Overloading Human Aortic Smooth Muscle Cells with Low Density Lipoprotein-Cholesteryl Esters Reproduces Features of Atherosclerosis in Vitro

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A B S T R A C T Human aortic smooth muscle cells accumulate only small amounts of cholesteryl esters in tissue culture, even when incubated for prolonged periods with high levels of plasma low density lipoprotein (LDL). This failure to overaccumulate LDL-cholesteryl esters is due to an LDL-mediated feedback suppression of the activity of the cell surface LDL receptor, a regulatory action that limits the rate at which the cells take up LDL. This regulatory system can be bypassed by incubating smooth muscle cells with LDL that has been given a strong positive charge by covalent linkage with N,N-dimethyl-1,3-propanediamine (DMPA-LDL). The unregulated uptake of DMPA-LDL produces a massive deposition of cholesteryl esters in the form of inclusions within the cell. These inclusions take up lipid stains and exhibit positive birefringence with formée crosses that are typical of liquid crystals of cholesteryl esters. By electron microscopy, the cholesteryl ester inclusions appear as homogeneous gray cytoplasmic lipid droplets. The current studies demonstrate that the unregulated uptake of LDL-cholesteryl esters by human aortic smooth muscle cells can reproduce in vitro the major biochemical and morphological alterations that occur within smooth muscle cells in vivo during the process of atherosclerosis.

INTRODUCTION

Early in human atherosclerosis large amounts of cholesteryl esters accumulate within smooth muscle cells of the arterial intima (1-3). These cholesteryl esters form globular inclusions with the following characteristics: (a) they take up lipid stains such as Oil Red 0 (1-3); (b) they exhibit positive birefringence of the type shown by pure liquid crystals of cholesteryl esters (4); and (c) they appear in the electron microscope as homogeneous electron dense spheres that may or may not be surrounded by a tripartite limiting membrane (3, 5).

Most investigators agree that the cholesterol component of the cholesteryl esters in smooth muscle cells originates from plasma lipoproteins (6). However, attempts to reproduce the pathologic changes of atherosclerosis in vitro by incubating cultured smooth muscle cells with large amounts of plasma lipoproteins from normal or hyperlipidemic animals or man have been disappointing. The maximal level of intracellular cholesteryl esters that can be attained by this approach (7-9) is much lower than the mas-

sive levels seen in naturally occurring or experimentally induced atherosclerosis (2, 6).

A possible explanation for the failure of large amounts of lipoproteins to produce a pathologic over-accumulation of cholesteryl esters in smooth muscle cells has emerged from recent studies of the regulation of lipoprotein uptake in cultured human fibroblasts (10–12). For fibroblasts to take up plasma lipoproteins in amounts sufficient to expand their intracellular cholesteryl ester pool, the cells must first bind the lipoprotein at a specific cell surface receptor site. This receptor is either absent or greatly deficient in cells from patients with homozygous familial hypercholesterolemia (FH).[1] In normal cells this receptor binds the major cholesterol-carrying lipoprotein in human plasma, low density lipoprotein (LDL). After its binding to the receptor, LDL is internalized by adsorptive endocytosis and delivered to lysosomes where its cholesteryl esters are hydrolyzed. The resulting free cholesterol is made available for plasma membrane synthesis and for regulatory actions. Any excess cholesterol that enters the cell through the LDL pathway is reesterified through an activation of a microsomal acyl-CoA:cholesterol acyltransferase and stored within the cell as cholesteryl esters. As cholesteryl esters begin to accumulate, the synthesis of the LDL receptor becomes suppressed and hence the cells limit their uptake of LDL. This feedback suppression of the synthesis of the LDL receptor prevents fibroblasts from accumulating massive amounts of cholesteryl esters even when the cells are exposed to high concentrations of LDL.

The above control mechanism can be overcome and an unregulated uptake of LDL can be achieved in fibroblasts by incubating the cells with LDL that has been rendered polycationic by the covalent attachment of N,N-dimethyl-1,3-propanediamine (DMPA) residues to the lipoprotein (13). This positively-charged DMPA-LDL binds to nonspecific negatively-charged sites on the cell surface from which it is taken up by the fibroblasts through a process that does not involve the physiologic LDL receptor (13).[2]

In the current studies, we have utilized the information learned from the metabolism of DMPA-LDL in fibroblasts to overload cultured human aortic smooth muscle cells with cholesteryl esters, thus reproducing an in vitro counterpart of the in vivo atherosclerotic lesion.

[1] *Abbreviations used in this paper*: DMPA, N,N-dimethyl-1,3-propanediamine; FCS, fetal calf serum; FH, familial hypercholesterolemia; LDL, low density lipoprotein; LPDS, lipoprotein-deficient serum.

[2] Basu, S. K., R. G. W. Anderson, J. L. Goldstein, and M. S. Brown. 1977. Metabolism of cationized lipoproteins by human fibroblasts: Biochemical and morphologic correlations. *J. Cell Biol.* In press.

METHODS

Aortic smooth muscle cells. The normal smooth muscle cell line was derived from intimal-medial segments of the thoracic aorta of a 6-mo-old human fetus and was obtained with informed consent of the parents. The FH homozygote smooth muscle cell line was derived from an 8-yr-old girl (M. C.) whose clinical features have been described previously (14). Segments of M. C.'s thoracic aorta were removed during aortic valve replacement in October 1976. With the informed consent of M. C.'s parents, these segments were used for tissue culture. The smooth muscle cells were cultured by the method of Ross (15) and used between the 4th and 10th passage. By phase contrast microscopy the cells were observed to grow in multiple overlapping layers, and by electron microscopy they showed myofilaments and dense bodies that are characteristic of aortic smooth muscle cells in culture (15).

Stock cultures were maintained in a humidified incubator (5% CO_2) at 37°C in 250-ml flasks containing 10 ml of growth medium consisting of Eagle's minimum essential medium (Grand Island Biological Co., Grand Island, N.Y., catalogue no. F-11) supplemented with penicillin (100 U/ml); streptomycin (100 µg/ml); 20 mM Tricine-chloride, pH 7.4; 24 mM $NaHCO_3$; 1% (vol/vol) nonessential amino acids; and 10% (vol/vol) fetal calf serum (FCS). For each experiment, on day 0 confluent monolayers of cells from stock flasks were dissociated with 0.05% trypsin-0.02% EDTA solution, and 1 × 10^5 cells were seeded into each 60-mm Petri dish containing 3 ml of growth medium with 10% FCS.

Lipoproteins. Human LDL ($d = 1.019$–1.063 g/ml) and lipoprotein-deficient serum (LPDS) ($d > 1.215$ g/ml) were obtained from the plasma of healthy subjects and prepared by differential ultracentrifugation (16). Calf LPDS (16), ^{125}I-labeled LDL (17), and DMPA-LDL (13) were prepared as described in the referenced articles.

Biochemical assays. Measurements of surface-bound, heparin-releasable ^{125}I-LDL (18) and degradation of ^{125}I-LDL (19) in smooth muscle cell monolayers were carried out as described for fibroblasts. The content of free and esterified cholesterol in smooth muscle cells was determined by gas-liquid chromatography (20). Protein was determined by the Lowry method (21).

Electron microscopy. Monolayers were fixed in situ for 30 min with 3% glutaraldehyde in 0.1 M sodium phosphate, pH 7.3, scraped from the dish, and centrifuged (5 min, 4°C, 12,000 rpm). Cells were postfixed with 2% OsO_4 in 0.1 M sodium phosphate, pH 7.3, dehydrated, embedded in araldite, and stained with uranyl acetate and lead citrate.

Oil Red 0 staining. Monolayers were grown on glass coverslips, fixed with 6% paraformaldehyde in 0.1 M sodium phosphate, pH 7.3, and stained with Oil Red 0 and double-strength Harris hematoxylin.

Polarizing light microscopy. Monolayers were grown on glass coverslips that were subsequently mounted on glass slides. To enhance birefringence, the slides were first warmed to 40–45°C for 10 min, which caused the initial birefringence to disappear, after which they were cooled to 10°C for 10 min, and examined immediately thereafter (4, 22). The cells were photographed under polarizing optics with a Zeiss Photomicroscope III (Carl Zeiss, Inc. New York).

RESULTS

The experiment in Fig. 1 compares the ability of native LDL and DMPA-LDL to produce an accumulation of cholesteryl esters in smooth muscle cells. Since
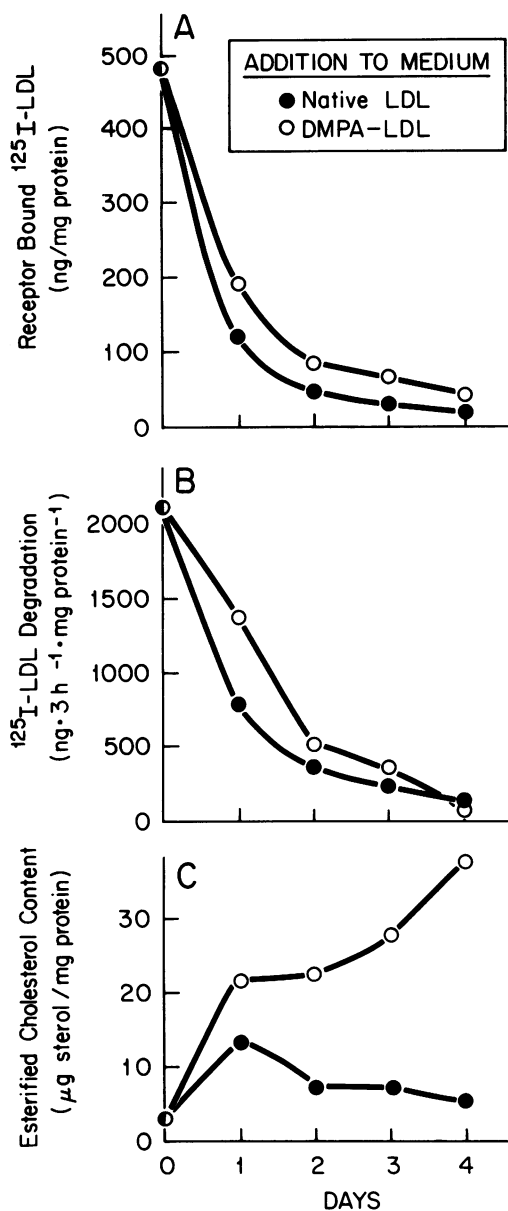
the cells had been subjected to prior incubation in
LPDS, the cholesteryl ester content at the beginning
of the experiment was less than 4 µg sterol/mg
cell protein (Fig. 1C, day 0). At the same time, the
number of LDL receptors was high and hence the
cells were able to bind a large amount of ^{125}I-LDL
(Fig. 1A) and degrade the lipoprotein at a rapid rate
(Fig. 1B). In the presence of this large number of
LDL receptors, the addition of native LDL produced
a fourfold increase in the cellular content of cholesteryl
esters on day 1. Thereafter, however, the number of
LDL receptors declined as indicated by the decreased
ability of the cells to bind and degrade ^{125}I-LDL
after the 1st day. Because of this decrease, the choles-
teryl ester content of the cells did not continue to
rise after day 1 and actually declined significantly

FIGURE 1 Changes in the ability of normal aortic smooth
muscle cells to bind (A) and degrade (B) ^{125}I-LDL and
changes in the cellular content of cholesteryl esters (C)
after incubation with native LDL (●) or DMPA-LDL (○).
On day 0, 1×10^5 cells were seeded into 60-mm Petri
dishes containing 3 ml of medium with 10% FCS. On days
4 and 7, the cells received fresh medium containing 10%
calf LPDS. On day 10 (zero time), the medium was replaced
with 2 ml of fresh medium containing 5% calf LPDS and
either 20 µg protein/ml of native LDL (●) or 5 µg protein/ml
of DMPA-LDL (○). Every 24 h throughout the experiment,
the medium was replaced with fresh medium containing
the same concentration of the indicated lipoprotein. Each
day a group of dishes was divided into two sets. In one set
(Panels A and B), each monolayer was washed twice with
medium containing 5% human LPDS, after which each dish
received 2 ml of medium containing 5% human LPDS and
10 µg protein/ml of ^{125}I-LDL (225 cpm/ng). After incubation
for 3 h at 37°C, the amount of ^{125}I-LDL bound to the cell
surface and hence accessible for heparin release (A) and the
amount of ^{125}I-LDL degraded to TCA-soluble material (B)
was determined. At zero time, more than 95% of the observed
binding and degradation represented specific, high affinity
processes as previously defined (25). For the second set of
dishes (panel C), cell monolayers were washed, harvested, and
pooled (three dishes per sample), and their content of
esterified cholesterol was determined. Each value represents
the average of duplicate incubations (A and B) or duplicate
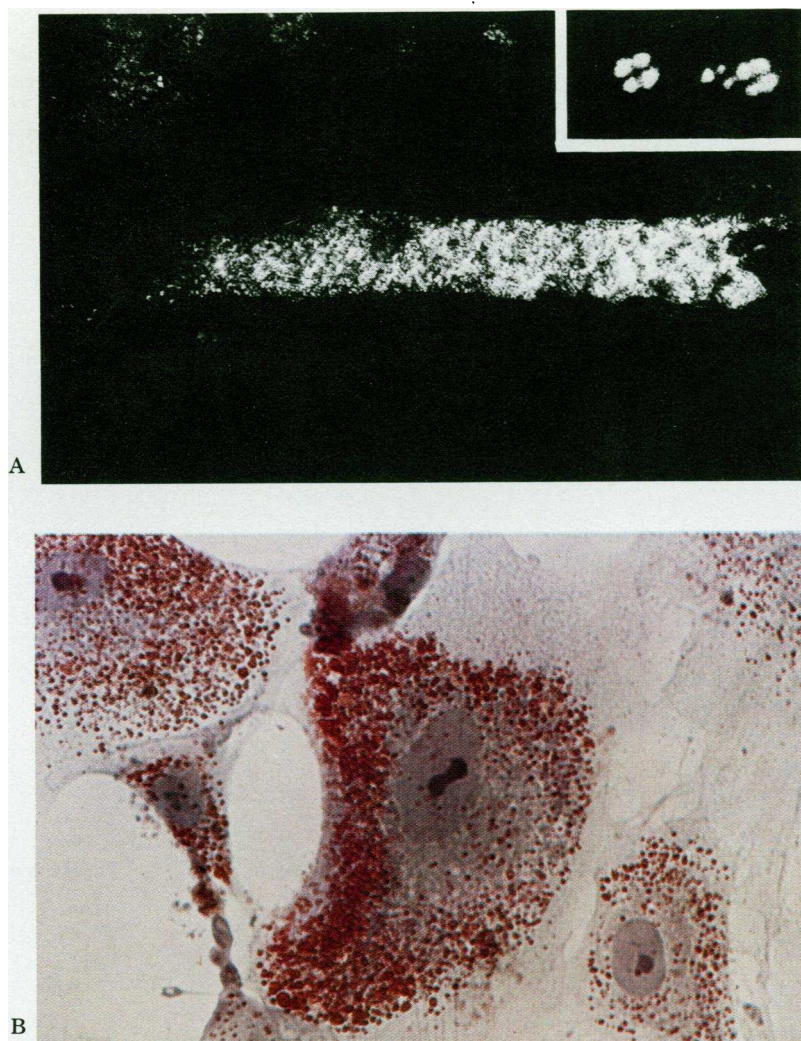samples (C).

FIGURE 2 Light microscopic appearance of normal aortic smooth muscle cells that were repeatedly incubated with DMPA-LDL. Monolayers were grown on coverslips and incubated with 10 μg protein/ml of DMPA-LDL in 2% FCS as described in the legend to Table I. On day 19, the coverslips were removed and either examined with polarizing microscopy (A) or stained with Oil Red 0 and hematoxylin (B). Magnifications: A, ×525; A Inset, ×3,520; B, ×600.

even though a constant level of native LDL was maintained in the culture medium. In other experiments, incubation of smooth muscle cells for 3 days with native LDL at concentrations as high as 300 μg protein/ml produced the same increase in the cellular content of cholesteryl esters as did 10 μg protein/ml (i.e., 7 μg sterol/mg cell protein).

When DMPA-LDL was used in place of native LDL, the number of LDL receptors was similarly reduced (Fig. 1A). However, the uptake of the polycationic lipoprotein was not dependent upon the LDL receptor, and hence the cellular content of cholesteryl esters increased progressively over the 4-day period (Fig. 1C).

When smooth muscle cells were grown for periods of more than 1 wk in the presence of DMPA-LDL, an even more massive increase in the content of cholesteryl esters was observed. This increase was similar whether the cells were exposed continuously to DMPA-LDL (data not shown) or whether they were subjected to several intermittent exposures of 48 h each (Table I). The cellular content of cholesteryl esters after intermittent exposure to DMPA-LDL was three-fold higher when the cells were grown in 2% FCS as compared to 10% FCS (Table I). When the cells were grown in 2% FCS, DMPA-LDL caused a 65-fold higher level of cellular cholesteryl esters than did an equal concentration of native LDL.

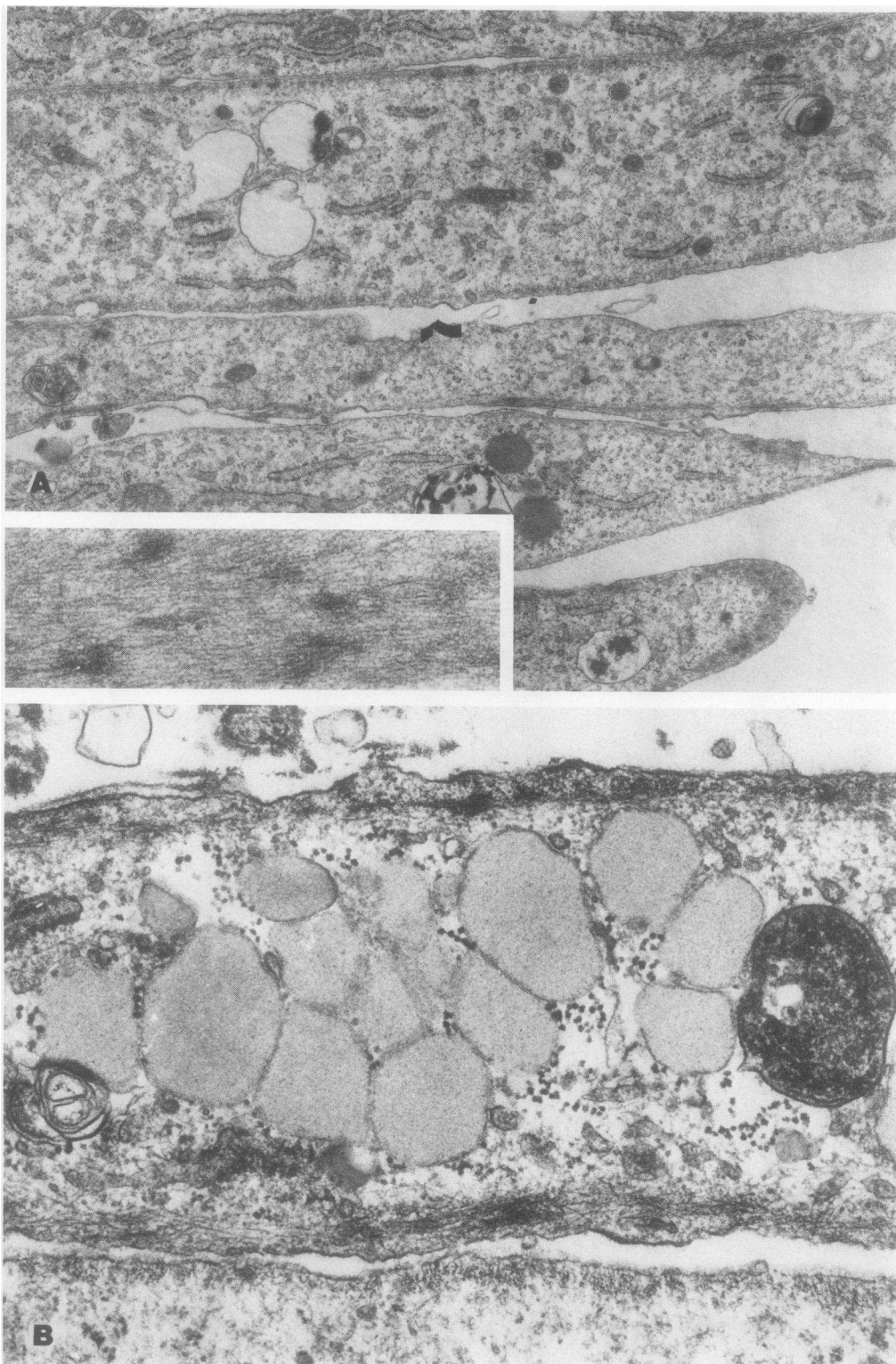FIGURE 3   Electron microscopic appearance of normal aortic smooth muscle cells that were repeatedly incubated with either native LDL (A) or DMPA-LDL (B). Monolayers were incubated with 10 μg protein/ml of either native LDL (A) or DMPA-LDL (B) in 2% FCS as described in the legend to Table I. On day 19, the cells were fixed in situ as described in Methods. Magnifications: A, ×14,600; B, ×42,200.

DMPA-LDL did not elevate the triglyceride content of the smooth muscle cells. In an experiment in which the cellular content of esterified cholesterol was 22-fold higher in cells exposed to 10 μg protein/ml of DMPA-LDL as compared with an equal amount of native LDL (370 vs. 17 μg sterol/mg protein), the content of cellular triglycerides was 82 and 72 μg/mg cell protein, respectively.

The massive accumulation of cholesteryl esters produced by DMPA-LDL could be visualized at the light and electron microscopic levels. In control cells incubated with native LDL, only occasional small lipid droplets were seen after staining with Oil Red 0 (data not shown). In contrast, cells incubated with the same concentration of DMPA-LDL showed large numbers of Oil Red 0 positive inclusions (Fig. 2B). The majority of these inclusions exhibited birefringence (Fig. 2A), and many showed typical formée crosses indicative of liquid crystals of cholesteryl esters (4, 22) (Fig. 2A, inset). The proportion of inclusions exhibiting formée crosses increased progressively for several days after DMPA-LDL was added to the culture medium, suggesting that the development of organized liquid crystals of cholesteryl ester may require a slow physical or chemical modification of the incoming DMPA-LDL by the cell.

The electron microscopic appearance of the cultured human aortic smooth muscle cells used in the current experiments was similar to that reported previously for human (23) and monkey aortic smooth muscle cells (24). In particular, the human smooth muscle cells showed abundant myofilaments (Fig. 3A, inset) and peripheral dense bodies (Fig. 3A). Ultrastructural examination of smooth muscle cells treated with DMPA-LDL showed that most of the cells had accumulated large numbers of homogeneous gray lipid droplets that were not surrounded by a typical tripartite limiting membrane (Fig. 3B). In contrast to this DMPA-LDL effect, smooth muscle cells treated with an equal amount of native LDL in the same experiments showed only an occasional neutral lipid droplet (Fig. 3A).

In the course of these experiments, we had the opportunity to study the metabolism of LDL in smooth muscle cells cultured from the thoracic aorta of a patient with homozygous FH. The binding, uptake, and degradation of ^{125}I-LDL were markedly deficient in the mutant cells (<2% of normal values). As a result, these mutant smooth muscle cells did not exhibit normal LDL-mediated suppression of 3-hydroxy-3-methylglutaryl CoA reductase activity or stimulation of cholesteryl ester formation. Despite their genetic lack of LDL receptors, the mutant smooth muscle cells accumulated cholesteryl esters to the same degree as did normal cells in the presence of DMPA-LDL (manuscript in preparation).

DISCUSSION

In the present studies, an unregulated uptake of LDL in aortic smooth muscle cells was achieved by enhancing the positive charge of the lipoprotein so that it entered the cells by a process not involving the physiologic LDL receptor. It is likely that within the athersoclerotic lesion a different mechanism is responsible for triggering the engorgement of smooth muscle cells with lipoprotein-bound cholesteryl esters. Nonetheless, it seems clear from the current data that when the LDL receptor regulatory system is bypassed and smooth muscle cells are forced to take up LDL in excess of their cholesterol requirements, they accumulate massive amounts of cholesteryl esters and develop morphologic abnormalities similar to those of the smooth muscle cells of the atherosclerotic lesion in vivo (1–5). These studies thus emphasize the critical role of the regulation of the LDL receptor in protecting aortic smooth muscle cells against an overaccumulation of cholesteryl esters when they are exposed to large amounts of native LDL.

ACKNOWLEDGMENTS

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