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Malondialdehyde alteration of low density lipoproteins leads to cholesteryl ester accumulation in human monocyte-macrophages

(cell surface receptors/blood platelets/atherosclerosis)

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ABSTRACT Glutaraldehyde treatment of ^{125}I-labeled low density lipoprotein (^{125}I-native-LDL) produced a modified LDL (^{125}I-glut-LDL) with a molecular weight of 10×10^6 or more. Malondialdehyde treatment of ^{125}I-native-LDL produced a product (^{125}I-MDA-LDL) with a molecular weight not appreciably different from that of the original lipoprotein. However, the electrophoretic mobility of MDA-LDL indicated a more negative charge than native-LDL. ^{125}I-MDA-LDL was degraded by two processes: a high-affinity saturable process with maximal velocity at 10–15 μg of protein per ml and a slower, nonsaturable process. The degradation of ^{125}I-MDA-LDL was readily inhibited by increasing concentrations of nonradioactive MDA-LDL but was not inhibited by acetylated LDL or native-LDL even at concentrations as high as 1600 μg of protein per ml. After exposure of native-LDL to blood platelet aggregation and release *in vitro*, 1.73 ± 0.19 nmol of malondialdehyde per mg of LDL protein was bound to the platelet-modified-LDL. No detectable malondialdehyde was recovered from native-LDL that had been treated identically except that the platelets were omitted from the reaction mixture.

After incubation with glut-LDL, MDA-LDL, or platelet-modified-LDL for 3 days, human monocyte-macrophages showed a dramatic increase in cholesteryl ester content whereas the cholesteryl ester content of cells incubated with the same concentration of native-LDL did not. Based on these experiments we propose that modification of native-LDL may be a prerequisite to the accumulation of cholesteryl esters within the cells of the atherosclerotic reaction. We further hypothesize that one modification of LDL *in vivo* may result from malondialdehyde which is released from blood platelets or is produced by lipid peroxidation at the site of arterial injury.

There is increasing evidence that the foam cells found in the atherosclerotic reaction are macrophages that are derived from blood-borne monocytes or from smooth muscle cells that have taken on many of the properties of macrophages (1–3). The hallmark of these cells is their high cholesteryl ester content (≥50% of total cellular cholesterol) (4, 5). Our objective has been to define the conditions and mechanisms leading to cholesteryl ester accumulation within these cells. We learned from experiments to be reported elsewhere that human monocytes contain very little cholesteryl ester (approximately 2% of total cellular cholesterol), and the conversion of the monocytes into macrophages *in vitro* did not appreciably increase their cholesteryl ester content. Moreover, these cells did not accumulate cholesteryl esters when incubated in high concentrations of low density lipoprotein (LDL).

The experiments reported here demonstrate that LDL must be modified before it will produce cholesteryl ester accumulation in human monocyte-macrophages. Based on these experiments we propose that one modification of LDL *in vivo* may result from an interaction with malondialdehyde which is released from blood platelets or is produced by lipid peroxidation at the site of arterial injury. We further propose that it is this modified LDL, rather than normal LDL, that leads to cholesteryl ester accumulation in the cells of the atherosclerotic reaction.

METHODS

Monocyte-Macrophages. Pure human monocytes were prepared from the blood of normal individuals as described (6). The cells were cultured in 30% autologous serum supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml) in either serumless medium (medium A; GIBCO, no. 320-1630) or in Dulbecco's modified Eagle's medium (GIBCO, no. 430-1600) supplemented with 24 mM $NaHCO_3$, 10 mM Hepes, insulin (8 μg/ml), and glucose (2 mg/ml) (medium B). Cytochemical, morphological, ultrastructural, and functional criteria were utilized to confirm that the cells were viable macrophages (6–8).

Fibroblasts. Normal human fibroblasts were grown in monolayer in Ham's F-12 medium containing 15% fetal calf serum, penicillin (50 units/ml), and streptomycin (50 μg/ml).

Platelets. Washed platelets were prepared according to the method of Hamberg *et al.* (9) from the blood of normal individuals who had not taken any drugs (including aspirin) during the preceding 14 days.

Modification of Proteins. LDL was prepared from the blood of normal subjects as described (10) and was radioiodinated by the method of McFarlane (11) as modified by Bilheimer *et al.* (12). The concentration of LDL is given in terms of its protein content. LDL modified by treatment with glutaraldehyde (glut-LDL) was prepared by incubating 1 μl of 10% glutaraldehyde in H_2O with 0.5 ml of LDL (10 mg/ml) at 4°C for 1 hr.

LDL modified by treatment with malondialdehyde (MDA-LDL) was prepared by incubating 0.25 ml of 0.2 M malondialdehyde, generated by acid hydrolysis of malonaldehyde bis(dimethyl acetal) (Aldrich), with 0.5 ml of LDL (10 mg/ml) at 37°C and pH 6.5 for 3 hr.

Platelet-modified-LDL was prepared as follows. A mixture of 5 mg of LDL, 6 mM arachidonic acid, and 12 mM $CaCl_2$ in 1 ml at pH 6.5 was preincubated in the dark for 5 min at 37°C, and then $3–6 \times 10^{10}$ washed platelets in 0.9 ml was added. The reaction was started by the addition of 10 units of human thrombin dissolved in 10 μl of isotonic saline and was continued in the dark at 37°C for 1 hr. The reaction mixture was centri-

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Abbreviations: LDL, low density lipoprotein; acetyl-LDL, LDL modified by acetylation with acetic anhydride; glut-LDL, LDL modified by glutaraldehyde; MDA-LDL, LDL modified by malondialdehyde; platelet-modified-LDL, LDL modified by blood platelets.

fuged at 90,000 × *g* for 60 min and the supernatant was removed to a dialysis bag.

LDL modified by treatment with acetic anhydride (acetyl-LDL) was prepared as described by Basu *et al.* (13).

Each of the LDL preparations described above was dialyzed for 16 hr at 4°C against 0.01 M phosphate/0.15 M NaCl/0.01% EDTA, pH 7.4. The lipoproteins were filtered through a 0.45-μm filter just prior to addition to the cells.

Assays. The proteolytic degradation of ^{125}I-labeled LDL preparations (^{125}I-native-LDL, ^{125}I-glut-LDL, and ^{125}I-MDA-LDL) was measured by assaying the amount of ^{125}I-labeled trichloroacetic acid-soluble (noniodide) material formed by the cells and excreted into the culture medium as described by Goldstein and Brown (14). Gel filtration was performed with Sepharose 4B. Lipoprotein electrophoresis was performed in Tris/barbital/sodium barbital, pH 8.0, on Sepraphore III cellulose polyacetate strips (Gelman Instrument Co., Ann Arbor, MI).

The free and esterified cholesterol contents of the cells were determined after the cells had been incubated with each lipoprotein (500 μg of protein per ml) for 3 days. On the day of harvest, control dishes (not exposed to the lipoprotein) were cooled to 4°C for 30 min and 500 μg of lipoprotein protein per ml was added. After 4 hr, the cells were washed three times in the dishes with phosphate-buffered saline containing 1% bovine serum albumin. The cells were scraped with a rubber policeman in phosphate-buffered saline and layered on 3% human serum albumin in 30-ml siliconized tubes. The tubes were centrifuged at 10,000 × *g* for 30 min and the supernatant was removed. The pellet was washed three times with phosphate-buffered saline and suspended in H_2O. The free and esterified cholesterol contents were determined by gas/liquid chromatography as described (10). The protein content was determined by the method of Lowry (15). The free and esterified cholesterol contents of cells that had never been exposed to the experimental lipoproteins did not differ from those of control cells which had only been exposed to these lipoproteins at 4°C, indicating that the washing procedure was highly effective.

Malondialdehyde was determined spectrophotometrically by using the thiobarbituric acid assay (16). The malondialdehyde content of LDL was determined after the LDL was precipitated and washed twice with heparin-manganese (17). The precipitate was incubated with 40% trichloroacetic acid in 1 M HCl (0.375 ml) and 0.1 M thiobarbituric acid/0.26 M Tris, pH 7.0 (0.2 ml), at 90°C for 30 min, diluted with 0.15 M NaCl (0.625 ml), and centrifuged at 2000 × *g* for 10 min; the supernatant was assayed for malondialdehyde.

RESULTS

Others (18–21) have shown that cultured arterial smooth muscle cells do not accumulate cholesteryl esters when incubated with high concentrations of normal LDL but do accumulate cholesteryl esters when incubated with LDL from hyperlipidemic animals. In the monkey, these hyperlipidemic LDL molecules were larger than normal LDL molecules (21, 22). In order to test whether particle size might be an important determinant of cholesteryl ester accumulation we set out to produce, by chemical means, LDL of increased molecular weight. In searching for a suitable method we considered the possibility that glutaraldehyde might form a Schiff base with the ε-amino group of lysines located on two different LDL molecules and thus serve to crosslink LDL and form high molecular weight polymers.

When ^{125}I-native-LDL was treated with glutaraldehyde and analyzed by gel filtration, the vast majority of the lipoproteins eluted in the void volume of the column, indicating a molecular weight of 10 × 10^6 or more compared to a molecular weight of 2 × 10^6 for native-LDL (Fig. 1). Electrophoresis of glut-LDL showed an intensely staining band at the origin and one that migrated just ahead of native LDL. In between these two bands there was a faint trail of stain, suggesting particles of different molecular weight or charge. Degradation of ^{125}I-glut-LDL was not significantly different from that of ^{125}I-native-LDL when equal amounts of protein were presented to the cells. ^{125}I-Native-LDL and ^{125}I-glut-LDL were degraded by two processes: a high-affinity saturable process with maximal velocity at 25–50 μg of protein per ml and a slower nonsaturable process. The degradation of ^{125}I-glut-LDL was readily inhibited by increasing concentrations of nonradioactive glut-LDL but was not inhibited by nonradioactive native-LDL even at concentrations as high as 1200 μg/ml (Fig. 2). After 3 days of incubation with glut-LDL, the cholesteryl ester content of the cells had increased to 28 μg of esterified cholesterol per mg of cell protein (43% of total cellular cholesterol) compared to 0.9 μg of esterified cholesterol per mg of cell protein (2.5% of total cellular cholesterol) in cells incubated with the same concentration of native-LDL (500 μg/ml).

It was concluded that modification of the size or charge of native-LDL was required in order to cause cholesteryl ester accumulation.

In considering mechanisms by which LDL could be modified at the site of the atherosclerotic reaction the important role of blood platelets in causing the proliferation of smooth muscle cells at such sites was recalled (23). Furthermore, it is known that, in the metabolism of arachidonic acid by blood platelets, 1 mol of malondialdehyde is produced per mol of thromboxane A_2 (9, 24, 25). Because malondialdehyde has a structure analogous to that of glutaraldehyde, LDL was incubated with malondialdehyde prepared from malonaldehyde bis(dimethyl acetal). In contrast to glut-LDL, MDA-LDL migrated in a single band on electrophoresis with a mobility indicating a more negative charge than native-LDL (Fig. 3). Moreover, on gel filtration on Sepharose 4B, only 3% of the ^{125}I-MDA-LDL ap-

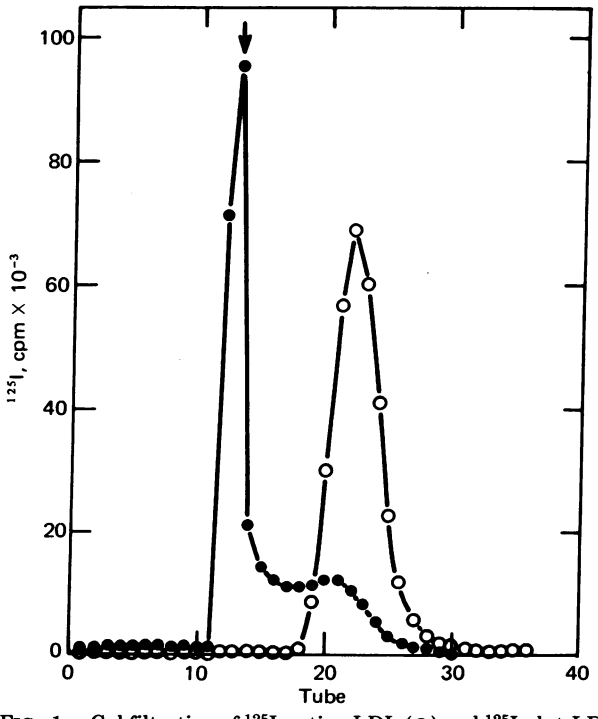

FIG. 1. Gel filtration of ^{125}I-native-LDL (○) and ^{125}I-glut-LDL (●) on Sepharose 4B; 0.9-ml fractions were collected and assayed. Arrow, void volume of the column.

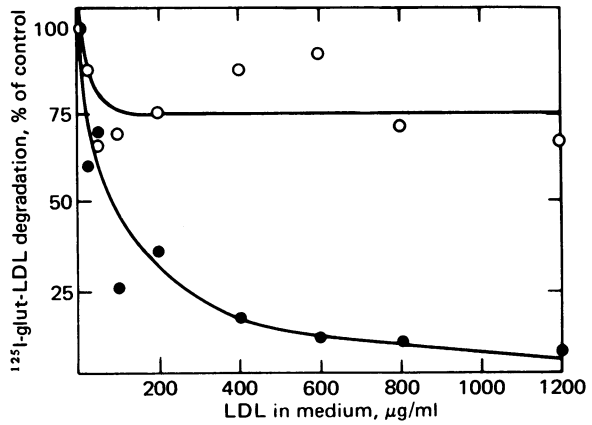

FIG. 2. Effect of nonradioactive glut-LDL (●) and native-LDL (○) on the degradation of ^{125}I-glut-LDL. Normal monocytes were cultured in 30% autologous serum in medium B for 8 days. The medium was removed, the cells were washed with medium B, and 1 ml of medium B containing 25 μg of ^{125}I-glut-LDL (165 cpm/ng) and the indicated concentration of either nonradioactive LDL was added. After incubation for 4 hr at 37°C, the amount of ^{125}I-labeled acid-soluble material in the medium was determined. The 100% value for the degradation of ^{125}I-glut-LDL in the absence of competing lipoproteins was 2.2 μg/4 hr per mg of protein.

peared in the void volume of the column and 97% eluted in the same position as native LDL. ^{125}I-MDA-LDL was degraded by two processes: a high-affinity saturable process with maximal velocity at 10–15 μg of protein per ml and a slower, nonsaturable process (Fig. 4). ^{125}I-MDA-LDL was degraded more rapidly than ^{125}I-native-LDL at protein concentrations of 25

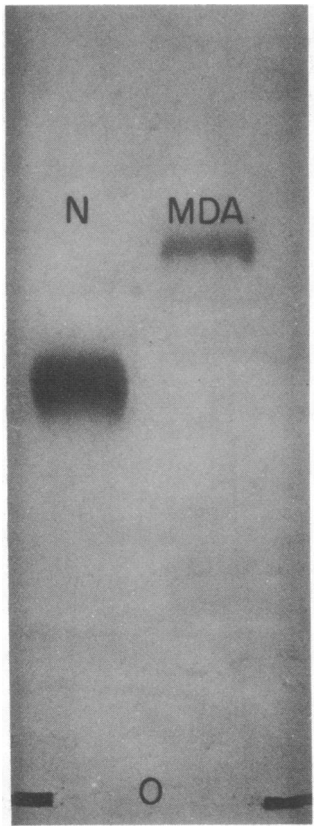

FIG. 3. Electrophoretic mobility of native (N) and MDA-LDL (MDA) in Tris/barbital/sodium barbital, pH 8.0, on Sepraphore III cellulose polyacetate strips; the strips were stained with Oil Red O. O, origin.

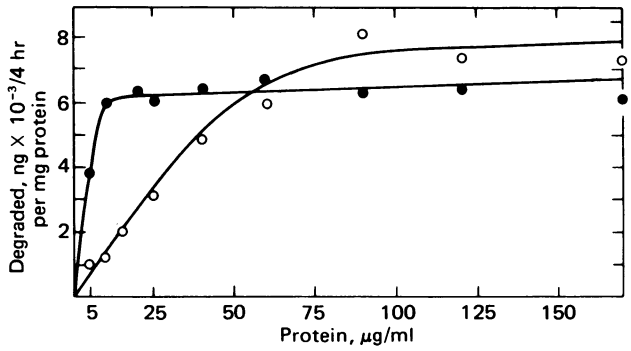

FIG. 4. Degradation of ^{125}I-MDA-LDL (●) and ^{125}I-native-LDL (○) by human monocyte-macrophages. Normal monocytes were cultured in 30% autologous serum in medium B for 4 days. The medium was removed, the cells were washed with medium B, and 1 ml of medium B containing the indicated concentration of either ^{125}I-MDA-LDL (165 cpm/ng protein) or ^{125}I-native-LDL (165 cpm/ng protein) was added. After incubation for 4 hr at 37°C, the amount of ^{125}I-labeled acid-soluble material in the medium was determined.

μg/ml or less. The degradation of ^{125}I-MDA-LDL was readily inhibited by increasing concentrations of nonradioactive MDA-LDL but was not inhibited by acetyl-LDL or native-LDL at concentrations as high as 1600 μg/ml (Fig. 5). Conversely, the degradation of ^{125}I-native-LDL was readily inhibited by increasing concentrations of nonradioactive native-LDL but, not by MDA-LDL at concentrations as high as 900 μg/ml (Fig. 6). Sodium heparin at a concentration of 1 mg/ml released approximately 66% of the ^{125}I-native-LDL bound to its receptor site on the surface of normal human fibroblasts (see figure 2 in ref. 26). In normal human monocyte-macrophages, sodium heparin at a concentration of 1 mg/ml inhibited the high-affinity degradation of ^{125}I-native-LDL by 66% but did not affect the degradation of ^{125}I-MDA-LDL. After 3 days of incubation with MDA-LDL, the cholesteryl ester content of the cells increased to 17 μg per mg of cell protein (26% of total cellular cholesterol) compared to 1.0 μg per mg cell protein (3% of total cellular cholesterol) in cells incubated with the same concentration of native-LDL (500 μg/ml).

High-affinity degradation of ^{125}I-native-LDL and ^{125}I-

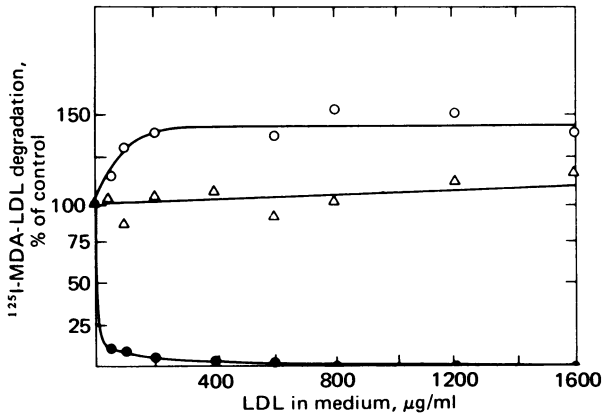

FIG. 5. Effect of nonradioactive MDA-LDL (●), native-LDL (△), and acetyl-LDL (○) on the degradation of ^{125}I-MDA-LDL. Normal monocytes were cultured in 30% autologous serum in medium A for 14 days. The medium was removed, the cells were washed with medium A, and 1 ml of medium A containing 25 μg of ^{125}I-MDA-LDL (152 cpm/ng protein) and the indicated concentration of one LDL was added. After incubation for 4 hr at 37°C, the amount of ^{125}I-labeled acid-soluble material in the medium was determined. The 100% value for the degradation of ^{125}I-MDA-LDL in the absence of competing lipoproteins was 7 μg/4 hr per mg of protein.

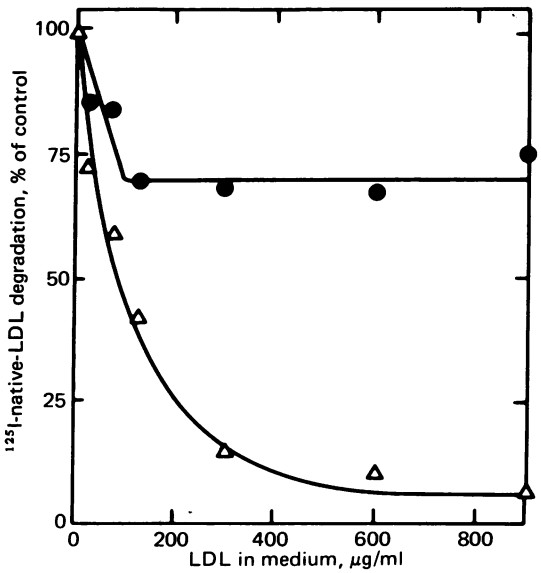

FIG. 6. Effect of nonradioactive native-LDL (△) and MDA-LDL (●) on the degradation of ^{125}I-native-LDL. Normal monocytes were cultured in 30% autologous serum in medium B for 4 days. The medium was removed, the cells were washed with medium B, and 1 ml of medium B containing 25 μg of ^{125}I-native-LDL (165 cpm/ng protein) and the indicated concentration of either LDL was added. After incubation for 4 hr at 37°C, the amount of ^{125}I-labeled acid-soluble material in the medium was determined. The 100% value for the degradation of ^{125}I-native-LDL in the absence of competing lipoproteins was 3.2 μg/4 hr per mg of protein.

MDA-LDL was studied in two additional cell types. The high-affinity degradation of ^{125}I-native-LDL by normal human fibroblasts was markedly increased by preincubation of the cells with the lipoprotein-deficient fraction of serum as reported by Brown and Goldstein (27). The same fibroblasts under the same experimental conditions failed to show any high-affinity degradation of ^{125}I-MDA-LDL, either before or after preincubation of the cells in the lipoprotein deficient-fraction of serum. Rat hepatocytes cultured in monolayers for 2 days did not demonstrate high-affinity degradation of either ^{125}I-native-LDL or ^{125}-I-MDA-LDL.

Because platelets produce malondialdehyde only at sites at which platelets aggregate and release, this mechanism seemed ideally suited to explain the focal nature of the atherosclerotic process. In order to test this hypothesis, normal human platelets were caused to aggregate and release *in vitro* in the presence of ^{125}I-LDL. The resulting lipoproteins were analyzed by gel filtration on Sepharose 4B. More than 90% of these radioactive lipoproteins were found to have the same molecular weight as ^{125}I-native-LDL. The elution profile from Sepharose 4B was like that of ^{125}I-MDA-LDL and hence different from ^{125}I-glut-LDL. When normal human monocyte-macrophages were incubated for 3 days with platelet-modified-LDL the cholesteryl ester content of these cells increased to 9 μg per mg of cell protein (16% of total cellular cholesterol) compared to 2 μg per mg of cell protein (3.8% of total cellular cholesterol) in cells incubated with the same concentration of native-LDL (500 μg protein/ml) that had been treated identically except that platelets were omitted from the reaction mixture.

In order to determine if malondialdehyde released from the platelets reacted with the LDL, platelet-modified-LDL was precipitated with heparin-manganese (17), washed, and subjected to acidic conditions in order to hydrolyze Schiff base bonds. The malondialdehyde released was measured spectrophotometrically; 1.73 ± 0.19 (mean ± SD; n = 5) nmol of malondialdehyde per mg of LDL protein was recovered from the platelet-modified-LDL. No detectable malondialdehyde was recovered from control LDL that had been treated identically except that the platelets were omitted from the reaction mixture.

DISCUSSION

Glutaraldehyde treatment produced LDL with a molecular weight of 10×10^6 or more (Fig. 1). Malondialdehyde treatment of LDL did not change the molecular weight of the lipoprotein but did alter its electrophoretic mobility (Fig. 3). Presumably, malondialdehyde formed intramolecular Schiff base bonds with the ε-amino group of lysines in LDL (28), thus modifying its charge. The following evidence suggests a distinctly different cell surface receptor for MDA-LDL: (*i*) ^{125}I-MDA-LDL was degraded by a high-affinity saturable process with maximal velocity at 10–15 μg of protein per ml (Fig. 4); (*ii*) native-LDL and acetyl-LDL failed to inhibit the degradation of ^{125}I-MDA-LDL (Fig. 5); (*iii*) MDA-LDL failed to inhibit the degradation of ^{125}I-native-LDL (Fig. 6); (*iv*) sodium heparin inhibited the high-affinity degradation of ^{125}I-native-LDL but not the high-affinity degradation of ^{125}I-MDA-LDL; (*v*) normal human fibroblasts demonstrated high-affinity degradation of ^{125}I-native-LDL but not ^{125}I-MDA-LDL.

From the experiments reported here and from experiments to be reported elsewhere, we have concluded that native-LDL is incapable of producing cholesteryl ester accumulation in human monocyte-macrophages.

We have demonstrated here that LDL modified with glutaraldehyde or malondialdehyde or by the action of blood platelets produces cholesteryl ester accumulation in human monocyte-macrophages. Goldstein *et al.* (29) demonstrated that acetyl-LDL produced cholesteryl ester accumulation in mouse peritoneal macrophages. If such modified LDL were generated at a site distant from the atherosclerotic reaction, it would probably be cleared from the circulation by the liver (30). However, if the modified LDL were produced at the site of the atherosclerotic reaction, it might be taken up by the monocyte-macrophages and smooth muscle cells with macrophage properties that are present at such sites (1–3). There is no known mechanism for the production of acetyl-LDL or glut-LDL at the site of the atherosclerotic reaction. However, malondialdehyde is known to be produced via the metabolism of arachidonic acid by blood platelets (9, 24, 25) and perhaps as well by lipid peroxidation (31, 32) at such sites. We propose that modification of native-LDL may be a prerequisite to the accumulation of cholesteryl esters within the macrophages and smooth muscle cells of the atherosclerotic reaction. We further hypothesize that one modification of LDL *in vivo* may result from an interaction with malondialdehyde which is released from blood platelets or is produced by lipid peroxidation at the site of arterial injury. Because MDA-LDL may be taken up by a receptor different from the native-LDL receptor, this hypothesis may provide an explanation for the accumulation of cholesteryl esters within the cells of the atherosclerotic reaction in receptor-negative homozygous patients with familial hypercholesterolemia.

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