B_O that amount bound in the absence of competitor. Competitors were native LDL (○), MDA-modified LDL (●), copper-oxidized LDL (●), MDA-modified human albumin (♦), native apo B-100 (△), MDA-modified apo B-100 (▲), and modified LDL isolated from plasma of patients with acute myocardial infarction (■).

of the other lipoproteins with phosphotungstic acid and magnesium. The LDL cholesterol levels in plasma samples with triglyceride levels < 400 mg/dl, were calculated using the Friedewald formula. The apo B-100 levels were measured as described previously (29). Protein concentrations were measured according to Lowry (30).

Cellular uptake of modified LDL. Murine J774 macrophages (American Type Culture Collection, Rockville, MD) were seeded in 35-mm dishes at a cell density of 2×10^6 cells/dish, grown for 24 h in 2 ml DMEM containing 10% FCS in a 10% CO_2 atmosphere and used as confluent monolayers. The cells were incubated for 24 h in serum-free DMEM containing 10 mg HSA/ml, and with native or modified LDL for 24 h in serum-free medium containing 10 mg HSA/ml. The plates were washed with PBS, fixed for 1 h with 4% formaldehyde in PBS, and then briefly immersed in 60% isopropanol. Cells were stained with 1% oil red O in 60% isopropanol for 1 h at 4°C, rinsed in PBS, and mounted for light microscopic evaluaton. Foam cells were identified as previously described (31).

Statistical analysis. The statistical significance of differences in plasma levels of MDA-modified apo B-100 levels between control subjects and patients was determined using the Mann–Whitney *U* test. The correlation between MDA-modified apo B-100 and cholesterol levels, age, or autoimmune antibody titers was determined with Pearsons' test.

Results

Characterization of monoclonal antibodies mAb-1H11 and mAb-13F6. The apo B-100 molecules of in vitro MDA-modified LDL used for calibration of the assay were found to contain on average 244 modified lysines (out of a total of 356). Modification of lysines resulted in a threefold increased electrophoretic mobility in 1% agarose gels (Fig. 1). SDS-PAGE revealed that its apo B-100 moiety was intact (not shown). MDA-modified LDL had a decreased affinity for the LDL receptor and an increased affinity for the scavenger receptor, as evidenced by its enhanced uptake by macrophages in vitro (10). The apo B-100 molecules of copper-oxidized LDL were found to contain an average 210 modified lysines, resulting in a 2.5-fold increased electrophoretic mobility (Fig. 1). Furthermore, its apo B-100 moiely was highly fragmented (not shown).

The association constant for the binding of mAb-1H11 to immobilized MDA-modified LDL was 10^9 M^{-1}, whereas its association constant for native LDL was < 10^7 M^{-1}. The maximal number of binding sites per apo B-100 molecule was ~ 2 (20×10^{11} bound antibody molecules per 12×10^{11} immobilized apo B-100 molecules). The specificity of mAb-1H11 was further derived from the inhibition of its binding to immobilized MDA-modified LDL in the presence of increasing concentrations of different soluble ligands (Fig. 2 *A*). 50% inhibition was obtained with 1 nM MDA-modified LDL, 50 nM copper-oxidized LDL, and 500 nM native LDL, respectively. Delipidation of LDL and reconstitution of the lipid environment of LDL yielded soluble apo B-100 detergent complexes, MDA-modification of which yielded immunoreactive material with the same affinity for mAb-1H11 as MDA-modified LDL (Fig. 2 *A*). 20-fold higher molar concentrations of MDA-modified albumin were required to obtain 50% inhibition. Up to 1 μM MDA-modified lysine did not affect mAb-1H11 binding (not shown). The specificity of mAb-1H11 for MDA-modified LDL was further confirmed by Western blot analysis (Fig. 1).

In contrast, Fig. 2 *B* illustrates that soluble MDA-modified LDL, copper-oxidized LDL, and native LDL, inhibited the binding of mAb-13F6 to immobilized MDA-modified LDL to the same extent. Cross-reactivity of mAb-13F6 with all ligands was further confirmed by Western blot analysis (Fig. 1).

Immunodetection of MDA-modified LDL in carotid atherosclerotic lesions. Endarterectomy specimens from patients with established carotid atherosclerosis displayed typical atheromatous plaques with necrotic cores containing lipid particles, cell debris, and interspersed calcium deposits. Microscopic evaluation of three representative lesions (Fig. 3) revealed lipid-like material staining with oil red O (not shown), which could be immunostained with mAb-1H11 (Fig. 3, A–C), but not with the irrelevant antibody mAb-17C8 (not shown). Staining with mAb-1H11 was inhibited with MDA-modified LDL but not with native LDL (not shown). Immunoreactive material was associated with smooth muscle cells (Fig. 3 *D*) and with macrophages (Fig. 3 *E*) as evidenced by specific staining of these cells. (not shown). MDA-modified LDL could also be demonstrated in the necrotic lipid core (Fig. 3 *F*).

Quantitation of MDA-modified apo B-100 in plasma. MDA-modified apo B-100 in plasma was quantitated using an immunoassay based on inhibition of the binding of mAb-1H11 to the wells of microtiter plates coated with MDA-modified LDL. The lower limit of detection was 0.05 mg/dl of MDA-modified LDL

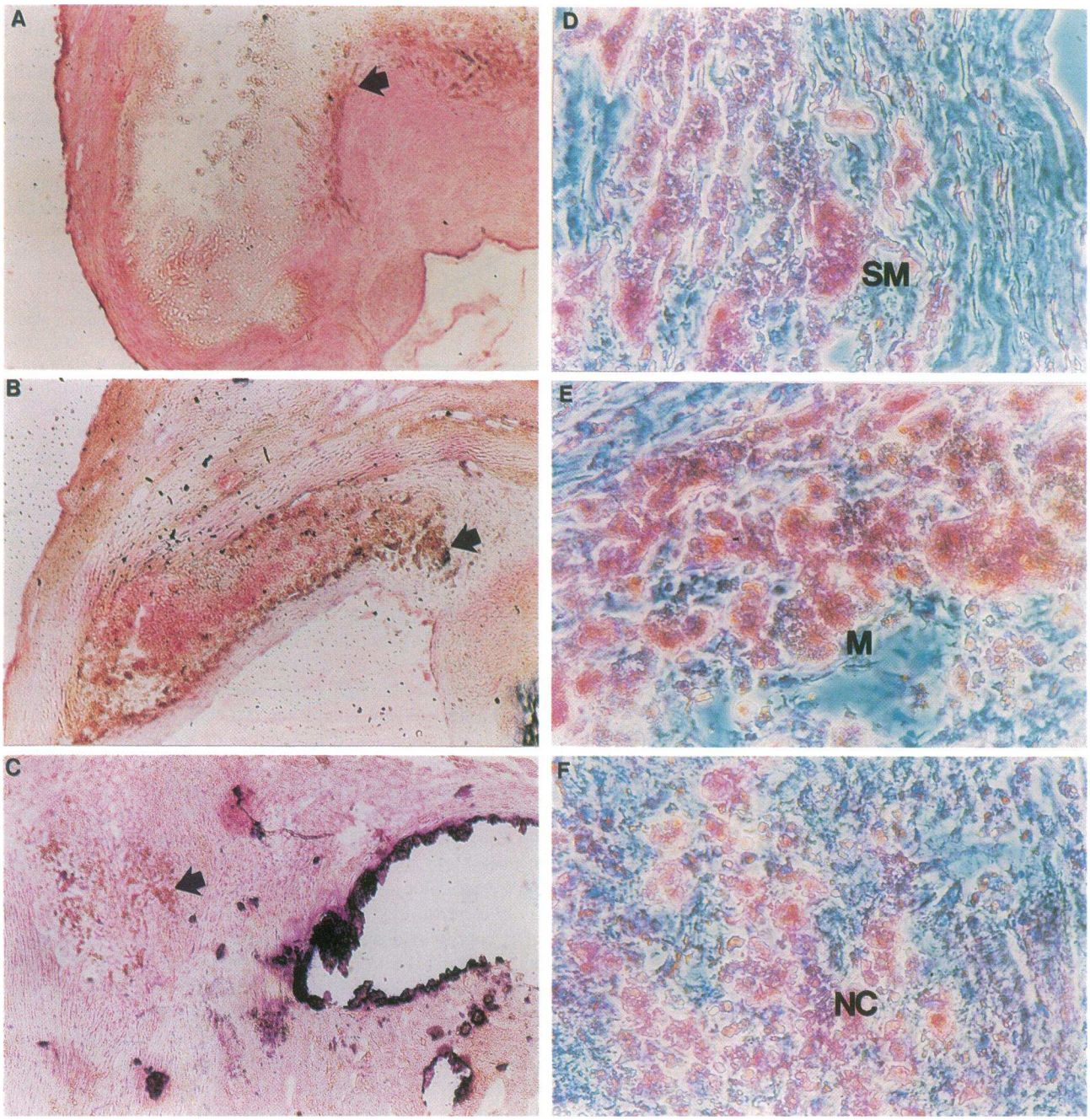

Figure 3. Light micrographs of three representative carotid endarterectomy specimens. (*A–C*) Light micrographs (×40) of tissue sections, immunostained with mAb-1H11. Immunostained areas are indicated with black arrows. (*D–F*) Phase contrast micrographs (×400) of the area indicated by the arrows in *A–C*. These sections show accumulation of MDA-modified LDL in or around smooth muscle cells (*SM*) and macrophages (*M*), and in the necrotic lipid core (*NC*).

in undiluted human plasma. Intra- and interassay coefficients of variation were 12 and 15%, respectively. When in vitro MDA-modified LDL were added to human plasma at a final concentration of 0.25 and 1 mg/dl, recoveries were 95 and 106%, respectively. The recoveries of in vitro MDA-modified LDL in plasma samples of control subjects and of patients were very similar.

Plasma levels of MDA-modified LDL in 44 plasma samples from control subjects were 0.19±0.02 mg/dl (mean±SEM). The control population was retrospectively subdivided in two groups based on age: those younger than 40 yr (27±1 yr, mean±SEM, n = 15) and those older than 40 yr (55±2 yr, n = 29). As expected, the LDL cholesterol and total apo B-100 levels were significantly lower in the younger group (120±5 and 58±2 mg/dl, respectively), than in the older group (180±12 and 70±5 mg/dl, respectively; $P < 0.001$ each). However, the MDA-modified LDL levels were very similar in both groups: 0.22±0.02 and 0.18±0.02 mg/dl, respectively (Table I).

MDA-modified LDL levels in 15 patients with chronic stable angina pectoris were 0.24±0.02 mg/dl and thus were not different from those in controls matched for LDL cholesterol (Table I).

Table I. Relevant Characteristics and Lipoprotein Values in Control Subjects and Patients Matched for LDL Cholesterol

		CSA		AMI				CA	
	Controls		*P* vs controls	Group I	*P* vs controls	Group II	*P* vs controls		*P* vs controls
	n = 29	*n* = 15		*n* = 24		*n* = 36		*n* = 22	
Age (yr)	55±2	77±4	<0.001	55±1	NS	72±1	<0.001	59±2	NS
HDL cholesterol (mg/dl)	47±3	56±3	NS	35±3	0.01	39±2	0.03	29±2	<0.001
LDL cholesterol (mg/dl)	180±12	180±12	NS	185±9	NS	173±5	NS	200±10	NS
Total apo-B100 (mg/dl)	70±5	67±4	NS	69±3	NS	67±4	NS	78±4	NS
MDA-modified apo B-100 (mg/dl)	0.18±0.02	0.24±0.02	NS	1.5±0.2	<0.001	1.4±0.1	<0.001	0.86±0.11	<0.001

The data represent means±SEM. CSA, chronic stable angina pectoris; AMI, acute myocardial infarction; CA, carotid artherosclerosis.

Plasma MDA-modified LDL levels in samples obtained from 60 patients early after the onset of acute myocardial infarction were 1.4±0.1 mg/dl ($P < 0.001$ vs controls matched for LDL cholesterol). The patient population was retrospectively subdivided in two groups based on age: group I with patients younger than 60 yr (55±1 yr, $n = 24$) and group II with patients older than 60 yr (72±2 yr, $n = 36$). The plasma LDL cholesterol levels and apo B-100 levels in the two groups were very similar and were also not different from those in the older control group (Table I). The HDL levels were, however, lower in the patients. MDA-modified LDL levels were significantly increased in patients matched for both age and LDL cholesterol (group I in Table I). The MDA-modified LDL levels were not different in the two groups of patients (Table I).

In 56 out of 60 plasma samples from patients with acute myocardial infarction the level was ≥0.5 mg/dl (mean value + 2 SD in control subjects and patients with chronic stable angina pectoris). These samples were considered to be positive. The frequency of positive samples in the patients was thus > 90%.

A set of 10 plasma samples of patients were thawed, assayed, and frozen again. The MDA-modified LDL levels in these samples were 1.4±0.1 mg/dl after the first thawing, 1.3±0.2 mg/dl after the second thawing, and 1.5±0.2 mg/dl after the third thawing.

Plasma MDA-modified LDL levels in 22 patients with carotid atherosclerosis were 0.86±0.11 mg/dl ($P < 0.001$ vs matched controls) (Table I). MDA-modified LDL levels again did not correlate with LDL cholesterol, total apo B-100, and HDL cholesterol levels or with age. The MDA-modified apo B-100 levels were significantly higher in patients with subacutely occluded carotid arteries (1.3±0.18 mg/dl) than in patients with similar carotid stenosis but without carotid artery occlusion (0.48±0.03 mg/dl; $P < 0.001$) (Table II). The frequency of positive samples was 30% for patients with extensive stenosis without subacute carotid artery occlusion and 90% for patients with similar stenosis, but complicated with subacute carotid artery occlusion.

Measurement of IgG autoantibody titers. The IgG autoantibody titers against MDA-modified LDL were 6.8±0.7 (mean±SEM) in controls, 8.6±1.0 in patients with chronic stable angina pectoris, 7.8±1.2 in patients with carotid atherosclerosis, and 7.5±0.3 in patients with acute myocardial infarction (all P = NS vs controls). The IgG antibody titers against MDA-modified LDL in plasma samples of carotid atherosclerosis patients with MDA-modified LDL levels > 0.5 mg/dl were 6.8±0.7 ($n = 12$) as compared to 8.8±1.6 ($n = 10$) in plasma samples of carotid atherosclerosis patients with MDA-modified LDL levels < 0.5 mg/dl (P = NS). Corresponding values were 6.6±0.4 ($n = 54$) for acute myocardial infarction patients with significantly elevated levels of MDA-modified LDL as compared to 8.4±0.4 ($n = 6$) for acute myocardial infarction patients with lower levels of MDA-modified LDL (P = NS).

Characterization of immunoreactive material. Six samples obtained from acute myocardial infarction patients with MDA-modified apo B-100 levels > 1 mg/dl were applied to a Superose 6HR 10/30 column. 77±4% (mean±SEM, $n = 6$) of the immunoreactive material was recovered in the LDL fractions. The elution profile of a representative sample is illustrated in Fig. 4. No immunoreactive material migrated in the serum albumin position. When in vitro MDA-modified LDL was added to a final concentration of 2 mg/dl in two plasma samples obtained from control subjects with MDA-modified LDL < 0.1 mg/dl, 80% of the immunoreactive material was recovered in the LDL fractions separated by gel-exclusion chromatography (data not shown). Agarose gel electrophoresis revealed that only the plasma of patients contained a LDL fraction with increased electrophoretic mobility (Fig. 5). Therefore, two pools of LDL of 10 patients each were applied to a mono Q-Sepharose ion-exchange column. The LDL was separated in two fractions: one that migrated in the position of native LDL (peak I in Fig. 6 and lane *5* in Fig. 5) and one that migrated in the position of in vitro MDA-modified LDL (peak II in Fig. 6 and lane *6* in Fig. 5). 74% of the immunoreactive material applied to the

Table II. Relevant Characteristics and Lipid Values in Stable and Unstable Carotid Atherosclerosis Patients

	Stable CA patients	Unstable CA patients	*P*
	n = 11	*n* = 11	
Age (yr)	58±2	60±3	NS
HDL cholesterol (mg/dl)	28±2	30±2	NS
LDL cholesterol (mg/dl)	200±14	200±9	NS
Total apo B-100 (mg/dl)	80±5	76±4	NS
MDA-modified apo B-100 (mg/dl)	0.48±0.03	1.3±0.18	<0.001

The data represent means±SEM. CA, carotid atherosclerosis.

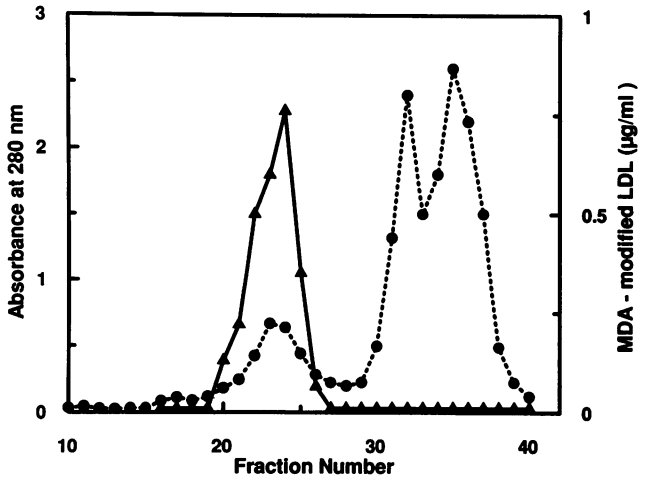

Figure 4. Gel filtration on a Superose HR 10/30 column of a citrated plasma sample obtained from a patient with acute myocardial infarction. The sample contained 880 µg MDA-modified LDL and its protein pattern was representative for six samples that were analyzed. 200 µl plasma was applied to the column equilibrated with 0.1 M citrate buffer, pH 7.4, containing 0.01% EDTA) and 10 IU/ml aprotinin; 0.5-ml fractions were collected. Absorbance at 280 nm (●) and MDA-modified LDL levels (micrograms/mililiter) (▲), measured in the mAb-1H11 based ELISA, are plotted vs the fraction number. The gel filtration column was calibrated with VLDL (*peak at fraction 17*), LDL (*peak at fraction 23*), HDL (*peak at fraction 32*), IgG (*peak at fraction 33*), and serum albumin (*peak at fraction 35*).

column was recovered in peak II (Fig. 6). The relative electrophoretic mobility (Fig. 5) of this fraction, its extent of lysine modification, and its reactivity in the mAb-1H11 based ELISA (Table III) was very similar to that of in vitro MDA-modified LDL. The apo B-100 moiety of the MDA-modified LDL isolated from the plasma was not fragmented (Fig. 6 *B*), its cholesterol/protein and phospholipid/protein ratios were somewhat higher than those of native LDL (Table III); its ratio of cholesterol to cholesterol esters and its level of unsaturated fatty acids were not different from that of native LDL and lysophosphatidylcholine was not detected (Table III). The characteristics of

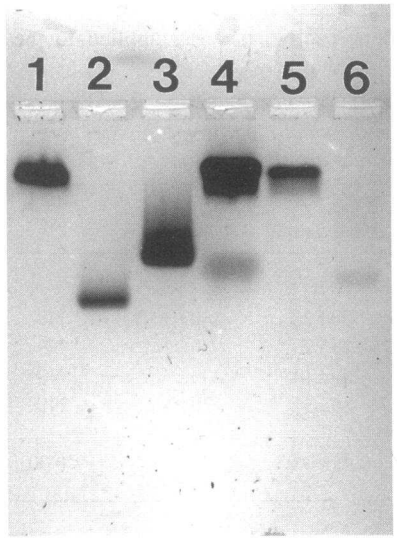

Figure 5. Agarose gel electrophoresis of native LDL (lane *1*), MDA-modified LDL (lane *2*) copper-oxidized LDL (lane *3*), LDL isolated from pooled plasma of patients with acute myocardial infarction (lane *4*). Lanes *5* and *6* represent two LDL subfractions isolated from pooled LDL of these patients by ion-exchange chromatography as illustrated in Fig. 6. Lipoproteins were stained with Coomassie brilliant blue. Protein contents in the gel were 15 µg in lanes *1–5* and 2 µg in lane *6*.

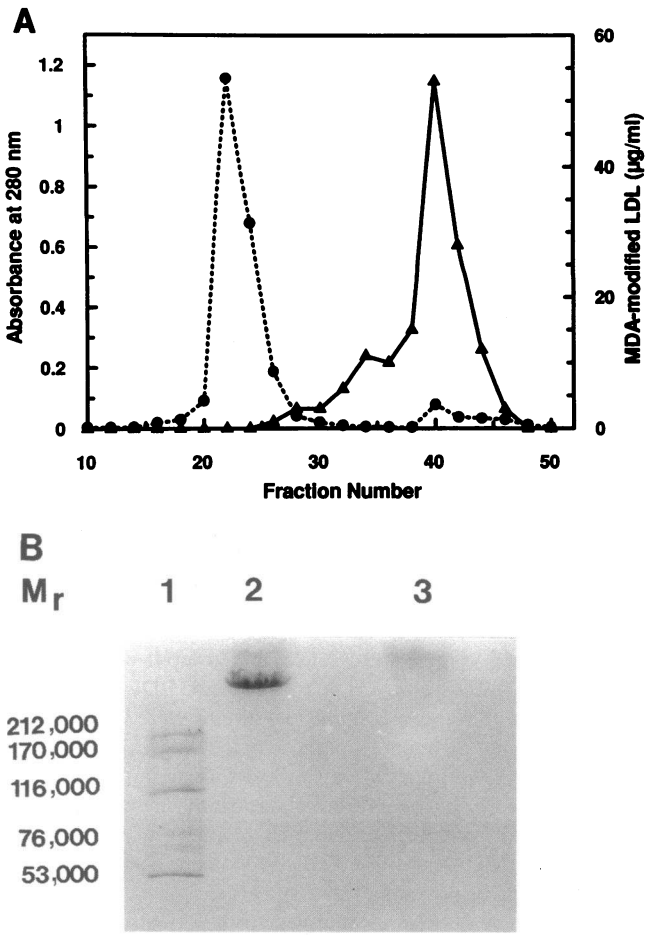

Figure 6. (A) Ion-exchange chromatography on a Mono Q-Sepharose column of pooled LDL from patients with acute myocardial infarction. LDL samples of 1 ml were applied to the column equilibrated with 20 mM Tris-HCl buffer, pH 8.2, containing 0.01% EDTA and 10 IU/ml of aprotinin. The NaCl gradient for separation was: from 0.0 to 0.3 M in fractions 14–38; from 0.3 to 1 M in fractions 39–50. Absorbance at 280 nm (●) and MDA-modified LDL levels (microgram/milliliter) (▲) are plotted vs the fraction number. (*B*) SDS-PAGE of LDL separated by ion-exchange chromatography. (Lane *1*) Protein calibration mixture containing myosin (M_r 212,000), α_2-macroglobulin (M_r 170,000), β-galactosidase (M_r 116,000), transferrin (M_r 76,000), and glutamic dehydrogenase (M_r 53,000); (lane *2*) LDL from peak 1; (lane *3*) LDL from peak II. Apolipoproteins were separated in a 4–15% gradient polyacrylamide gel under nonreducing conditions. Proteins were stained with Coomassie brilliant blue. Protein contents in the gel were 4 µg in lane *2* and 1 µg in lane *3*.

the LDL fraction that was isolated from pooled LDL of patients by affinity chromatography on immobilized mAb-1H11 were very similar to those of the fraction peak II that was isolated by ion-exchange chromatography, whereas the characteristics of the peak I fraction were very similar to those of LDL isolated from pooled LDL of control subjects by affinity chromatography on immobilized mAb-13F6.

Foam cell generation in J774 macrophages. Fig. 7 represents light micrographs of J774 macrophages incubated with 10 µg native LDL separated from pooled LDL of control subjects by affinity chromatography on mAb-13F6 (Fig. 7 *A*), with 10 µg in vitro MDA-modified LDL obtained by incubation of the

Table III. Characteristics of Native and of MDA-modified LDL Separated of Pooled LDL from Patients with Acute Myocardial Infarction by Ion-exchange Chromatography

	Native LDL	Modified LDL
Protein/antigen ratio*	>100	1.1
Reactivity with mAB-1H11‡ (C_{50} in nM)	500	1.2
Relative electrophoretic mobility§	1	2.5
Malondialdehyde (mol/mol protein)‖	4	100
Blocked lysines¶	7	192
Cholesterol/protein ratio*,**	2.2	3.6
Free cholesterol:cholesterol ester ratio**	0.41	0.37
Fatty acids**		
18:1 (%)	13	10
18:2 (%)	34	35
20:4 (%)	15	7
Phospholipid/protein ratio*,‡‡	0.42	0.66
Phosphatidylcholine/sphingomyelin ratio‡‡	1.6	1.9

The results represent means for two preparations starting from pooled LDL of 10 patients. * Protein concentration was measured in Lowry assay, whereas antigen concentration was determined in mAb-1H11–based ELISA calibrated with a standard in vitro MDA-modified LDL preparation. ‡ C_{50} represents concentration of ligand that is required to inhibit for 50% the binding of mAb-1H11 to immobilized MDA-modified LDL. § LDL was subjected to electrophoresis in 1% agarose gels and the mobility of the fractions was measured relative to native LDL, isolated from the plasma of control subjects by density gradient ultracentrifugation. ‖ Malondialdehyde was quantitated in LDL samples by thiobarbituric acid reactive substances reaction. ¶ The percentage of blocked lysines was calculated from the number of MDA molecules bound per molecule apo B-100, assuming that two lysines on apo B-100 were blocked by each bound MDA molecule. ** Levels of free cholesterol, cholesterol esters, and fatty acids were determined by HPLC. ‡‡ Phospholipid levels were measured by two dimensional chromatography on Silica plates.

native LDL with malondialdehyde (Fig. 7 *B*), or with 10 μg MDA-modified LDL isolated from pooled LDL of patients by affinity chromatography on mAb1H11 (Fig. 7 *C*). Native LDL contained < 10-blocked lysines per molecule, with corresponding values of 244 for in vitro MDA-modified LDL and 192 for MDA-modified LDL isolated from the plasma of patients. Incubation with either in vitro or in vivo MDA-modified LDL resulted in the accumulation of lipid droplets (stained with oil red O) in the cytoplasm of these macrophages. Addition of mAb-4E4, a monoclonal antibody that stimulated the scavenger receptor–mediated uptake of in vitro MDA-modified LDL (10), markedly enhanced foam cell generation both by in vitro and in vivo MDA-modified LDL (not shown).

Discussion

mAb-1H11 which reacts with MDA-modified LDL but not with native LDL was used for the detection of MDA-modified LDL in plasma and in atheromatous tissue. The antibody reacted with an epitope that was fully exposed in soluble MDA-modified apo B-100 detergent complexes, indicating that it is directed against the protein moiety of modified LDL.

mAb-1H11 detected elevated levels of immunoreactive material in the plasma of patients with carotid atherosclerosis or acute myocardial infarction, but not of patients with stable angina pectoris. After separation of plasma proteins by gel filtration, > 75% of the immunoreactive material applied to the column was recovered in the LDL fraction, whereas no immunoreactivity comigrated with albumin (Fig. 4). Agarose gel electrophoresis revealed an LDL fraction with increased electrophoretic mobility in the LDL of patients with acute myocardial infarction, but not in the LDL of control subjects (Fig. 5), which could be isolated with a yield of 74% by ion-exchange chromatography on a mono Q-Sepharose column (Fig. 6). This material was similar to in vitro MDA-modified LDL standard preparations as in terms of electrophoretic mobility in agarose gels,

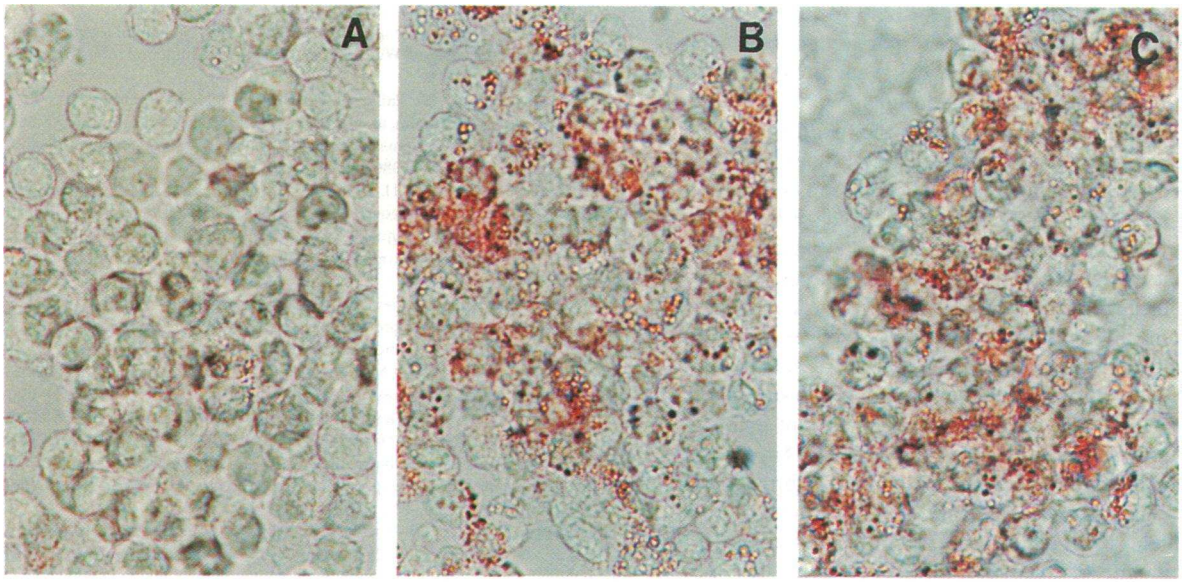

Figure 7. Light micrographs (×400) of J774 macrophages incubated with 10 μg native LDL, affinity purified on mAb-13F6 from pooled LDL of control subjects (*A*), with 10 μg MDA-modified LDL, obtained by in vitro MDA modification of LDL used in *A* (*B*), or with 10 μg MDA-modified LDL affinity purified on mAb-1H11 from pooled LDL of patients (*C*). The lipid droplets in the cytoplasm were stained with oil red O and appear as brown spots.

number of thiobarbituric acid reactive substances, and the affinity for mAb-1H11.

The titer of MDA-modified LDL specific autoimmune antibodies was previously found to correlate with the progression of carotid atherosclerosis (14). In the present study, this titer was found to be somewhat higher in patients than in control subjects, but the differences were not statistically significant. Furthermore, the levels of specific autoimmune antibodies were very similar in patients with and without elevated levels of MDA-modified LDL. This suggests that the observed differences in MDA-modified LDL levels are not due to differences in levels of autoimmune antibodies or that there are increased levels of immune complexes present in the plasma of patients.

An LDL subfraction has previously been isolated by ion-exchange chromatography (25) from plasma of fasting control persons. This more electronegative fraction, in an amount ranging from 5 to 20% of the total LDL, had a low affinity for high affinity LDL receptors of fibroblasts and a low immunoreactivity with monoclonal antibodies that recognize epitopes of apo B-100 close to the LDL receptor recognition domains, but was not taken up more avidly by macrophages. The characteristics of this LDL subfraction were comparable to these of an oxidation-labile subfraction isolated by Shimano et al. (26). In the present study, the more electronegative LDL was characterized by a higher number of blocked lysines, resulting in uptake by J774 macrophages and foam cell generation (Fig. 7). The levels of the modified LDL fraction in the plasma of control subjects were $\leq 0.1\%$ of total LDL, whereas they increased to 2% of total LDL in patients with unstable carotid atherosclerosis and patients with acute myocardial infarction.

The increased electrophoretic mobility, the increased lysine modification, the increased cholesterol/protein ratio, and the resulting increased uptake by macrophages of the LDL isolated from the plasma of patients are very similar with these of LDL extracted from atherosclerotic lesions (11, 12). Furthermore, the levels of linoleate and oleate and of lysophosphatidylcholine were unaltered, suggesting that LDL detected in the plasma of patients might represent LDL released from the arterial wall after rupture of atherosclerotic plaques (32, 33). Indeed mAb-1H11 detected immunoreactive material in or around foam cells (both macrophages and smooth muscle cells) and in the necrotic core of human carotid atherosclerotic lesions.

The finding that the protein moiety of the modified LDL is not fragmented, suggests that the modified LDL does not originate from extensive metal ion–induced oxidation of LDL. It may however be generated by malondialdehyde released by oxidation of arachidonic acid present in LDL. Indeed, it has been shown that interaction with malondialdehyde or 4-hydroxynonenal, another product of prostaglandin synthesis, modifies LDL to an extent that it is recognized by scavenger receptors (18, 34, 35). Ischemic injury, associated with increased production of free radicals during oxidative stress, may result in the activation of not only the cyclooxygenase-dependent pathway of prostaglandin synthesis in endothelial cells (36), but also in increased production of noncyclooxygenase-derived prostaglandin F_2-like compounds, F_2-isoprostanes (37, 38) that are strong inducers of platelet aggregation. Activated platelets may then produce large amounts of aldehydes, further enhancing the modification of LDL that infiltrated from the plasma into the intima of atherosclerotic lesions.

In conclusion, the current study presents direct evidence for the occurrence in plasma of MDA-modified LDL that may be atherogenic and suggests that increased plasma levels of MDA-modified LDL may constitute a marker for unstable atherosclerotic disease.

Acknowledgments

We thank Dr. P. Declerck for advice and help with the production of the monoclonal antibodies and M. Verstreken for technical assistance. The tissue specimens and plasma samples of the carotid atherosclerosis patients were obtained from Dr. R. Suy, Department of Vascular Surgery, and the plasma samples of patients with acute myocardial infarction and with stable angina pectoris from Dr. S. Janssens and Dr. F. Van de Werf, Department of Cardiology, at the University Hospitals, Leuven, Belgium. Phospholipid levels were determined by Dr. P. Van Veldhoven, Department of Pharmacology, Leuven, Belgium.

This study was supported by the Nationaal Fonds voor Geneeskundig Wetenschappelijk Onderzoek (Project 3.0103.92) and by the Interuniversitaire Attractiepolen.

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