Modified low density lipoprotein isolated from atherosclerotic lesions does not cause lipid accumulation in aortic smooth muscle cells

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Abstract Foam cells in atherosclerotic lesions are derived not only from blood monocytes but also from smooth muscle cells (SMC). To better understand the mechanisms by which SMC may become lipid-laden, we have studied the catabolism by cultured rabbit aortic SMC of LDL derived from atherosclerotic lesions (A-LDL) previously shown to be chemically modified. A-LDL was isolated either from homogenates of atherosclerotic plaques in human aortas by affinity chromatography and gel filtration, or from nonhomogenized extracts of plaque minces by ultracentrifugation and gel filtration. Internalization of A-LDL by SMC or fibroblasts appeared to be mediated primarily via the LDL receptor since: *1*) either unlabeled LDL or A-LDL could inhibit the degradation of ^{125}I-labeled A-LDL or of ^{125}I-labeled LDL, *2*) the uptake of both A-LDL and LDL, as estimated by their abilities to stimulate cholesterol esterification, was reduced in cells in which LDL receptor expression was down-regulated; and *3*) the uptake of both [^{3}H]cholesteryl ether-labeled A-LDL and LDL by normal fibroblasts was significant and could be inhibited by excess LDL, but was negligible in receptor-negative fibroblasts. At saturating concentrations of lipoproteins, maximum cholesterol esterification in SMC was greater for LDL than for A-LDL. Over a 48-h incubation, A-LDL, like LDL, was unable to induce cellular cholesteryl ester accumulation. Cross-competition studies suggested that either the affinity of A-LDL for the LDL receptor was less than that of LDL, or that some particles in A-LDL are not internalized by SMC. The latter alternative was supported by the observations that some A-LDL particles had undergone aggregation, especially at high concentrations, and that aggregated forms of A-LDL or plasma LDL failed to be internalized and degraded by SMC. Collectively, these results are consistent with recognition of some of the A-LDL particles by the LDL receptor, but also suggest that, at least under in vitro conditions, A-LDL is unlikely to induce lipid accumulation in SMC resulting in SMC-derived foam cells. —**Hoff, H. F., J. M. Pepin, and R. E. Morton.** Modified low density lipoprotein isolated from atherosclerotic lesions does not cause lipid accumulation in aortic smooth muscle cells. *J. Lipid Res.* 1991. **32:** 115–124.

Supplementary key words lipoproteins • cholesteryl ester • atherosclerosis • LDL receptor • degradation • cholesterol esterification

Atherosclerotic lesions are characterized by foam cells that represent not only lipid-laden macrophages (1) but also lipid-laden SMC (2). Although chemical modification of LDL has been shown to enhance uptake by macrophages in vitro (3), relatively little is known about the mechanisms leading to lipid-loading of SMC. Incubation of SMC in culture with unmodified LDL does not lead to lipid-loading, because the LDL receptors on these cells readily down-regulate (4). However, accumulation of cholesteryl esters does occur in cells, believed to be SMC, explanted from the subendothelial space of human atherosclerotic lesions when incubated with LDL complexed to latex particles or connective tissue elements (5). Uptake of these complexes is suggested to be mediated by nonspecific phagocytosis. Consistent with this explanation, synthetic cholesteryl ester-rich emulsions free of protein, and cholesteryl ester-rich droplets isolated from atherosclerotic lesions or derived from lysed lipid-laden macrophages, are internalized by SMC in culture, presumably by phagocytosis (6).

To further define the potential mechanisms contributing to SMC lipid-loading, we have investigated whether LDL extracted from human atherosclerotic lesions, termed A-LDL, can be catabolized by, and can induce lipid-loading in, SMC. We have previously shown that relative to LDL, A-LDL has an increased electrophoretic mobility (7–9), a lower protein to cholesterol ratio (9), and lower hydrated density (9), a reduced linoleate and arachidonate content (7), and a highly fragmented apoB component. Furthermore, A-LDL demonstrated an increased uptake relative to LDL by mouse

Abbreviations: SMC, smooth muscle cells(s); LDL, low density lipoprotein; A-LDL, artery-derived LDL; LPDS, lipoprotein-deficient serum; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; HNE, 4-hydroxynonenal; TCA, trichloroacetic acid; EDTA, disodium ethylenediaminetetraacetic acid.

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peritoneal macrophages (8, 9) by a mechanism that appeared distinct from the scavenger receptor. We propose that since A-LDL is an in vivo-derived form of chemically modified LDL (7–9), it may have unique interactions with SMC that may facilitate cellular accumulation of cholesteryl esters.

MATERIALS AND METHODS

Materials

[1-^{14}C]Oleic acid (48 mCi/mmol) was purchased from ICN Pharmaceuticals, Inc. (Irvine, CA), and [1,2,6,7-^{3}H(N)] cholesteryl oleate (82.7 Ci/mmol), [1,2-^{3}H(N)]cholesterol (55 Ci/mmol), and sodium [^{125}I]iodide were from New England Nuclear (Boston, MA). [^{3}H]Cholesteryl oleyl ether was synthesized from [1,2-^{3}H(N)]cholesterol and oleyl alcohol by the method of Halperin and Gatt (10). Oleic acid, cholesteryl oleate, bovine serum albumin (fatty acid-free), gentamycin, leupeptin, pepstatin, cholesterol oxidase, cholesteryl esterase, horseradish peroxidase, and bovine pancreatic trypsin (T-8642), and polyethylene glycol (20,000 mol wt), were purchased from Sigma Chemical Co. (St. Louis, MO). Tissue culture plates were from Costar (Cambridge, MA). DMEM (Cat. #430-1600), penicillin–streptomycin solution, and fetal calf serum were obtained from Gibco Laboratories (Grand Island, NY). LPDS was prepared from human serum by ultracentrifugation at d 1.21 g/ml. Spectra/Por dialysis tubing was obtained from Spectrum Medical Industries, Inc. (Los Angeles, CA). Centriprep 30 concentrators were purchased from Amicon Corps. (Danvers, MA).

Isolation of lipoproteins from aortic tissue

Human aortas (arch to iliac bifurcation) with raised fatty fibrous and complicated atherosclerotic lesions were obtained at autopsy within 12 h of death from individuals over 40 years of age. The tunica intima of raised lesions was dissected away from the underlying tunica media at its natural cleavage plane, rinsed briefly in PBS, and finely minced.

Procedure A: Tissue was prepared and A-LDL was isolated as previously described (8, 9). Briefly, this consisted of homogenizing minces of intima with a Polytron tissue disrupter (Brinkmann Instruments, Westbury, NY). A supernatant fraction of this extract, obtained by low speed centrifugation (10,000 *g*), was then subjected to affinity chromatography on an anti-LDL-Sepharose column to isolate a fraction containing immunoreactive apolipoprotein B, followed by gel filtration chromatography (Bio-Gel A-15m column) to isolate a fraction co-eluting with plasma LDL. Average yield of A-LDL using this procedure was about 20 μg apoB per g wet weight of intima. Seven separate batches of A-LDL were prepared for this study using procedure A.

Procedure B: In this procedure minced aortic tissue preparations were extracted for 18 h at 4°C without homogenization in PBS also containing 1.5 mM Na_2EDTA, 35 μM leupeptin, 5 μM pepstatin, 50 μg/ml gentamycin, 10 mM ϵ-aminocaproic acid, 0.002% chloramphenicol, 100 KIU/ml aprotinin, and 20 μM vitamin E. The extract was centrifuged at 25,000 *g* for 1 h at 4°C; the supernatant was filtered through glass wool to remove any large lipid droplets and subjects to ultracentrifugation for 18 h at 260,000 *g* at 4°C following adjustment of its solvent density with NaBr to d 1.10 g/ml. The floated fraction was dialyzed against the extraction buffer and then subjected to gel exclusion chromatography on Sephacryl 400 HR (column dimensions 87 × 1.5 cm) at a flow rate of 1 ml/min. The retained fraction that co-eluted with LDL was pooled and designated A-LDL. Average yield of apoB using this procedure was about 20 μg apoB per tissue wet weight. Five separate batches of A-LDL were prepared for this study using procedure B. Based on previous studies (8) and more recent unpublished studies, no differences were found in the structural and functional properties of A-LDL isolated from tissues obtained either 12 h after death or immediately after surgery.

Lipoprotein isolation and modification

LDL was isolated from fresh (<24-h-old) plasma, obtained from the Cleveland Clinic Blood Bank, by differential ultracentrifugation as the $1.019 < d < 1.063$ g/ml fraction as described by Hatch and Lees (11). LDL was acetylated by repetitive additions of acetic anhydride (12) and then extensively dialyzed against 150 mM NaCl, 1.5 mM Na_2EDTA, pH 7.4. The extent of lysyl modification was measured by changes in 2,4,6-trinitrobenzene sulfonic acid reactivity (13). Routinely, >60% of the lysyl residues of LDL were modified. LDL, acetyl-LDL, and A-LDL were labeled with ^{125}I following the iodine monochloride procedure of McFarlane (14) as modified by Bilheimer, Eisenberg, and Levy (15); the specific activity of labeled preparations was generally 100–200 cpm/ng protein.

Lipoproteins were also labeled with [^{3}H]cholesteryl oleyl ether by first incorporating the [^{3}H]cholesteryl ether into high density lipoproteins following the lipid dispersion technique of Morton and Zilversmit (16). [^{3}H]Cholesteryl ether-labeled high density lipoprotein (100 μg cholesterol) was then incubated with the lipoprotein of interest (2 mg of cholesterol) plus partially purified lipid transfer protein (500 μl of CM-cellulose fraction). After incubation at 37°C for 6 h, the radiolabeled lipoprotein was re-isolated by ultracentrifugation as the $d < 1.063$ g/ml fraction.

LDL was induced to aggregate by modifying with HNE, a major propagation product of lipid peroxidation (17), as previously published (18). Briefly, 500 μg LDL protein/ml was incubated with 5 mM HNE for 5 h at

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37°C. Approximately 90–95% of the particles could be precipitated by centrifugation at 10,000 *g*. LDL (1 mg protein/ml) was oxidized by dialysis in the presence of 10 μM $CuSO_4$ for 24 h at 20°C, rather than at 37°C as previously published (19). The oxidized sample was then subjected to centrifugation at 10,000 *g* and the supernatant and precipitated fractions were collected. Ethanol-denatured LDL was prepared by incubating LDL (2 mg protein/ml) with absolute ethanol 1:1 (v/v) for 15 min and subsequently dialyzing the lipoprotein overnight against 0.15 M NaCl, 0.3 mM EDTA, pH 7.4. A-LDL was induced to aggregate by vortexing for 30 sec at 20°C at full power using a Vortex-Geinie Mixer (Scientific Products, McGaw Park, IL) as described by Khoo et al. (20) or by concentrating using a Centriprep 30 concentrator or dialysis using Spectra/Por dialysis tubing (3,500 molecular weight cutoff) and polyethylene glycol (20,000 molecular weight).

LDL and A-LDL particles were observed by transmission electron microscopy after staining with phosphotungstic acid (21). Determination of the number of aggregated A-LDL and LDL particles was assessed in a field of 400 to 1000 particles present in a final print at 25,000 × magnification. Lipoprotein total cholesterol was determined enzymatically with Reagent Set (Boehringer, Mannheim, Indianapolis, IN), a modification of the method of Roeschlau, Bernt, and Gruber (22). Unesterified and esterified cholesterol values were determined by the procedure of Gamble et al. (23). Protein was determined by the Peterson modification of the procedure of Lowry et al. (24) using human serum albumin as a standard.

Cell interaction studies

Preparation of cultured cells. Arterial SMC were obtained by explant culture of the medial layer from juvenile rabbit thoracic aorta (25) or from bovine aortas obtained at a slaughter house. Cells grown out of medial explants were suspended in a solution of trypsin and EDTA and subcultured. The SMC used in these studies were from the fourth to sixth passage. The cells were grown in a 1:1 mixture of Dulbecco-Vogt and Ham's F-12 media containing 10% fetal calf serum. The media were changed twice a week. Normal (GM 5757) and LDL-receptor-deficient (GM 1915B) fibroblasts were obtained from the NIGMS Human Genetic Mutant Cell Repository (Camden, NJ) and cultured as described above for SMC.

Stimulation of cholesterol esterification. Stimulation of cholesterol esterification in SMC by lipoproteins was measured as previously described in macrophages (8, 9). Essentially, SMC grown in 24-well plates were incubated with [^{14}C]oleate-albumin, and the incorporation of radiolabeled oleate into cholesteryl oleate was determined after the separation of lipids by thin-layer chromatography.

Lipoprotein uptake by cells. Measurement of cellular uptake of lipoproteins was performed in SMC as was previously reported in macrophages (9). Briefly, lipoproteins, whose lipid core was labeled with [^{3}H]cholesteryl oleyl ether (10), were incubated with cells for the time intervals indicated and the amount of cell-associated label was determined after extensive washing.

Lipoprotein degradation in cells. The degradation of ^{125}I-labeled LDL, acetyl LDL, and A-LDL by SMC was measured essentially as described by Goldstein et al. (12). Wells contained ^{125}I-labeled lipoproteins ± unlabeled lipoproteins, 6 mg/ml bovine serum albumin, and DMEM medium in a total volume of 0.25 ml. At the indicated time, the medium was removed, the cells were briefly rinsed with phosphate-buffered saline, and the combined medium plus rinse was subjected to 10% TCA precipitation. The extent of degradation was calculated from the TCA-soluble, noniodide radioactivity. All values were corrected for the TCA-soluble, noniodide radioactivity present in identical wells which lacked cells. Determinations were made in triplicate, unless otherwise stated, and were expressed as means ± SD, unless otherwise indicated.

RESULTS

In preliminary studies we determined that SMC degrade A-LDL by a high affinity mechanism and that A-LDL isolated by procedures A or B do not differ in their uptake and degradation by SMC. To determine whether A-LDL was recognized by the LDL receptor on SMC, we assessed whether A-LDL uptake, degradation, and subsequent stimulation of cholesterol esterification was reduced in SMC in which the LDL receptor had been down-regulated. As seen in **Table 1**, when LDL or A-LDL was incubated with rabbit SMC that had been pre-incubated with LDL, stimulation of cholesterol esterification was dramatically reduced relative to control cells. In addition, pre-incubation of SMC with A-LDL was just as effective as LDL in down-regulating the cellular response to these lipoproteins. Similar results were obtained with bovine SMC (not shown).

The role of the LDL receptor in A-LDL uptake was further investigated by constrasting A-LDL metabolism in fibroblasts from a normal subject with those from a homozygous familial hypercholesterolemic (FH) subject. The uptake of A-LDL and LDL was found to be nearly the same in the normal cells, but essentially undetectable in FH cells (**Table 2**, Experiment 1). Similar results were obtained in a separate study using cholesterol esterification as the experimental end point (Table 2, Experiment 2). Furthermore, the uptake of labeled LDL and A-LDL by normal fibroblasts was markedly inhibited by a 10-fold excess of either unlabeled A-LDL or LDL (Table 2, Experiment 1).

To further characterize the catabolism of A-LDL by SMC, we performed cross-competition degradation stud-

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TABLE 1. Effect of down-regulating the LDL receptor on cholesterol esterification induced by A-LDL and LDL in arterial smooth muscle cells

Additions to Pre-incubation Media	Lipoprotein Used for Stimulation	Stimulation of Cholesterol Esterification
		nmol cholesteryl ester/mg cell protein/6 h
None	LDL	2.5 ± 0.4
None	A-LDL	2.6 ± 0.4
LDL	LDL	0.2 ± 0.2
LDL	A-LDL	0.9 ± 0.2
A-LDL	LDL	0.2 ± 0.1
A-LDL	A-LDL	0.7 ± 0.1

SMC were cultured for 24 h in 10% LPDS-containing media followed by an additional 24 h with LPDS in the absence or presence of LDL or A-LDL (100 μg cholesterol/ml). Stimulation of cholesterol esterification was then measured during a subsequent 6-h incubation of SMC with LDL or A-LDL (100 μg cholesterol/ml) and [^{14}C]oleate. The extent of cholesterol esterification that occurred in cells incubated in the absence of lipoprotein has been subtracted from the values presented. A-LDL was isolated by Procedure A. Values are expressed as a mean ± SD for duplicate determinations.

ies. The degradation of ^{125}I-labeled A-LDL was inhibited by up to 80% by excess unlabeled A-LDL and LDL (**Fig. 1a**). LDL was just as effective as A-LDL in inhibiting the degradation of ^{125}I-labeled A-LDL. Although unlabeled A-LDL was able to inhibit the degradation of ^{125}I-labeled LDL, it was not as effective as unlabeled LDL, as indicated by the more shallow slope of the competition curve (Fig. 1b). These results suggest that A-LDL is recognized by the LDL receptor but with lower affinity than LDL. This is consistent with the observation that at

TABLE 2. Lipoprotein uptake and cholesterol esterification in normal and LDL receptor-negative fibroblasts

Experiment	Unlabeled Lipoprotein	Lipoprotein Uptake	Cholesterol Esterification
		pmol cholesterol/μg cell DNA/6 h	*pmol cholesterol/μg cell DNA/6 h*
Experiment 1			
With normal fibroblasts:			
LDL		330 ± 38	
LDL	LDL	73 ± 3	
LDL	A-LDL	99 ± 0	
A-LDL		318 ± 3	
A-LDL	LDL	47 ± 11	
A-LDL	A-LDL	58 ± 15	
With FH fibroblasts:			
LDL		38 ± 5	
A-LDL		72 ± 16	
Experiment 2			
With normal fibroblasts:			
None			40 ± 3
LDL			210 ± 29
A-LDL			270 ± 16
Acetyl-LDL			20 ± 2
With FH fibroblasts:			
None			30 ± 3
LDL			50 ± 4
A-LDL			50 ± 1
Acetyl-LDL			50 ± 4

Fibroblasts were pre-incubated for 24 h in 10% LPDS-containing media. For experiment 1, lipoproteins (20 μg lipoprotein cholesterol/ml) were labeled with [^{3}H]cholesteryl ether and the uptake by fibroblasts was measured in the presence and absence of 200 μg unlabeled lipoprotein/ml. Uptake is expressed as pmoles lipoprotein cholesterol and was calculated from the cellular ^{3}H content and the initial specific activity of the lipoprotein. In Experiment 2, performed with different lipoproteins than those used in Expt. 1, the stimulation of cholesterol esterification elicited by 100 μg lipoprotein cholesterol/ml was measured as described in the Methods. A-LDL was isolated by Procedure A. "FH" fibroblasts refers to LDL receptor-negative fibroblasts. Values are expressed as mean ± SD of duplicate determinations.

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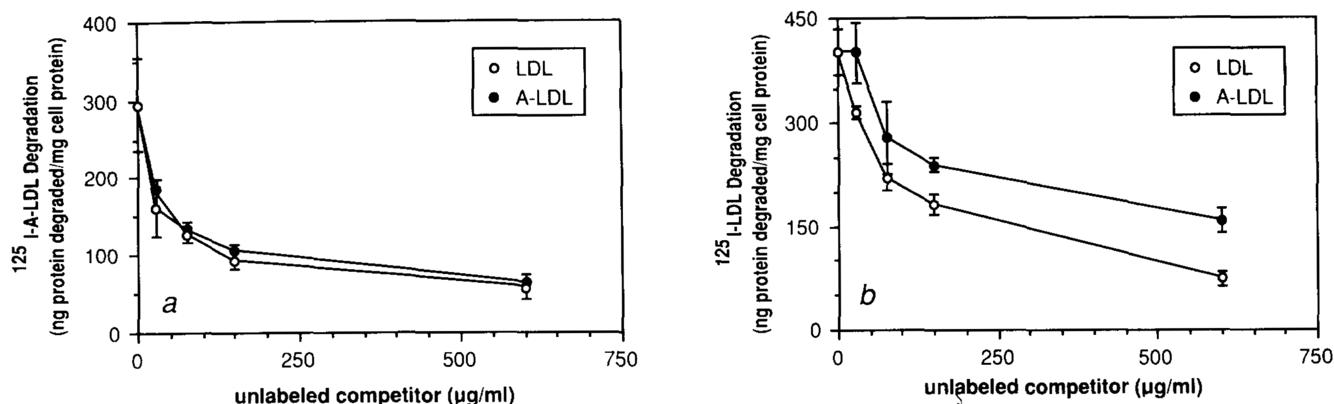

Fig. 1. Degradation of ^{125}I-labeled A-LDL and ^{125}I-labeled LDL by SMC in the absence and presence of unlabeled A-LDL and LDL. Confluent secondary cultures of SMC were pre-incubated for 24 h in media containing 10% LPDS. Cells were then incubated with 15 µg protein/ml of ^{125}I-labeled A-LDL isolated by procedure B (panel a) or ^{125}I-labeled LDL (panel b) for 4.5 h at 37°C in the presence of either unlabeled A-LDL or unlabeled LDL at 0 to 40-fold excess concentrations (0 to 600 µg protein/ml). Intracellular degradation of lipoproteins was determined as described in Methods.

a concentration of 15 µg protein/ml (i.e., zero competitor in Fig. 1), LDL degradation was 400 ng protein/mg cell protein/4.5 h, whereas A-LDL degradation was only 300. However, since the protein content of A-LDL was shown to be lower than that of LDL (9), when expressed as molar concentrations, such differences in affinities almost disappeared. SMC degradation of A-LDL from different batches ranged from 50 to 100% that of LDL (not shown) at equivalent concentrations. This difference in degradation was not due to poor intracellular degradation of A-LDL, since the fraction of cell-associated, undegraded A-LDL was similar to that for LDL (**Table 3**).

Further insights into the mechanisms of A-LDL metabolism by SMC were gained from a study of the concentration dependence of cholesterol esterification. As seen in **Fig. 2**, the apparent affinities of SMC for A-LDL and LDL were nearly the same (half-maximum ~25 µg lipoprotein cholesterol/ml). However, despite similar affinities, the maximum esterification elicited by LDL was more than 50% higher than that for A-LDL. This result suggests that A-LDL is less effectively internalized at higher lipoprotein concentrations.

The most likely explanation for the differences in the degradation and esterification of A-LDL and LDL (Figs. 1 and 2) is that some particles in A-LDL preparations are not recognized by the LDL receptor. Consistent with this, based on data from our laboratory and others (26–28), A-LDL is chemically modified and, at least a portion, is recognized by a non-LDL receptor on macrophages in culture. It is anticipated that this A-LDL would be recognized by the LDL receptor with much lower affinity. Likewise, since A-LDL was recently shown by us to be easily aggregated (H. F. Hoff, J. M. Pepin, and R. E. Morton unpublished observation), it is possible that aggregated A-LDL is poorly catabolized by the SMC. As seen in **Fig. 3a**, some aggregation of A-LDL could be seen by transmission electron microscopy after negative staining. In a field containing over 1200 particles, 65 aggregates containing 2 to 7 particles (mean of 3, median of 2) were observed. Thus, about 15% of the particles were aggregated, mainly as dimers and trimers. In a similar field of LDL particles about 5% were aggregated. However, aggregation was enhanced greatly when A-LDL was concentrated from 100 µg/ml to 2 mg/ml for its use in com-

TABLE 3. Processing of A-LDL and LDL by smooth muscle cells

^{125}I-Labeled Lipoprotein Added	Lipoprotein Degradation	Cell-Associated Lipoprotein
	ng lipoprotein protein/mg cell protein	*ng lipoprotein protein/mg cell protein*
LDL	3053 ± 91	557 ± 32
A-LDL	2077 ± 58	443 ± 21

After a 24-h pre-incubation of SMC in media containing 10% LPDS, the cells were incubated with ^{125}I-labeled LDL or A-LDL (16 µg lipoprotein cholesterol) in a total media volume of 0.5 ml. After 16 h, the media were removed and the cells were rapidly rinsed three times with media, released from the plate by trypsin/EDTA (6 and 20 mg/100 ml, respectively, for 5 min, 37°C followed by the addition of 1 ml of 10% FCS in media), and collected by centrifugation. The extent of lipoprotein degradation (noniodide, TCA-soluble radioactivity in the media) and the amount of undegraded lipoprotein associated with the cells (cellular TCA-precipitable radioactivity) were determined as described in the Methods. A-LDL was isolated by Procedure A. Values are mean ± SE (n = 4–6). Cell-free degradation values have been subtracted from those shown.

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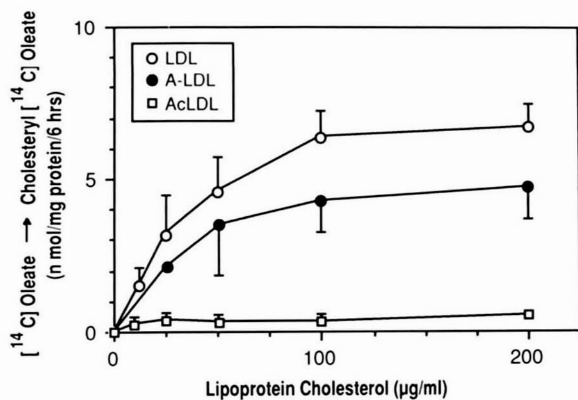

Fig. 2. Stimulation of cholesterol esterification in SMC induced by A-LDL and LDL. Secondary cultures of SMC were pre-incubated for 24 h in media containing 10% LPDS. Lipoproteins ranging in concentration between 0 and 200 µg cholesterol/ml were incubated with SMC for 6 h at 37°C and the incorporation of [^{14}C]oleate into cholesterol oleate was determined as described in Methods. A-LDL was isolated by procedure A. Each determination represents the mean of duplicate determinations.

petition and dose-response studies (Fig. 3b). Four hundred out of 430 particles viewed were in an aggregated state even when excluding extremely large aggregates (1 to 10 microns) that precipitated from solution.

To assess whether aggregated lipoproteins are recognized by SMC, we compared the degradation of ^{125}I-labeled LDL that had been induced to aggregate by several methods with that of unmodified ^{125}I-labeled LDL. These forms of aggregated LDL consisted of: *1*) LDL modified by HNE, a major propagation product of lipid peroxidation (17,18); *2*) the precipitate formed after LDL was oxidized by Cu^{2+} (19); and *3*) LDL denatured by treating with 50% ethanol. When each of these aggregated forms of ^{125}I-labeled LDL was incubated with SMC, none was measurably degraded (**Fig. 4a**). Cell-associated radiolabel for ^{125}I-labeled HNE-modified LDL was also low (not shown), suggesting that the poor degradation of aggregated LDL was due to poor uptake rather than poor intracellular processing. The soluble fraction of oxidized LDL, e.g., 10,000 *g* supernatant fraction, was also studied to assess whether its degradation by SMC differed from that of LDL. SMC degradation of soluble oxidized LDL was only about one-half that of LDL but greater than that of the insoluble fraction of oxidized LDL. In one study, ^{125}I-labeled A-LDL was aggregated by vortexing (20), which resulted in the precipitation of 70% of the label after centrifugation at 10,000 *g*. As seen in Fig. 4b, degradation of aggregated ^{125}I-labeled A-LDL was less than half that of the original A-LDL sample, which was degraded at about the same rate as ^{125}I-labeled LDL in this experiment. Degradation of ^{125}I-labeled acetyl LDL was less than half that of ^{125}I-labeled LDL. This latter result suggests a small contribution by the scavenger receptor, recently shown to be expressed on rabbit SMC under certain conditions (26),

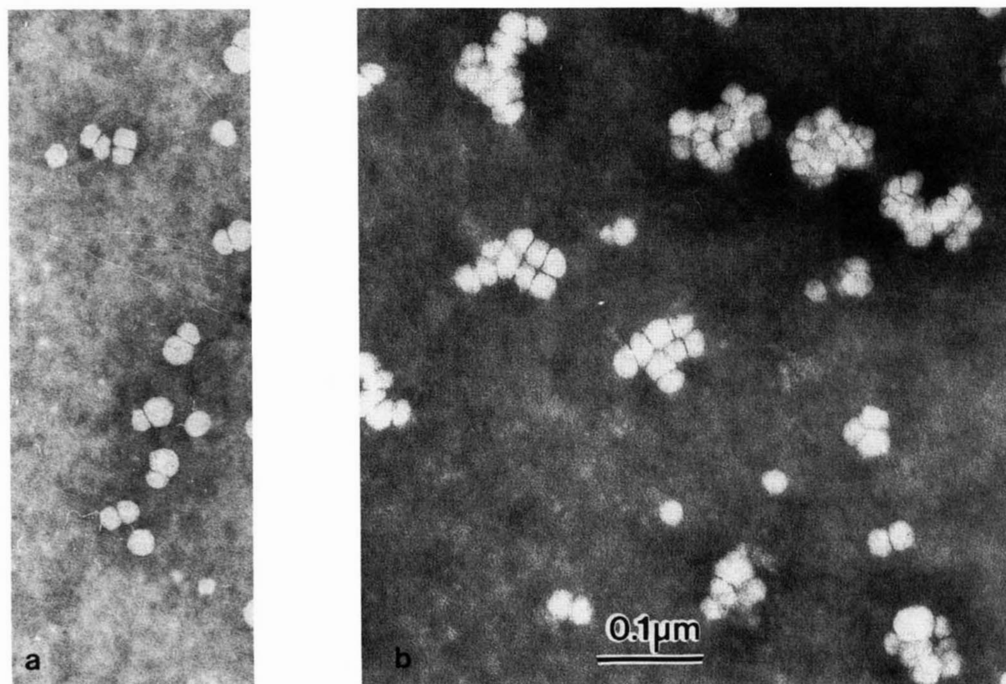

Fig. 3. Electron micrographs of A-LDL. Electron microscopy of A-LDL was performed following negative staining with 2% phosphotungstate as previously described (21). Fig. 3a illustrates the properties of A-LDL upon its isolation, and Fig. 3b shows A-LDL particles after concentration to 2 mg/ml. Both micrographs are at same magnification.

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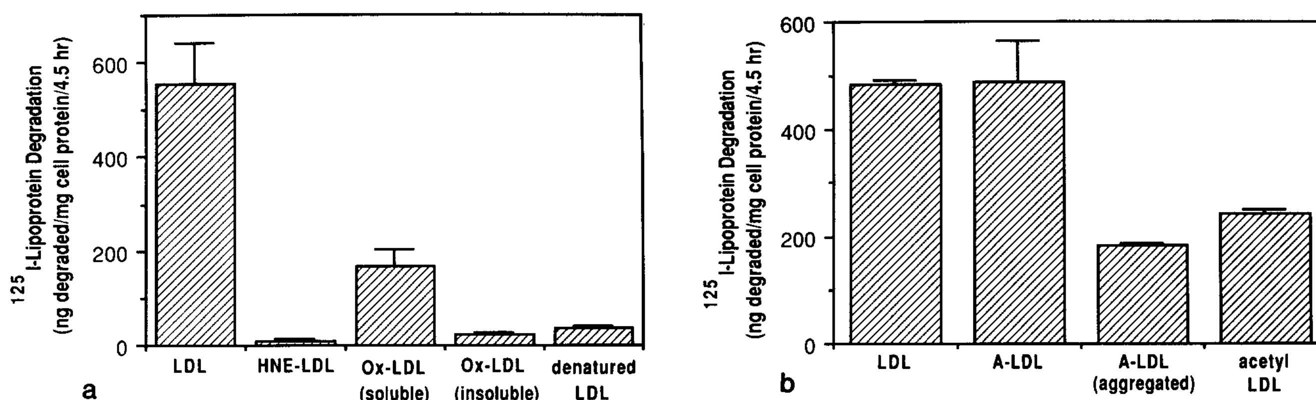

Fig. 4. Degradation of native and aggregated lipoproteins by SMC. Secondary cultures of SMC were pre-incubated for 24 h in media containing 10% LPDS. ^{125}I-labeled native LDL, A-LDL, or aggregated forms of these lipoproteins, were prepared as described in the Methods and added at a final concentration of 25 μg protein/ml to SMC for 4.5 h at 37°C. Intracellular degradation was determined as described in the Methods. Non-standard abbreviations are: ox-LDL, copper oxidized LDL; denatured LDL, ethanol-treated LDL.

to total uptake of A-LDL by SMC. This is consistent with the results of a separate experiment showing that the degradation of ^{125}I-labeled A-LDL is reduced only 11% by the presence of a 30-fold excess of acetyl LDL (458 ± 37 vs. 514 ± 12 mg protein degraded/mg cell protein/4.5 h). Collectively, these results suggest that the observed lower catabolism of A-LDL by SMC may have been due to partial aggregation of the A-LDL particles.

In addition to the foregoing short-term studies, we have also assessed whether long-term incubations of SMC with A-LDL could induce lipid loading in vitro, and therefore, perhaps potentiate SMC foam cell formation in vivo. SMC were incubated with unlabeled or radiolabeled lipoproteins for up to 48 h. As seen in **Fig. 5**, the degradation of both ^{125}I-labeled A-LDL and ^{125}I-labeled LDL tended to plateau at time points ≥ 32 h. The rate of degradation of A-LDL was lower than that of LDL for all time intervals. Additionally, neither lipoprotein was capable of inducing cholesteryl ester accumulation in SMC after a 48-h incubation (**Table 4**). However, both lipoproteins increased cellular free cholesterol by ~20%. Together, these results are consistent with the uptake of both lipoproteins occurring via the LDL receptor, and also suggests that incubation of SMC with A-LDL does not appear to induce appreciable lipid loading.

DISCUSSION

Several lines of evidence from this study suggest that the modified form of LDL present in the arterial lesion, A-LDL, is internalized by SMC via the LDL receptor. For example, down-regulation of the LDL receptor by pre-incubation with LDL dramatically reduced lipoprotein-mediated cholesterol esterification for both LDL and A-LDL. Notably, pre-incubation of cells with A-LDL was just as effective as LDL in down-regulating the LDL receptor. The uptake of A-LDL and LDL, as estimated by the stimulation of cholesterol esterification, was also greatly reduced in fibroblasts that lack functional LDL receptors as compared to normal fibroblasts. Furthermore, cross-competition degradation studies showed that excess LDL or A-LDL could inhibit the degradation of either ^{125}I-labeled A-LDL or ^{125}I-labeled LDL. Although excess LDL and A-LDL were equally effective inhibiting the degradation of labeled A-LDL, excess LDL was more effective than A-LDL in inhibiting the degradation of labeled LDL. Even though this latter result suggests that the affinity of binding of A-LDL to the LDL receptor is lower than that of LDL, such a conclusion is not supported by the data which indicate that the affinities of A-LDL and LDL are similar (Fig. 2, and Fig. 1 when

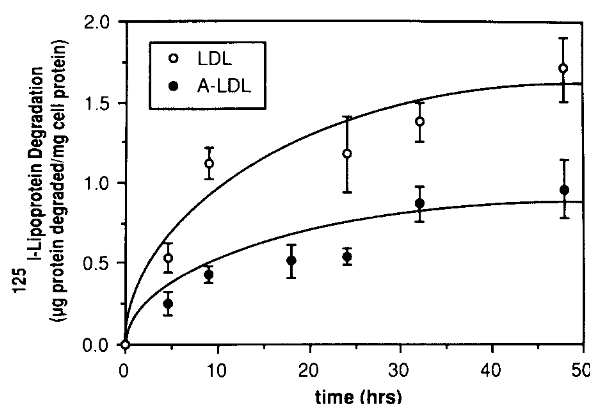

Fig. 5. Time-dependent increase in degradation of ^{125}I-labeled A-LDL and ^{125}I-labeled LDL by SMC. Secondary cultures of SMC were pre-incubated for 24 h in media containing 10% LPDS. Radiolabeled lipoproteins were incubated with confluent SMC at a concentration of 15 μg protein/ml for periods ranging from 0 to 48 h. Intracellular degradation of lipoproteins was determined as described in Methods. A-LDL was isolated by procedure B.

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TABLE 4. Effects of LDL and A-LDL on smooth muscle cell cholesterol content

Lipoprotein Added	μg Total Cholesterol/ mg Cell Protein	μg Free Cholesterol/ mg Cell Protein	μg Esterified Cholesterol/ mg Cell Protein
None	45.1 ± 0.7	31.8 ± 0.6	13.3 ± 0.7
LDL	49.4 ± 2.2	38.5 ± 0.8	10.9 ± 1.7
A-LDL	53.4 ± 0.8	39.2 ± 3.2	14.2 ± 2.3

Smooth muscle cells were pre-incubated for 24 h in 10% LPDS in media, then incubated in media containing the indicated lipoprotein at 100 μg cholesterol/ml. After 24 h, the incubation media were replenished with the same reagents and the incubations were continued for a total of 48 h. The cellular content of total and free cholesterol was measured enzymatically as described in the Methods. Esterified cholesterol was calculated as the difference between these two values. Values shown are the mean ± SD (n = 4-6).

converted to molar concentrations). Rather, the data indicate that these lipoproteins differ in the maximum cellular response they can elicit (Figs. 4 and 5).

The reasons why A-LDL facilitates a lower maximum response in SMC are not completely clear. Since, in a related, unpublished study, we have observed that A-LDL has an enhanced tendency to aggregate and that this aggregation is facilitated by high A-LDL concentrations, we speculate that this property of A-LDL may alter its interaction with SMC, especially at higher concentrations. We have observed here that the ability of SMC to degrade ^{125}I-labeled LDL aggregated by several different procedures, e.g., ethanol-denatured, oxidized, or treated with HNE, was significantly decreased compared to native LDL. In addition, A-LDL that was aggregated by vortexing was also degraded to half the rate of the original A-LDL sample.

Thus, we propose that the decreased cellular catabolism of A-LDL by SMC, which is most easily discernible at higher A-LDL concentrations, is due to the formation of A-LDL aggregates that do not interact readily with SMC. The interactions of unaggregated and aggregated forms of LDL and A-LDL with SMC appear to be the direct opposite of their interactions with macrophages in culture. Uptake and degradation of A-LDL was previously shown by us to be greater than that of LDL in mouse peritoneal macrophages (9). Aggregated forms of LDL were also shown to be degraded to a greater degree than unaggregated LDL in macrophages (20, 29). Recently, we have found that aggregates of A-LDL induced by concentration or vortexing were degraded to a greater degree than unaggregated A-LDL in macrophages (H. F. Hoff, J. M. Pepin, and R. E. Morton, unpublished observations).

The reduced uptake of aggregated forms of LDL by SMC also appears to contradict the data of others that demonstrate that aggregated LDL associated with connective tissue components can increase the cholesterol content of SMC (5). However, these investigators did not rule out the possibility that most of the cholesterol was derived from aggregated LDL adhering to the cell surface. Additionally, although these authors state that their primary cultures of human intimal cells were SMC, they also have not ruled out the presence of tissue macrophages in their preparations which could have contributed to their results. In a recent study, Davis and Bowyer (30) showed that proteolytically digested β-very low density lipoprotein could induce cholesteryl ester accumulation in SMC. However, again the possibility that this cell-associated lipid had not been internalized was not ruled out.

Although the data from the present study suggest that A-LDL and LDL interact with receptors on cultured SMC with similar affinities, we cannot rule out the possibility that A-LDL represents a mixture of particles with divergent affinities for the LDL receptor whose mean is similar to that of LDL. Data from several studies suggest that some particles in A-LDL have undergone oxidation (7, 26–28) resulting in recognition by the scavenger receptor (27).Assuming that the extent of oxidation varies widely, individual particles in A-LDL may interact with the LDL receptor in a wide range of affinities as well as not at all. Although the scavenger receptor on SMC pre-incubated with LPDS for 24 h would not be extensively expressed (31), the observation by us that acetyl LDL was degraded by SMC at about one-half that of LDL suggests that some scavenger receptor activity is present which could affect the overall interpretation of the relative affinities of A-LDL and LDL. Finally, published studies of A-LDL extracted from human grossly normal aortic intima (32) and unpublished observations by us on A-LDL from atherosclerotic lesions revealed the presence of apoE on A-LDL. This would increase the affinity of the lipoprotein for the LDL receptor compared to LDL (4). Thus, mixtures of particles of A-LDL with both increased and decreased affinities for the LDL receptor might give an average affinity similar to that of LDL. Additional studies are needed in which A-LDL is further separated into functionally homogeneous fractions before the contributions of each subfraction to the total A-LDL sample can be ascertained.

The studies reported here on the time-dependent degradation of ^{125}I-labeled A-LDL and LDL show that this degradation approaches a maximum at later time intervals, and imply that A-LDL cannot induce SMC-

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derived foam cell formation. This was confirmed by measurements of the mass of cholesterol accumulating. At the moment, however, we cannot exclude the possibility that within the atherosclerotic lesion there may be A-LDL subspecies that can induce lipid loading in these cells but, due to their rapid catabolism, are absent from our A-LDL isolates. Likewise, we cannot exclude the possibility that human SMC may respond to A-LDL differently than the rabbit cells studied herein, or that cultured SMC may interact differently with A-LDL than those in vivo. In this regard, Campbell et al. (33) demonstrated that the uptake and processing of LDL differed between SMC in the contractile state, e.g., primary cultures, and those in the synthetic state, e.g., secondary cultures. They demonstrated that SMC in the synthetic stated degraded LDL at much lower rates than SMC in the contractile state. However, when grown in hyperlipemic serum, much greater lipid accumulation occurred in SMC in the synthetic state than in the contractile state.

Several groups have demonstrated that lipid loading of SMC can be achieved after an incubation with cell debris from atherosclerotic lesions (34), or with cholesteryl ester droplets derived from lipid-laden macrophages or lipid emulsions free of protein (6). It remains unclear as to why aggregates of LDL are not internalized by SMC, whereas lipid droplets and/or cell debris are readily catabolized. Nevertheless, based on our observations, we conclude that the SMC foam cells in atherosclerotic lesions are more likely to be formed by internalization of products from lysed macrophage-derived foam cells than from the direct uptake of LDL accumulated in the arterial intima, regardless of whether the LDL is modified or not. Such a scenario would also be consistent with the typical pathology of lesion progression where SMC foam cells occur after lipid laden macrophages (2).

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REFERENCES

1. McGill, H. C., Jr. 1968. Fatty streaks in the coronary arteries and aorta. *Lab. Invest.* **18:** 560-570.
2. Geer, J. C. 1965. Fine structure of human aortic intimal thickening and fatty streaks. *Lab. Invest.* **14:** 1764-1781.
3. Brown, M. S., and J. L. Goldstein. 1983. Lipoprotein metabolism in the macrophage: implications for cholesterol deposition in atherosclerosis. *Annu. Rev. Biochem.* **52:** 223-261.
4. Goldstein, J. L., and M. S. Brown. 1977. Low-density lipoprotein pathway and its relation to atherosclerosis. *Annu. Rev. Biochem.* **46:** 897-930.
5. Orekhov, A. N., V. V. Tertov, D. N. Mukhim, V. E. Koteliansky, M. A. Glukhova, K. A. Khashimov, and V. N. Smirnov. 1987. Association of low-density lipoprotein with particulate connective tissue matrix components enhances cholesterol accumulation in cultured subendothelial cells of human aorta. *Biochim. Biophys. Acta.* **928:** 251-258.
6. Wolfbauer, G., J. M. Glick, L. K. Minor, and G. H. Rothblat. 1986. Development of the smooth muscle foam cell: uptake of macrophage lipid inclusions. *Proc. Natl. Acad. Sci. USA.* **83:** 7760-7764.
7. Hoff, H. F., and J. W. Gaubatz. 1982. Isolation, purification, and characterization of a lipoprotein containing apoB from the human aorta. *Atherosclerosis.* **42:** 273-297.
8. Clevidence, B. A., R. E. Morton, G. West, D. M. Dusek, and H. F. Hoff. 1984. Cholesterol esterification in macrophages: stimulation by lipoproteins containing apoB isolated from human aortas. *Arteriosclerosis.* **4:** 196-207.
9. Morton, R. E., G. A. West, and H. F. Hoff. 1986. A low density lipoprotein-sized particle isolated from human atherosclerotic lesions is internalized by macrophages via a non-scavenger-receptor mechanism. *J. Lipid Res.* **27:** 1124-1134.
10. Halperin, G., and S. Gatt. 1980. The synthesis of cholesteryl alkyl ethers. *Steroids.* **35:** 39-42.
11. Hatch, F. T., and R. S. Lees. 1968. Practical method for plasma lipoprotein analysis. *Adv. Lipid Res.* **6:** 2-63.
12. Goldstein, J. L., Y. K. Ho, S. K. Basu, and M. S. Brown. 1979. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proc. Natl. Acad. Sci. USA.* **76:** 333-337.
13. Habeeb, A. F. S. A. 1966. Determination of free amino groups in proteins by trinitrobenzenesulfonic acid. *Anal. Biochem.* **14:** 328-336.
14. McFarlane, A. S. 1958. Efficient trace-labelling of proteins with iodine. *Nature.* **182:** 53-57.
15. Bilheimer, D. W., S. Eisenberg, and R. I. Levy. 1972. The metabolism of very low density lipoprotein proteins. I. Preliminary in vitro and in vivo observations. *Biochim. Biophys. Acta.* **260:** 212-221.
16. Morton, R. E., and D. B. Zilversmit. 1981. A plasma inhibitor of triglyceride and cholesteryl ester transfer activities. *J. Biol. Chem.* **256:** 11992-11995.
17. Jürgens, G., J. Lang, and H. Esterbauer. 1986. Modification of human low-density lipoprotein by the lipid peroxidation product 4-hydroxynonenal. *Biochim. Biophys. Acta.* **875:** 103-114.
18. Hoff, H. F., J. O'Neil, G. M. Chisolm, T. B. Cole, O. Quehenberger, H. Esterbauer, and G. Jürgens. 1989. Modification of LDL with 4-hydroxynonenal induces uptake of LDL by macrophages. *Arteriosclerosis.* **9:** 538-549.
19. Steinbrecher, U. P. 1987. Oxidation of human low density lipoprotein results in derivatization of lysine residues of apolipoprotein B by lipid peroxide decomposition products. *J. Biol. Chem.* **262:** 3603-3608.
20. Khoo, J. C., E. Miller, P. McLaughlin, and D. Steinberg. 1988. Enhanced macrophage uptake of low density lipoprotein after self-aggregation. *Arteriosclerosis.* **8:** 348-358.
21. Hoff, H. F., J. D. Morrisett, and A. M. Gotto. 1973. Interaction of phosphatidylcholine and apolipoprotein alanine: electron microscopic studies. *Biochim. Biophys. Acta.* **296:** 653-660.
22. Roeschlau, P., E. Bernt, and W. Gruber. 1974. Enzy-

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matische Bestimmung des Gesamt-Cholesterins im Serum. *Z. Klin. Chem. Klin. Biochem.* **12:** 403–407.
23. Gamble, W., M. Vaughan, H. S. Kruth, and J. Avigan. 1978. Procedure for determination of free and total cholesterol in micro- or nanogram amounts suitable for studies with cultured cells. *J. Lipid Res.* **19:** 1068–1070.
24. Peterson, G. L. 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal. Biochem.* **83:** 346–356.
25. Ross, R. 1971. The smooth muscle cell. II. Growth of smooth muscle in culture and formation of elastic fibers. *J. Cell Biol.* **50:** 172–186.
26. Daugherty, A., B. S. Zweifel, B. E. Sobel, and G. Schonfeld. 1988. Isolation of low density lipoprotein from atherosclerotic vascular tissue of Watanabe heritable hyperlipemic rabbits. *Arteriosclerosis.* **8:** 768–777.
27. Shaikh, M., S. Martini, J. R. Quiney, P. Baskerville, A. E. La Ville, N. L. Browse, R. Duffield, P. R. Turner, and B. Lewis. 1988. Modified plasma-derived lipoproteins in human atherosclerotic plaques. *Atherosclerosis.* **69:** 165–172.
28. Ylä-Herttuala, S., W. Palinski, M. E. Rosenfeld, S. Parthasarathy, T. E. Carew, S. Butler, J. L. Witztum, and D. Steinberg. 1989. Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. *J. Clin. Invest.* **84:** 1086–1095.
29. Suits, A. G., A. Chait, M. Aviram, and J. W. Heinecke. 1989. Phagocytosis of aggregated lipoprotein by macrophages: low density lipoprotein receptor-dependent foam-cell formation. *Proc. Natl. Acad. Sci. USA.* **86:** 2713–2717.
30. Davis, J. B., and D. E. Bowyer. 1989. Macrophages modify β-VLDL by proteolysis and enhance subsequent lipid accumulation in arterial smooth muscle cells. *Atherosclerosis.* **77:** 203–208.
31. Pitas, R. E. 1990. Expression of the acetyl low density lipoprotein receptor by rabbit fibroblasts and smooth muscle cells. Up-regulation by phorbol esters. *J. Biol. Chem.* **265:** 12722–12727.
32. Ylä-Herttuala, S., O. Jaakkola, C. Ehnholm, M. J. Tikkanen, T. Solakivi, T. Särkioja, and T. Nikkari. 1988. Characterization of two lipoproteins containing apolipoproteins B and E from lesion-free human aortic intima. *J. Lipid Res.* **29:** 563–572.
33. Campbell, J. H., L. Popadyne, P. J. Nestel, and G. R. Campbell. 1983. Lipid accumulation in arterial smooth muscle cells. Influence of phenotype. *Atherosclerosis.* **47:** 279–295.
34. Hashimoto, S., H. Wong, D. Blissard, and D. Anderson. 1988. Cholesteryl ester accumulation in smooth muscle cells after uptake of necrotic products from atherosclerotic lesions. *Exp. Mol. Pathol.* **49:** 196–205.

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